VIRAL AND IMMUNOLOGICAL CHARACTERISATION
OF MURINE CYTOMEGALOVIRUS-INDUCED
MYOCARDITIS

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SUMMARY

The aim of this thesis was to characterise and examine various mechanisms involved in the development of murine cytomegalovirus (MCMV)-induced myocarditis with a view to develop potential therapeutic regimes. MCMV induces acute and chronic myocarditis in BALB/c mice, which are genetically susceptible to MCMV, and low levels of acute disease in resistant C57BL/6 mice. The role of virus was studied using the laboratory strain of MCMV, K181, and a wild isolate of MCMV, G4. Whilst MCMV is capable of directly killing myocytes, infectious virus is not detectable in the heart beyond day 6 post-infection (p.i.). Myocytes undergo low levels of apoptosis following virus infection, indicating that apoptosis is not a major cause of the necrosis observed in this disease. The inflammatory infiltrate was identified as being predominantly CD8+ T cells in the foci during the acute and chronic phases of disease in both susceptible and resistant mouse strains. CD4+ T cells and B cells were identified throughout the myocardium in both mouse strains, however, only susceptible BALB/c mice mount a macrophage and neutrophil response in the heart following infection. Antiviral therapy was trialed with three drugs known for their potency against CMV. Early administration of antiviral drugs successfully reduced both acute and chronic myocarditis, however, no benefit was observed when treatment commenced after the establishment of cardiac disease. Antiviral therapy effectively reduced myocarditis exacerbation following MCMV reinfection. The role of the immune system was also examined. Lipopolysaccharide (LPS) administration exacerbated MCMV-induced myocarditis in susceptible BALB/c mice, and induced normally resistant C57BL/6 mice to develop chronic myocarditis. Cytokine levels were influenced by LPS, with increases in TNF observed, however, TNF administration to MCMV-infected mice only increased myocarditis in the C57BL/6 resistant strain. The importance of cytokines in MCMV-induced myocarditis was highlighted by examining levels of several cytokines in the heart following MCMV infection. Differences in cytokine production in the heart were observed between the acute and chronic phase of disease, as well as between susceptible and resistant mice.

These results suggest that whilst the presence of MCMV is critical for the development of myocarditis, the immune system is the major cause of ongoing myocardial inflammation and necrosis. Early clearance of virus reduces the severity of disease, as
observed in the antiviral drug studies. The ability of the immune system to clear virus is also important in the subsequent disease course. The type of cellular and cytokine response to MCMV infection ultimately dictates the severity of acute disease and whether chronic myocarditis develops. These findings have important implications for the treatment of clinical myocarditis. The treatment of virus infection with antiviral drugs together with immune system modulation may lead to more effective treatment regimes for human myocarditis.
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<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethylcarbazole</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BrDU</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CB3</td>
<td>Coxsackievirus B3</td>
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<td>cidofovir</td>
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<td>CO₂</td>
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<td>CR</td>
<td>complement receptor</td>
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<td>ddH₂O</td>
<td>double distilled water</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbant assay</td>
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<td>EMCV</td>
<td>encephalomyocarditis virus</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>FIC</td>
<td>fractional inhibitory concentration</td>
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<td>forward</td>
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<td>g</td>
<td>gram</td>
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<td>microgram</td>
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<td>gB</td>
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<td>ganciclovir</td>
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<td>granulocyte macrophage-colony stimulating factor</td>
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<td>glycoprotein</td>
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<td>HCMV</td>
<td>human cytomegalovirus</td>
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<td>H&amp;E</td>
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<td>HEPES</td>
<td>hydroxyethylpiperazine-N' -2-ethanesulfonic acid</td>
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<td>HIS</td>
<td>hyperimmune serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>ie</td>
<td>immediate early</td>
</tr>
<tr>
<td>IFN α/β</td>
<td>interferon alpha/beta</td>
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<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
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<tr>
<td>μl</td>
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<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
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</tr>
<tr>
<td>LPS</td>
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<tr>
<td>M</td>
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<td>MCMV</td>
<td>murine cytomegalovirus</td>
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<tr>
<td>MCP</td>
<td>monocyte chemotactic protein</td>
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<tr>
<td>MEF</td>
<td>mouse embryo fibroblast</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NMS</td>
<td>normal mouse serum</td>
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<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PFA</td>
<td>foscarnet</td>
</tr>
<tr>
<td>pg</td>
<td>picograms</td>
</tr>
<tr>
<td>pi</td>
<td>post-infection</td>
</tr>
<tr>
<td>rev</td>
<td>reverse</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription – polymerase chain reaction</td>
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<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>STE</td>
<td>sodium/tris/EDTA</td>
</tr>
<tr>
<td>TAE</td>
<td>tris/acetate/EDTA</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell type 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>UWA</td>
<td>University of Western Australia</td>
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CHAPTER 1

General Introduction
1.1 HERPESVIRIDAE
The herpesvirus family shares a number of characteristics including a double stranded linear DNA genome within an icosahedral capsid, an amorphous tegument and an outer membrane envelope with surface glycoproteins. Herpesviruses share other significant properties, including enzymes for nucleic acid synthesis and metabolism, as well as at least one protease and a number of protein kinases. The viruses have similar nuclear synthesis of viral DNA and assembly of capsids which leads to the production of infectious progeny accompanied by irreversible destruction of the infected cell. The Herpesviruses also develop a latent infection of the host, defined operationally by the inability to detect infectious particles and by the possibility of inducing a recurrent infection (Roizman & Sears, 1987).

The herpesvirus family has been divided into the α, β and γ herpesviruses, based on the difference in the size and base composition of their genomes and other unique biological properties (Roizman et al., 1981). The Alphaherpesvirinae subfamily includes herpes simplex type 1 and type 2 virus and varicella-zoster virus, with a genome size ranging from 125 to 152 kilobases (kb). Gammaherpesvirinae has two members, Epstein-Barr virus and Human Herpesvirus-8 (HHV-8). The Betaherpesvirinae subfamily contains Cytomegalovirus (CMV) with the largest genome of the herpesvirus family being 230 kb (Herpesvirus Study Group, 1992; Rawlinson et al., 1996).

1.1.1 Cytomegalovirus
CMV was initially named “salivary gland virus” due to its recovery from this tissue (Goodpasture, 1925; Rowe et al., 1956), but was later renamed “cytomegalovirus” due to the enlarged appearance, or cytomegalia, of host cells with viral intranuclear inclusions (Goodpasture, 1925). CMV is a ubiquitous agent that commonly infects many species including mice, rats, pigs, horses and humans (Alford & Britt, 1990). Assignment to the herpesvirus family was based on the morphology of the viral particle, the chemical composition of the virion, and the characteristics of the intranuclear inclusion body present in infected cells.
1.1.2 Human Cytomegalovirus

Human cytomegalovirus (HCMV) has been difficult to isolate and characterise due to its slow replicative cycle, low virus yield in vitro, complex structure and high species-specificity. HCMV is the major cause of mental retardation and congenital deafness due to infection in utero (Stagno et al., 1982; Rasmussen, 1990). HCMV is endemic in the Western world. Depending on the age of the individual, the immune system and associated other (neoplastic) diseases, serological evidence for HCMV can be found in up to 80% of cases (Maisch et al., 1993). The virus is spread by close contact with infected secretions or by the introduction of infected blood or organs into the host. Although HCMV infections are asymptomatic in most healthy individuals, cardiac manifestation of cytomegalovirus has been reported and is associated with high mortality particularly where there is underlying immunosuppression (Biton & Hermun, 1989; Wink & Schmitz, 1980; Gonwa et al., 1989). CMV infection is also a major cause of serious illness in immunocompromised patients, such as transplant recipients and AIDS patients (Glenn, 1981; Wreghitt et al., 1988; Fuller, 1992; Fernando et al., 1994; Herskowitz et al., 1994; Schoppel et al., 1998). Congenital CMV infections remain the leading viral cause of congenital malformations in the developed world. Congenital illness ranges from no clinical disease through to prematurity, encephalitis, deafness, haematological disorders and death (Trincado & Rawlinson, 2001).

With the enormous increase in the application of transplantation techniques that has occurred in the past two decades, we have learned much more about how HCMV manifests itself clinically during immunosuppression. In the first three to four months after transplantation HCMV infection syndromes such as mononucleosis, unexplained leukopenia, pneumonia, and, rarely, hepatitis may occur (Ho, 1982).

In the last ten years, HCMV infection has emerged as an important contributor in the development of inflammatory heart diseases such as myocarditis and dilated cardiomyopathies (Maisch et al., 1993; Friman et al., 1995; Friman & Fohlman, 1997). HCMV infection of vascular endothelial cells may also contribute to the development of atherosclerosis (Melnick et al., 1994; Woodroffe et al., 1995; Adam et al., 1997; Coles et al., 1998). Although the majority of patients with myocarditis eventually recover, HCMV-associated myocarditis in immunosuppressed patients carries a mortality rate of 60% (Ng et al., 1997). In 30 to 60% of patients, myocarditis progresses to dilated
cardiomyopathy, often resulting in the need for heart transplantation (Fallon, 1988; Peters & Poole-Wilson, 1991; Olsen, 1992; Martino et al., 1994). HCMV remains to be an important pathogen in human populations causing significant disease.

1.1.3 MCMV as a model for HCMV infection

Due to the strict host species-specificity of CMV, a direct animal model for HCMV infection has not been established (Hudson, 1979). However, animal models have been developed for guinea pig, bovine, rat, equine and murine CMV (MCMV) (Alford & Britt, 1990; reviewed in Staczek, 1990). Infection with MCMV is an appropriate animal model for the study of pathogenesis of HCMV for a number of reasons. MCMV closely resembles HCMV in tissue tropism (Mocarski et al., 1990) and produces similar pathological sequelae, such as the development of pneumonitis, hepatitis and myocarditis (Jordan, 1978; Olver et al., 1994; Lawson et al., 1989). MCMV also produces a latent infection in multiple organs in mice, such as the spleen, liver, kidney, heart, lung, brain and salivary glands (Collins et al., 1993), and is capable of being reactivated (Bevan et al., 1996). Likewise, CD8+ T cells have been shown to be important for viral clearance in both the mouse and human (Ho, 1980; Reddehase et al., 1987; Quinnan et al., 1982; Grob et al., 1987).

1.1.4 Molecular biology

MCMV has a linear double-stranded genome of 230 kilobases (kb), consisting of long and short unique regions flanked by inverted repeat sequences, predicted to code for 170 genes (Rawlinson et al., 1996). Once susceptible cells are infected, MCMV DNA is synthesised using viral DNA polymerase (Griffiths & Grundy, 1987). Transcription of mRNA is sequentially expressed, giving rise to immediate-early (0-2 hours p.i.), early (2-24 hours p.i.) and late (after 24 hours p.i.) proteins that are classified as α, β or γ respectively (Griffiths & Grundy, 1987; Rasmussen, 1990). Proteins that are synthesised during the immediate-early or early times are generally associated with regulation of viral replication, whereas the late proteins are involved in structural elements of the virion (Rasmussen, 1990).

The immediate-early (IE) genes encode at least three groups of proteins (Rawlinson et al., 1996). These genes are transcribed and translated prior to the onset of viral DNA
and protein synthesis. The resulting proteins are believed to function as transactivators of transcription of viral and cellular genes (Rasmussen, 1990; Stenberg & Kerry, 1995). The envelope glycoproteins of CMV serve as dominant antigens for both the cellular and humoral immune response. As many as eight glycoproteins are estimated to be present on the envelope of HCMV (Kim et al., 1976) of which four glycoproteins (gB, gH, gL and gM) have been found to be conserved among all herpesviruses, including HCMV and MCMV (Rapp et al., 1992; Xu et al., 1992; 1994b; Scalzo et al., 1995a; Rawlinson et al., 1996).

Glycoprotein B (gB) of CMV is made up of disulphide linked molecules of 55-58 kDa and 116 kDa (Rasmussen, 1990), and expression is restricted to the late phase of replication (Rapp et al., 1992). Currently, the function of gB is unknown, however, it is thought to play a role in viral entry and cell fusion since these roles have been established for gB of the Herpes Simplex Virus (HSV) (Cai et al., 1988).

The second major glycoprotein of MCMV, gH, is a single gene product of 87 kDa (Xu et al., 1992; 1994a) and is believed to be involved in intracellular spread of the virus and initiation of virus infection of cells (Rasmussen, 1990). Glycoprotein L of MCMV is 46 kDa and is thought to complex with gH to facilitate the correct folding and surface expression of gH (Xu et al., 1994a). Both of these glycoproteins whilst being vital for virus entry, replication and spread in host cells, also represent targets for the host’s immune response.

1.1.5 Immune response and autoimmunity

In normal individuals, infection with HCMV is usually asymptomatic, however, a mononucleosis-like syndrome can develop accompanied by fever, fatigue and the development of hepatitis (Rasmussen, 1990). After the acute infection has resolved, CMV remains latent in multiple cell types and tissues without apparent illness. Severe disease can develop in immunocompromised patients resulting in pneumonitis, mononucleosis, hepatitis, encephalitis, retinitis, colitis, vasculitis and myocarditis (Alford & Britt, 1990; Merigan & Resta, 1990; Woodroffe et al., 1995). Tissue damage is caused by direct viral infection, replication and ultimate lysis of the target cell. In some cases the immune response to CMV can itself augment the pathology of the disease and indeed may be responsible for establishing autoimmune diseases. It is
highly probable that CMV can trigger autoimmune diseases because it elicits a strong cellular immune response and viral antigens persist for long periods in target cells (von Herrath & Oldstone, 1995). CMV has been shown to induce the production of many cytokines and this may also lead to the stimulation of autoreactive lymphocytes (Gribaudo et al., 1993; Van Parijs & Abbas, 1998).

Studying MCMV infection in the mouse has provided valuable insights into the possible mechanisms involved in CMV-induced autoimmunity. MCMV-induced pneumonitis is believed to be immunopathologically-mediated with uncontrolled recruitment of T cells to the lung (Grundy et al., 1987b; Chapman et al., 1994). These T cells may either recognise a CMV-encoded non-structural protein or respond to the deposition of autoreactive antibodies (Chapman et al., 1994). In the MCMV-induced hepatitis model, autoantibodies against liver-specific lipoprotein have been observed suggesting that such lipoproteins may be a likely candidate autoantigen in chronic active hepatitis (Bartholomaeus et al., 1983; 1987; Olver et al., 1994). MCMV-induced myocarditis is associated with autoantibodies to cardiac myosin (Bartholomaeus et al., 1988). These autoantibodies and subsequent disease may be induced by cross reactive epitopes between MCMV and heart tissues (Lawson et al., 1990; 1992; 2000).

1.2 MYOCARDITIS

The cardiovascular disease, myocarditis, is characterised by inflammation and necrosis of cardiac muscle. The disease ranges from a transient inflammation to a fulminant syndrome with manifestation that may include heart failure, arrhythmias and sudden death. Interest in myocarditis has been generated by the linkage of this disease with the subsequent development of dilated cardiomyopathy, a disease which accounts for approximately 25% of all heart failures in North America (Sole & Liu, 1993).

Myocarditis may be diagnosed either clinically or histopathologically, but most cases are mild and pass unrecognised (Friman et al., 1995). By means of clinical examination and currently available tests, such as serial electrocardiograph recordings and serum markers of myocyte lesion, a clinical diagnosis of acute myocarditis may be made with reasonable certainty in most patients. The usually benign condition of acute myocarditis should be differentiated from sub-acute to chronic myocarditis, which presents with cardiac insufficiency and maybe in the process of developing into dilated
cardiomyopathy (Mason et al., 1995). A histopathological diagnosis of the disease is made either by endomyocardial biopsy or following autopsy (Why et al., 1994).

The true incidence of myocarditis is unknown, but up to 10% of routine post-mortem examinations show histological evidence of myocarditis (Saphir, 1941; Gore & Saphir, 1947; Gravanic & Sternby, 1991). Histologically defined disease has been diagnosed in 17-30% of patients with clinically suspected myocarditis, and in 40-60% of patients with acute dilated cardiomyopathies (Zee-Cheng et al., 1984; Marboe & Fenoglio, 1988; Peters & Poole-Wilson, 1991). Many cases of myocarditis are overlooked because of the focal nature of this disease, the limited number and size of tissue specimens taken at the time of biopsy and the timing of the biopsy in relation to disease progression (Marboe & Fenoglio, 1988; Ng et al., 1997). In an attempt to standardise the diagnosis of this disease, the Dallas Classification System was introduced (Aretz et al., 1987). This classification was proposed by eight cardiac pathologists (the Dallas panel) (Aretz, 1987). This classification divides patients into four distinct subgroups, classified as fulminant, acute, chronic active, or chronic persistent myocarditis, which in turn determines the type of therapy to be given (Liebermann et al., 1993).

Non-infectious causes of myocardial inflammation include drugs (cocaine, Adriamycin), chemicals (carbon monoxide), autoimmune diseases (systemic lupus erythematosus, Kawasaki disease), or hypersensitivity reactions (Drucker & Newburger, 1997). Infectious causes of myocardial inflammation are numerous and the aetiology can be difficult to elucidate. The majority of myocarditis cases are considered to be initiated by infectious agents (Fonataine et al., 1995; O'Connell et al., 1995) (Table 1.1). Viral myocarditis has been reported in 20-65% of patients who subsequently develop dilated cardiomyopathy (Olsen, 1992; Sole & Liu, 1993). The viruses most frequently associated with myocarditis include picornaviruses, orthomyxoviruses and herpesviruses (Friman et al., 1995; Penninger et al., 1996; Huber, 1997). Viral infections are implicated in the aetiology of patients with myocarditis where anti-viral antibodies or viral genomes can be detected (Huber, 1997).

Despite diagnostic and technical advances in medicine, the treatment of patients with myocarditis remains primarily supportive. As myocarditis causes significant myocardial dysfunction, there is an increase in the work of breathing and a decrease in gas
Table 1.1  Major infectious agents associated with clinical cases of myocarditis.

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Bacteria</th>
<th>Protozoa</th>
<th>Other</th>
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<tbody>
<tr>
<td>Coxsackievirus</td>
<td>Beta-haemolytic streptococci</td>
<td><em>Toxoplasma gondii</em></td>
<td><em>Trichinella spiralis</em> (worm)</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td><em>Borrelia burgdorferi</em> (Lyme disease)</td>
<td><em>Trypanosoma cruzi</em></td>
<td><em>Fusarium</em> (fungus)</td>
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<td>Varicella Zoster</td>
<td>Mycoplasma</td>
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<td>Epstein Barr</td>
<td>Chlamydia</td>
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<td>Influenza</td>
<td>Staphylococcus</td>
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<tr>
<td>HIV</td>
<td><em>Coxiella burnetti</em> (Q-fever)</td>
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<td>Adenovirus</td>
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<td>ECHO</td>
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<td>Poliovirus (before vaccine)</td>
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</table>

From Huber, 1997.
exchange. Patients are therefore put on an increased fraction of inspired oxygen, in order to improve oxygenation. Standard medications used to treat congestive heart failure associated with myocarditis include diuretics, inotropic agents, afterload-reducing agents and anticoagulants. These treatments are all designed to maintain heart function, rather that treat myocarditis. Further research is critical in order to elucidate appropriate treatments for this disease and researchers have turned to animal models to investigate the pathogenesis and mechanisms of myocarditis.

1.2.1 Animal models of viral myocarditis
Although a number of animal species such as primate, pig, dog, rabbit, guinea pig and rat have been used in myocarditis research, most animal models utilise the murine species (Huber, 1997). Experimental models predominantly involve either immunisation of animals with heart specific proteins emulsified in adjuvant, or infection of animals with agents associated with causing myocarditis in humans.

Reovirus-induced acute viral myocarditis in mice is characterised by a mild inflammatory infiltrate with marked necrosis (Hassan et al., 1965; Stangl et al., 1987). Studies on reovirus-induced myocarditis in SCID mice (Sherry et al., 1993) and macrophage inflammatory protein-1α knockout mice (Sherry et al., 1998) demonstrated that this disease is not immune cell-mediated. Reovirus is believed to induce disease by directly infecting myocytes (Baty & Sherry, 1993).

Encephalomyocarditis virus (EMCV), which is a member of Cardiovirus of the family Picornaviridae, causes acute myocarditis occasionally with fatal outcome in humans and various animal species. DBA/2 mice infected with EMCV show marked dilation and hypertrophy of the heart in the chronic stage of myocarditis, which is characterised by lesions similar to those seen in dilated cardiomyopathy in humans (Matsumori et al., 1982; Kanda et al., 1995). EMCV-induced myocarditis is believed to be a primarily autoimmune disease. EMCV infection is accompanied by IgG autoantibodies against the heart constituent vimentin, which are present from day 5 post infection onwards (Sato et al., 1994). Infection of nu/nu BALB/c mice with EMCV results in a reduction in the severity of myocarditis when compared to nu/+ mice (Kishimoto et al., 1986). The autoimmune phenomena of this disease may also be exacerbated by cytokines, intended to help eliminate the virus. For example, antibody against TNF has a
therapeutic effect on the development of myocarditis (Yamada et al., 1994), whereas administration of the proinflammatory cytokine IL-6 reduces myocardial damage in mice (Kanda et al., 1996).

In humans, the group B Coxsackieviruses have been identified as the viral agent most frequently associated with viral myocarditis (Sanders, 1963; Smith, 1970). Lerner and colleagues (Lerner & Wilson, 1973) first demonstrated that 14 day old mice, inoculated intraperitoneally with Coxsackievirus B3 (CB3) develop severe inflammatory heart muscle disease. The lesions observed in infected mice are histologically similar to those observed in humans (Dec et al., 1985; Herskowitz et al., 1985; Godman et al., 1952). The primary lesion consists of scattered necrotic foci surrounded by mononuclear cells throughout the ventricles and, to a lesser extent, in the atria. Later, the necrotic foci are replaced with connective tissue and calcification, and a diminishing number of mononuclear cells (Rose et al., 1992). Susceptibility to the development of CB3-induced myocarditis depends on several factors including the strain of virus (Gauntt et al., 1984; Chow et al., 1991), and the mouse strain (Wolfram et al., 1986), age (Grodums & Dempstar, 1959; Khatib et al., 1980) and sex (Huber et al., 1981). Certain strains of mice develop a chronic disease, in which inflammation continues beyond day 9 post infection.

CB3 is a member of the picornavirus family, which are small, nonenveloped viruses containing a single positive-sense RNA strand within a protein icosahedral capsid (Chatterjee, 1988; Huber, 1997). Picornaviruses are able to immediately translate viral proteins after infecting host cells because they use their viral positive RNA strands as the mRNA template. Different strains of CB3 infect and replicate more efficiently in certain tissues. Cardiotropic (myocarditis-inducing) CB3 employ cell surface molecules as receptors which may be heart specific, while hepatotropic (hepatitis-inducing) or beta-tropic (diabetes-inducing) CB3 variants use molecules specific for hepatocytes or beta cells, respectively (Huber, 1997). CB3 infection of murine myocytes can clearly cause direct cell death without involving any form of host defence (Herzum et al., 1994; McManus et al., 1993). The immune response in infections with highly lytic viruses is more likely to be protective. Less lytic CB3 infections of myocytes can be combatted with interferon α/β, NK cells, neutralising antibody, activated macrophages and the release of nitric oxide (reviewed in Huber, 1997). However, such an immune response
to CB3 infection may also be contributing to myocardial damage. The release of nitric oxide from activated macrophages can induce damage directly to myocytes (Lowenstein et al., 1996). Likewise, cytokines such as TNF can induce direct damage or recruit NK cells and cytolytic T cells to sites of viral infection (Finkel et al., 1992; Huber, 1997).

Autoimmunity is also a frequent result of experimental CB3 infections. Research has demonstrated the presence of autoantibodies to cardiac isoforms of myosin after CB3 infection which correlate to animal susceptibility to viral myocarditis (Wolfgram et al., 1986; Neu et al., 1987a). Cellular autoimmunity also occurs and is mediated by CD8+ cytolytic T lymphocytes (CTL) (Huber & Lodge, 1984). These CTL specifically lyse normal syngeneic myocytes but are inactive against various other tissues. These autoimmune T cells can effectively transfer myocarditis to recipient mice in the absence of virus (Huber et al., 1987). Interestingly, while both humoral and cellular immunopathogenic mechanisms can cause myocardial disease, the same mechanisms may not function in all genetic mouse strains. Studies demonstrate that DBA/2 mice infected with CB3 develop an exclusively humoral immunopathogenic myocarditis while BALB/c mice develop a predominantly cell-mediated disease (Huber & Lodge, 1986). Other strains, such as A/J mice, have a mixed pattern in which both autoimmune T cells and autoantibodies are active in disease (Lodge et al., 1987).

Whilst the genetic factors determining whether cellular or humoral autoimmunity arises in myocarditis are currently not well understood, evidence suggests that the differentiation into either Th1 or Th2 CD4+ cell responses in a particular mouse strain is important, particularly in the development of chronic disease. Therefore, as expected, cytokines can modulate whether mice develop CB3-induced myocarditis. Administration of exogenous IL-1 or IL-2 augments disease in CB3-infected susceptible mice, while blocking IL-1 receptors inhibits the development of myocarditis (Neumann et al., 1993; Huber et al., 1994). Mice that are normally resistant to the development of chronic myocarditis with CB3 infection can be induced to develop myocarditis and autoantibodies against cardiac myosin if treated with LPS, TNF or IL-1 in conjunction with CB3 infection (Lane et al., 1991; 1992). The development of CB3-induced myocarditis into the autoimmune disease is most likely a multi-facetted phenomenon, with many researchers developing conceptual models (Fig. 1.1). The CB3-induced
A conceptual scheme for the pathogenesis of autoimmune myocarditis induced by CB3 virus or cardiac myosin. From Rose, 1996.
myocarditis animal model is an excellent model for researching this disease and continues to provide exciting insights into autoimmune mechanisms of pathogenesis.

It is becoming widely accepted that the chronic forms of myocarditis are associated with post-infectious autoimmunity. This is clearly demonstrated by clinical and experimental findings such as the observation of myocyte-reactive T cells and circulating antibodies to various myocardial autoantigens (Maisch et al., 1982; Neumann et al., 1990; Kashahara et al., 1994). Post-infectious autoimmune myocarditis in certain mouse strains is triggered by cardiac myosin that is exposed to the immune system after virus-mediated myocyte damage (Rose et al., 1988). When purified cardiac myosin is injected into mice without virus, inflammatory heart disease, similar to post-infectious myocarditis is observed (Neu et al., 1987c). This inflammation is not observed when skeletal muscle myosin is given to mice, suggesting that the pathogenic epitopes are specific for the cardiac myosin isoform (Neu et al., 1987b). Cardiac myosin induced myocarditis offers a virus-free system with a defined autoantigen in which to study pathogenic autoimmune mechanisms.

Cardiac myosin is one of a number of α-helical coiled-coil molecules including tropomyosin, vimentin and laminin. The α-helical coiled-coil molecules share similar structural features, and cross-react with bacterial and viral proteins with α-helical coiled-coil structures such as streptococcus, shigella, trypanosoma and CB3/4 (Lahesmaa et al., 1991; Cunningham et al., 1992; 1993; Felix et al., 1993). T cells are necessary for the development of disease in this model, with myosin-immune T cells capable of transferring myocarditis in SCID mice (Smith & Allen 1991; Pummerer et al., 1995). Furthermore, depletion of CD4+ and CD8+ T cells from susceptible mice inoculated with myosin diminishes myocarditis. This evidence suggests that both T cell subsets are important in the pathogenesis of this disease (Pummerer et al., 1991; Smith & Allen, 1991; Penninger et al., 1993).

The murine model of CMV myocarditis has been established using the K181 strain of MCMV (Lawson et al., 1989; 1992). Sublethal MCMV infections of adult BALB/c mice induce inflammation in murine cardiac tissue ranging from focal lymphocyte infiltration to intense, diffuse infiltration and necrosis associated with MCMV-inclusion
bearing myocytes (Bartholomaeus et al., 1988). Although viral infection of cardiac myocytes accompanies myocarditis at days 7 and 10 post-infection, myocarditis is also observed in MCMV-infected mice at days 28 and 56 post-infection in the absence of detectable virus (Lawson et al., 1990). MCMV-induced myocarditis is evident in two distinct phases, the acute stage, approximately day 5 to 14 and a second, chronic phase, after day 21 and persisting out at least to 100 days post-infection (Fig. 1.2). Like CB3-induced myocarditis, MCMV-induced myocarditis produces autoantibodies to cardiac myosin, suggesting autoimmune mechanisms are responsible for driving the disease into the chronic phase. Several studies have strengthened the link between CMV and myocarditis. CMV genes have been detected by in situ hybridisation in patients with diagnosed myocarditis (Wu et al., 1994; Maisch et al., 1993). Because of this growing evidence for HCMV-induced myocarditis in man (Tayler-Wiedeman et al., 1991; Friman et al., 1995), this model is providing invaluable insights into the pathogenic nature of the equivalent human disease.

1.3 PATHOGENESIS

1.3.1 Viral mechanisms

Several pathogenic mechanisms have been suggested to account for the inflammation and necrosis found in virally-induced myocarditis. The most obvious mechanism would be direct viral damage to cardiac myocytes leading to necrosis and the subsequent development of a protective inflammatory immune response (Klingel & Kandolf, 1993; McManus et al., 1993). Evidence supporting this mechanism has been found in various animal models. CB3 infection in murine myocytes can clearly cause direct cell death without involving any immune mechanism of host defence (Herzum et al., 1994; McManus et al., 1993). During the early stage of CB3 disease, virus can be readily obtained from the heart tissue and it is likely that the virus itself induces significant cytopathic alterations in the myocytes (Rose et al., 1992). In situ hybridisation studies in CB3-infected mice demonstrated that viral infection led directly to myocyte necrosis and that the inflammatory foci were consistently associated with virus in both the acute and chronic phases of myocarditis (McManus et al., 1993; Kandolf et al., 1993; Klingel & Kandolf, 1993). In these cases, the cellular infiltrate served a predominantly protective role, suggesting that the presence of virus was necessary for the development of chronic inflammation.
Figure 1.2  MCMV-induced myocarditis in infected BALB/c mice. From Lenzo J.C., Fairweather, D., Cull V., Shellam G.R. & James (Lawson) C.M., 2002. Characterisation of murine cytomegalovirus myocarditis: cellular infiltration of the heart and virus persistence. *Journal of Molecular and Cellular Cardiology* 34(6): 629-640.
In the MCMV model of myocarditis, virus titres in the heart are extremely low, indeed 100-fold lower than CB3 titres (Rose et al., 1986; Fairweather et al., 1998). Infectious MCMV can only be isolated from cardiac tissue during the acute phase of disease and accumulating evidence suggests that direct viral damage of the myocytes is not the major pathological mechanism. Where the CB3 model shows myocyte damage before the appearance of an inflammatory infiltrate, no such damage is observed in the MCMV model (Fairweather et al., 2001). The low levels of infectious virus measured in the heart of susceptible mouse strains during acute MCMV infection indicates that high titres of virus in the heart are not necessary for the development of chronic inflammatory heart disease. Indeed, higher virus titres induced by treatment of mice with the immunosuppressive drug cyclosporin A, did not lead to enhanced inflammation of the heart (Lawson et al., 1989). Whilst replicating virus is not found in the heart beyond the acute stage of disease, recent research has shown that viral DNA (iel and gB genes) is present in the heart throughout the chronic phase. Viral nucleic acid was also detected in the hearts of resistant mice, in the absence of disease (Fairweather D., Lenzo J.C., Cull V., Shellam G.R. and Lawson C.M., unpublished observations). Whilst MCMV infection is undoubtably the trigger for myocarditis in this model, it appears unlikely that virus replication is the pathological mechanism that drives the disease into the chronic phase.

1.3.2 Immune cells

The immune response may also contribute to tissue damage by inappropriately attacking cardiac myocytes. Woodruff first demonstrated a role for T lymphocytes in the pathogenesis of murine myocarditis using depletion of T lymphocytes with either antithymocyte serum or thymectomy (Woodruff & Woodruff, 1974). Irradiation led to a decrease in mortality and a decrease in the inflammatory cell infiltrate after CB3 infection. As a consequence of these findings, considerable research has been conducted to determine the role of immune cells in the immunopathogenesis of viral myocarditis.

In the CB3 model of myocarditis, severe damage to the heart becomes evident only 5 days after infection, when cellular infiltration is first observed. The increased proportion of CD8\(^+\) lymphocytes during the evolution of chronic myocarditis suggests that cytotoxic T cells are involved in the pathologic process (Rose et al., 1992). Further research has shown that two distinct populations of cytotoxic T cells are produced.
following CB3 infection of mice. One population being virus specific was found to have the CD4+ phenotype, whilst the second population reacted to glycoproteins induced after infection that are not encoded by the viral genome (Huber & Job, 1983a; Guthrie et al., 1984; Huber & Job, 1983b). Thus observed immunopathological changes induced by these T lymphocytes resulted from the immune response to the virus.

In the MCMV model of myocarditis, the role of T lymphocytes in the development of disease was assessed by Lawson and colleagues (Lawson et al., 1989). T cell depletion in nu/nu mice inhibits the induction of myocarditis when compared to nu/+ mice given the same virus dose. This suggests that the inflammatory response observed in the heart is largely T cell-dependent.

Recently it was found in the EMCV model using gene knockout mice in the background of a normally robust myocarditic response that depletion of CD4+ T lymphocytes caused a small but significant decrease in the inflammatory cell infiltrate at 14 days post-infection. However, the decrease in myocarditis was not observed either side of this time point (Opavsky et al., 1999). Infection of the susceptible A/J knockout mice that lack CD8+ T lymphocytes did not have a significant change in myocarditis levels. Interestingly, in A/J mice that lacked both CD4+ and CD8+ cells there was a marked decrease in inflammatory cell infiltrate (Opavsky et al., 1999). Studies conducted in gene targeted mice showed that CB3 infection of mice that lacked CD4+ T lymphocytes had a marked increase in myocardial inflammation (Henke et al., 1995). In mice that lacked CD8+ cells, there was no significant change in myocarditis levels. However, antibody-mediated depletion of CD8+ cells in CD4+ knockout mice led to a decrease in myocardial inflammation (Henke et al., 1995). This finding is controversial and researchers must take caution when drawing conclusions from such studies as gene knockout mice and monoclonal antibody depleted mice provide differing results.

The depletion of individual T cell subsets from susceptible mice infected with MCMV also yields interesting results. CD8+ T cells are more important than CD4+ T cells in the development of acute MCMV-induced myocarditis (Fairweather et al., 2001). It has been shown in the CB3 model of myocarditis that susceptibility is related to the activation of T cells expressing the γδ TCR (Huber et al., 1992; 1996; 1999). Huber and
colleagues have shown that different subpopulations of γδ T cells, based on their Vγ/Vδ use, were activated in the resistant C57BL/6 versus the susceptible B1.Tg.Eα mouse strain following infection with CB3. It is thought that γδ T cells exert their effect by modulating CD4+-T cell responses, skewing a response to either the Th1 or Th2 phenotype. This modulation may be cytokine driven, with γδ T cells producing either IL-4 or IFN-γ, depending on the viral infection and the subset that is activated. Recent work has shown that whilst IFN-γ/IL-4 production by γδ T cells is important in CD4+ T cell activation, there are clearly other mechanisms involved (Huber et al., 2001). Mounting evidence suggests that T cells expressing the γδ receptor clearly play an important role in modulating the immune response and ultimately conferring resistance or susceptibility to viral myocarditis.

NK cells are also important in disease induction. Using the MCMV model, depletion of NK1.1+ cells from mouse strains (C57BL/6 and BALB.B6-CmvI) that are normally resistant to myocarditis leads to increases in disease comparable to levels found in susceptible mice (Fairweather et al., 2001). This finding suggests that prevention of myocarditis in this model involves NK cells, as the only genetic difference between the congeneric BALB.B6-CmvI and the susceptible BALB/c strain is the NK gene complex (Forbes et al., 1997). The precise mechanism involved in the NK1.1+ cell-mediated protection is currently unknown but effective viral clearance associated with the production of cytokines from activated T cells may reduce the development of autoreactive T and B cell responses (Bachmann et al., 1998). NK cell activity in the CB3 model is also important. Mice treated with anti-asialo GM-1 to decrease NK activity show increased viral content and extensive myocarditis and necrosis in the heart (Godney & Gauntt, 1986; Godney & Gauntt, 1987).

The influx of immune cells into the myocardium following infection may be partly mediated by the release of chemokines at the site of virus infection. Chemokines are a group of small (8-14 kDa) structurally related molecules that regulate cell trafficking of various types of leukocytes (reviewed in Zlotnik & Yoshie, 2000). Chemokines can induce surface expression of adhesion molecules in leukocytes, thus promoting their attachment to endothelial cells (Ley, 1996). Chemokines have recently been shown to play an important role in viral myocarditis. Production of MIP-2 is enhanced in C3H/He
mice following infection with EMCV and treatment of these mice with an antibody against MIP-2 increased survival and decreased myocarditis (Kishimoto et al., 2001). A similar result was observed in the CB3 myocarditis model (Kishimoto et al., 2000).

### 1.3.3 Autoimmunity

Favourable evidence supports the hypothesis that autoimmune mechanisms are involved in inducing myocarditis following viral infection. Several observations from the various animal models and human patients have indicated that the post-viral immune response may be the driving force behind persisting disease. Firstly, infectious virus can be isolated from cardiac tissue only during the early phase of disease (Woodruff, 1980; Lawson et al., 1990). Whilst viral nucleic acid may persist, no infectious virus is present in most cases of chronic myocarditis (Fairweather D., Lenzo J.C., Cull V., Shellam G.R. & Lawson C.M., unpublished observations). Secondly, most patients with myocarditis develop autoantibodies to heart muscle and these autoantibodies are also observed in animal models (Lauer et al., 1994; Lawson et al., 1992; Wolfgram et al., 1985). Finally, a proportion of patients with myocarditis respond favourably to treatment with immunosuppressive drugs (Lieberman et al., 1991).

The concept of autoimmunity has been extensively studied in the CB3 model of myocarditis. The most persuasive evidence is the finding that CB3-infected mice produce autoantibodies specific for cardiac myosin, and that cardiac myosin can induce an inflammatory response in the heart of uninfected mice. Mice with progressive myocarditis have significant titres of IgG antibody to mouse heart antigens. Absorption experiments showed that this antibody was specific for heart muscle and failed to cross-react with skeletal muscle. Immunohistochemical studies revealed that the most prominent antigen to which the autoantibodies were directed was the heavy chain of alpha-cardiac myosin (Alvarez et al., 1987). A possible explanation for this autoimmune response is that upon infection with CB3 virus, cardiac myosin is released from myocytes following necrosis. This molecule is normally intracellular, highly insoluble and is associated with a tolerized immune response. However, following myocyte necrosis, processing and presentation of myosin antigens is upregulated. The myosin molecule is processed and presented in association with class II MHC on antigen presenting cells to CD4+ T helper cells, thus allowing the stimulation of a potential autoimmune response (Rose et al., 1996). In addition to autoantibody
production, there is also a marked cellular response to cardiac myosin. Clearly, T cells are important in breaking tolerance to self-antigens, and in the development of autoimmune disease (Smith & Allen, 1993; von Herrath & Oldstone, 1995).

Similar findings have been observed in the MCMV model, with autoantibodies of the IgG class being detected. Whilst these autoantibodies react with the contractile proteins troponin, tropomyosin and myosin, the dominant reactivity of late immune sera is to the heavy chain of cardiac myosin (O'Donoghue et al., 1990). T cells have been shown to be necessary for the development of myocarditis in both CB3 and MCMV-induced myocarditis (Lawson et al., 1989; Craighead et al., 1992; Henke et al., 1995), indicating that immunological mechanisms are necessary for the development of disease. Furthermore, autoreactive T cells (Huber, 1997) and autoantibodies (Lawson et al., 1992; Gaunt et al., 1995) induced by viral infection are capable of transferring myocarditis to recipient mice, suggesting an autoimmune disease process (Rose & Bona, 1993).

1.3.4 Cytokines

Whilst it is believed that myocytes are directly damaged by the cytotoxic effects of cytolytic immune cells, T cells may produce tissue injury via the production of cytokines. Studies carried out in CB3 and MCMV mouse models have shown that myocarditis is greatly enhanced when bacterial LPS is co-administered with the viral inoculum. Indeed, mice that are normally resistant to this disease develop myocarditis similar to that seen in the susceptible strains when they are treated with LPS (Lane et al., 1991; Lenzo et al., 2001b). Since a major action of LPS is the induction of various cytokines such as interferon γ (IFNγ) and TNF, it is a possibility that cytokine production induces or augments myocardial damage.

TNF triggers the release of practically all known mediators of inflammation such as cytokines and all metabolites of arachidonic acid, with final tissue pathology ranging from haemorrhagic necrosis to extensive local fibrosis. TNF can damage the host by inducing and enhancing, often in association with IFNγ, the production of reactive oxygen derivatives, such as nitric oxide (NO) (Ferrari, 1999). TNFα production can be
stimulated by IL-1, which is known to stimulate cytokine production from macrophages and play a role in lymphocyte activation.

TNF and IL-1, when administered to CB3-infected mice, results in a similar disease as that seen with LPS treatment of CB3-infected mice (Lane et al., 1993). In fact, the development of autoimmune myocarditis is associated with infiltration of the heart by inflammatory cells that secrete the cytokines TNF as well as IL-1. This local secretion contributes to the elevated levels observed in the serum and such a local production of TNF and IL-1 is believed to promote the induction of the autoimmune disease (Lane et al., 1993). Similarly, an increase in systemic TNF is observed during the acute phase of MCMV-induced myocarditis (Lenzo et al., 2001b). Such pro-inflammatory cytokines may cause the recruitment of additional lymphocytes to the heart tissue. The recruited inflammatory T cells, upon activation by the cytokine secreting monocytes, may in turn secrete IFNγ to augment cytokine synthesis by the monocytes, leading to a perpetual cycle of inflammation and autoimmunisation (Lane et al., 1993). TNF also plays an important role in host defence against virus infection. In studies carried out in TNF gene deficient mice, EMCV infection resulted in decreased survival rate when compared to normal mice (Wada et al., 2001). Clearly a delicate balance must be reached in order to prevent virus infection by TNF and to reduce the inflammation in the myocardium.

The cytokine IL-2 is predominantly associated with the activation of T cells. It has also been shown to play a role in the activation of B cells and NK cells. T cell activation is thought to play a crucial role in many viral infections and the subsequent inflammation that may follow. As myocarditis is associated with activated T cells and inflammation of the myocardium it is important to elucidate the role of IL-2 in this disease. In the myosin-induced myocarditis model, susceptible C3H, IL-2 knockout mice developed severe disease. However, in IL-2 gene knockout mice on a resistant C57BL/6 background, very little myocarditis was observed, leading to the conclusion that IL-2 has no essential role for the development of autoimmune heart disease (Grassl et al., 1997). The examination of IL-2 mRNA transcripts revealed that IL-2 was expressed in the hearts of rats only during the initial inflammatory phase of experimental autoimmune myocarditis. Levels decreased during the maximum inflammatory phase and disappeared by the recovery phase (Okura et al., 1997). These results suggest that Th1 cells produce IL-2, which may cause further T cell activation and subsequent
inflammation. Antibody blocking of the IL-2 receptor in the CB3 myocarditis model did not exert any effect on myocardial inflammation, again suggesting a limited role for IL-2 in this disease.

IL-4 is the prototypic Th2-type cytokine. It stimulates Th2 T cell development, activates B cells, induces MHC class II expression on B cells, promotes allergic reactions, induces Ig class switching to IgE and IgG1 in mice and recruits Th2 cells to the site of inflammation (Finkelman et al., 1990; Hickey et al., 1999; Nelms et al., 1999). A recent study examining experimental autoimmune myocarditis in A/J mice found that blocking IL-4 with an anti-IL-4 monoclonal antibody reduced the severity of myocarditis. This reduction in severity was associated with a shift from a Th2-like to a Th1-like phenotype (Afanasyeva et al., 2001). Controversy exists, however, extensive studies using other models of autoimmune disease have led to the conclusion that Th1 responses promote autoimmune processes, whereas Th2 responses may suppress them (Liblau et al., 1995; Mosmann & Sad, 1996). However, mounting evidence suggests that myocarditis is an autoimmune disease with a Th2-like phenotype in which IL-4 plays a major role.

IL-6 is a pleiotropic cytokine that is involved in a variety of biological activities, including the ability to stimulate B-cell differentiation, activate T cells to differentiate, activate macrophages and NK cells (Hirano et al., 1986; Lots et al., 1988; Mule et al., 1990; Luger et al., 1989). IL-6 is considered to be a major inducer of the acute phase response, promoting early adaptive immune responses in the thymus, spleen and heart muscle (Kanda et al., 1996). In addition, IL-6 has been shown to suppress inflammation in several animal models (Ulich et al., 1991; Barton & Jackson, 1993), an effect that is attributed to the inhibition of IL-1β and TNF production. IL-6 is reported to play a role in reducing TNF levels in vitro (Aderka et al., 1981) and in vivo (Schindler et al., 1990). A study examining the effect of IL-6 on EMCV-induced myocarditis showed that IL-6 reduces myocardial damage. Neutralising antibody to the virus was significantly increased in the IL-6-treated mice, most likely due to IL-6 being essential for the maturation of B cells to antibody forming cells (Kanda et al., 1996).

The concept that IL-10 acts as an anti-inflammatory molecule was suggested primarily by studies showing inhibition of the synthesis of a large range of pro-inflammatory
cytokines by different cells, particularly of the myocyte lineage (reviewed in Pretolani, 1999). IL-10 has been shown to inhibit the production of IL-1α, IL-1β, IL-3, IL-6, IL-8, TNF, G-CSF, GM-CSF and IL-18 (Moore et al., 1993; Goldman et al., 1997). These cytokines play a critical role in the activation of granulocytes, monocytes/macrophages, NK cells, T and B cells and in their recruitment to the sites of inflammation. IL-10 is therefore a critical cytokine in the orchestration of the inflammatory response. The role of IL-10 in myocarditis was examined in the EMCV mouse model of disease (Nishio et al., 1999). Recombinant IL-10 administered to infected mice on the day of virus infection was shown to suppress inflammation without altering virus replication in the acute phase of disease. The favourable effect of IL-10 treatment may be explained by several mechanisms. Firstly, IL-10 can suppress TNF release by macrophages and lymphocytes. Secondly, IL-10 can inhibit IL-2 production by lymphocytes. IL-2 has been shown to reduce myocardial damage if administered in the first week after infection with CB3. However, administration thereafter exacerbated the disease (Kishimoto et al., 1994). Finally, IL-10 can reduce NO production by inhibiting macrophage function (Cunha et al., 1992). In the EMCV model, NO production was enhanced by viral infection, and the inhibition of NO attenuated myocardial lesions (Wang et al., 1997). Studies in CB3 infected mice showed that CB3 causes activation of host IL-10 during the course of infection (Schmidtke et al., 2000). Whilst the inhibition of inflammation may be seen as advantageous in myocarditis this down regulation and delay in the proinflammatory cytokine response may have deleterious effects. The subsequent specific immune cell functions such as the antigen presenting function of macrophages and the development of Th1 cells may be impaired. By these means, the CB3 induced IL-10 may undermine the specific immune response and facilitate the spread of virus in the infected host that could lead to a persistent virus infection with ensuing chronic myocarditis.

IL-18 is a recently described member of the IL-1 cytokine family and was initially defined as an IFN-γ-inducing factor (Ushio et al., 1996). Subsequent studies have elucidated a broad array of effector functions beyond lymphocyte activation that implicate IL-18 as an important regulator of both innate and acquired immune responses (McInnes et al., 2000). IL-18 has been implicated in autoimmune inflammatory diseases because of its ability to modulate the activity of Th1/Th2 cells, B cells, NK cells,
macrophages and dendritic cells. It can promote the expression of TNF, GM-CSF and IFN-\(\gamma\) as well as upregulating inducible nitric oxide synthase (iNOS) (reviewed in McInnes et al., 2000). The possible role of IL-18 in inflammatory disease has led researchers to examine its role in myocarditis. A recent study examined the effects of exogenous administration of IL-18 on survival and myocarditis in mice infected with EMCV (Kanda et al., 2000). Treatment commencing with viral infection dramatically increased survival rates and reduced myocardial inflammation. However, beneficial effects were lost when IL-18 treatment commenced later in infection, indeed, survival rates decreased compared to saline controls when treatment commenced 5 days p.i. Clearly, IL-18 plays an important role in early viral clearance and establishing immune responses, however, detrimental effects are observed if IL-18 persists in infection.

NK cells have been shown to be present and indeed necessary for the development of myocarditis. IFN\(\gamma\), the major cytokine product of the Th1 cell population, is a strong activation signal for NK cells. IFN\(\gamma\) also induces the cell surface expression of class II MHC molecules on the myocytes, a characteristic finding in human myocarditis (Herskowitz et al., 1988). It is therefore possible that IFN\(\gamma\) contributes to myocardial damage. Recent research examined a model of CB3-induced myocarditis in which transgenic mice express IFN-\(\gamma\) in the pancreas, the primary site for CB3 replication (Horwitz et al., 2000). In this model, IFN-\(\gamma\) expressed solely in the pancreas effectively ablated the development of myocarditis following CB3 infection. The authors believe that protection was a direct consequence of reduced viral replication in the pancreas and systemic spread of CB3 to the heart. Clearly, IFN-\(\gamma\) has beneficial anti-viral effects early in infection, however, down stream effects of IFN-\(\gamma\) may prove detrimental in myocarditis.

IFN\(\gamma\) and TNF, which are produced during virus infection, transcriptionally activate iNOS. This form of NO synthase, which is not constitutively present, becomes expressed in macrophages and many other cell types via immunological stimuli which leads to the production of large amounts of NO for an extended period (Moncado & Higgs, 1993). NO itself is a signalling and effector molecule with diverse roles. It can defend the host against diverse pathogens, including bacteria, fungi, parasites and viruses (Nathan, 1995; DeGroote & Fang, 1995). However in recent years, NO has been
implicated in the pathogenesis of a wide variety of immunologically-mediated disorders such as septic shock, cardiac allograft rejection, diabetes, arthritis, autoimmune encephalomyelitis and chronic granulomatous colitis (Petros et al., 1991; Yang et al., 1994; Corbett et al., 1993; McCartney-Francis et al., 1993; Cross et al., 1994; Grisham et al., 1994). Accumulating evidence suggests that it is possible that the excessive production of NO by iNOS is responsible for the development of myocardial lesions as well as for functional changes in myocardial contractility during the active phase of disease. However, some studies point to a protective role for NO and iNOS, indicating a worsening in myocarditis upon inhibition of NOS.

iNOS is induced in mice infected with CB3 virus and macrophages expressing iNOS have been detected in the hearts and spleens. Such infected mice have increased titres of virus and a higher mortality when fed NOS inhibitors (Lowenstein et al., 1996). Mice with a disrupted gene for NOS, hence lacking NOS, clear CB3 virus more slowly than wild-type parental mouse strains, resulting in more severe myocarditis (Zaragoza et al., 1998). Inhibition of iNOS has also been shown to reduce the severity of myocarditis in the rat experimental autoimmune myocarditis model (Hirono et al., 1997). Whilst evidence points to an important role for NO and iNOS in viral myocarditis, conflicting reports indicate that continuing research is required to further elucidate this mechanism.

1.3.5 Complement

The innate immune response is not only important in providing rapid host defence, but may also influence the subsequent development of the adaptive immune response. In addition to the previously discussed early acting cytokines and NK cells, complement plays an important role in the innate immune response to virus infection. The activation and subsequent binding to complement receptors can initiate and regulate the adaptive immune response via antigen trapping, B cell activation and Ig class switching (reviewed in Carroll, 1998). Complement has recently been shown to be critical for the induction of experimental autoimmune myocarditis (Kaya et al., 2001). Depletion of C3 in the induction of disease reduced myocarditis, however such depletion later in disease was not as effective. Decreased myocarditis was accompanied by a decrease in the proinflammatory cytokines TNF and IL-1 and total IgG. Blocking the complement receptors CR1 and CR2 also reduced myocarditis together with a decrease in TNF, IL-1 and total IgG. Clearly, complement plays a critical role in innate immunity and may
influence the severity of myocarditis by affecting both the production of autoantibodies to cardiac myosin and proinflammatory cytokines.

1.3.6 Molecular mimicry

Autoimmunity plays a major role in myocarditis with many researchers believing that the release of cardiac myosin due to viral damage causes an immune response against the heart tissue. A second, or contributing mechanism could be antigenic mimicry between the infectious agent and heart proteins. In this case, the immune response to the infectious agent also recognises normally expressed tissue antigens.

Evidence of molecular mimicry in viral myocarditis is mounting. A three-way cross reactivity has been observed between heart antigens, CB3 and group A streptococcus M5 (Cunningham et al., 1992), and neutralising monoclonal antibodies to CB3 which cross-react with cardiac myosin have been found to be pathogenic in mice (Gauntt et al., 1995). Various viral peptides have been used in this animal model as vaccines in an attempt to prevent the development of autoimmune myocarditis following viral infection (Huber et al., 1993). Preimmunisation with the various peptides either resulted in partial protection from viral induced myocarditis or exacerbation of the severity of myocarditis when compared to mice that were not immunised. This result highlights the importance of cross-reactive immune responses to infectious agents and cardiac myosin in the development of heart disease.

Mimicry may also be an important mechanism in MCMV-induced myocarditis. In this model it was found that some neutralising monoclonal antibodies (IgG2b subclass) directed against structural determinants of MCMV react with cardiac myosin (Lawson et al., 1991). This suggests that there is possible mimicry between the virus and cardiac myosin in the genesis of myocarditis following MCMV infection. Analysis of sequence homologies between cardiac myosin and MCMV have revealed a region of six out of nine amino acids in the large tegument protein M48 of MCMV and murine cardiac myosin heavy chain (residues 869-877; Fig. 1.3) (Lawson, 2000). This peptide sequence is identical in the rat and human CMV large tegument protein and in the rat and human cardiac myosin molecules with predicted high α helix amphiphilicity (Lawson, 2000). Further work must be performed in this disease model before the antigenic determinant is classified as a proven mimic in the induction of autoimmunity. By gaining insights
MCMV M48

Cardiac myosin heavy chain

(α, 871-879)

Figure 1.3 Sequence alignment of viral mimic.
into the nature of mimics we can better understand the pathogenesis and mechanisms of myocarditis.

1.4 THERAPY

Despite considerable advances in medicine and a greater understanding of myocarditis, treatment for this disease remains primarily supportive. The therapy regime is focused on the myocardial dysfunction and includes such measures as oxygenation and the administration of inotropic agents to increase cardiac output. Clearly, a treatment for the myocardial inflammation is required and this currently forms the focus of intense research.

Several types of agents have been trialed for therapeutic efficacy in myocarditis, including anti-inflammatory drugs, non-steroid anti-inflammatory drugs, immunosuppression agents, immunomodulatory agents and antiviral drugs. Each has shown promising yet limited efficacy. Researchers have examined the effect of corticosteroids on the inflammation of the myocardium with results remaining unclear. A single injection of cortisone in the early stage of infection with CB3 virus increases both the severity of myocardial damage and the incidence of lethal disease in mice (Kilbourne et al., 1956). Indeed, it is widely accepted that cortisone enhances the susceptibility of many animals to viral infection and commonly potentiates the severity of the disease. Nonetheless, there are several clinical reports of the successful use of corticosteroids (Garrison & Swisher, 1953; Ainger, 1964; Voigt, 1968). Data suggests that the timing of corticosteroid administration in the disease course is an important factor in determining the efficacy. Corticosteroids given in the early stage aggravate the course of acute viral myocarditis, but they may not have detrimental effects if given when neutralising antibody titre levels against virus is high, although they are not expected to have a beneficial effect (Tomioka et al., 1986).

Nonsteroid anti-inflammatory drugs are used to treat arthralgia and myalgia that may accompany viral infection. Their main mechanism of action is to block cyclooxygenase and lipoxygenase activity and the subsequent formation of the inflammatory mediators prostaglandin E2 and thromboxane A2. One such drug is ibuprofen. When CB3 infected BALB/c mice are treated with ibuprofen, myocardial inflammation and necrosis in the acute stage of disease worsened, without affecting viral titres (Costanzo-
Nordin et al., 1985). This seemingly contradictory result was possibly due to the immunomodulating properties of ibuprofen on prostaglandin-sensitive mononuclear cells.

Data from experiments with animals and humans suggest that corticosteroids have an immunosuppressive effect and hence have been used in transplantation recipients to reduce rejection rates (Strom, 1984). As viral myocarditis is believed to develop into an autoimmune disease, several research groups have trialed corticosteroid therapy in an attempt to reduce the immunopathological damage to the myocardium (reviewed in Maisch et al., 1993). Corticosteroid therapy has proven to be of limited benefit and is restricted to autoreactive chronic forms of clinical myocarditis, when enteroviral or CMV persistence is excluded.

Cyclosporin A, a fungal metabolite with immunosuppressive properties has made rejection after heart transplant manageable. Therefore, cyclosporin A was thought to be suitable for the treatment of autoreactive forms of myocarditis when an associated defective T cell suppressor function is present (Maisch et al., 1993). In the EMCV virus myocarditis model, cyclosporin A, when given early, increased mortality significantly, and later administration left mortality unchanged with a tendency towards deterioration (Matsumori & Kawai, 1989).

Modulating the immune system to reduce damage to the myocardium has been extensively studied by several groups. Matsumori et al., (1991) investigated the effect of anti-IL2 receptor monoclonal antibodies in the EMCV model. It was believed that blocking acquisition of membrane receptors for IL2, which would prevent T cell activation, should reduce inflammation. However, there was no observed decrease in inflammation or mortality.

IL-2, a cytokine released from activated T cells, in particular Th1 T cells, plays an important role in the immune system by inducing differentiation and proliferation of T cells and B cells, the proliferation of NK cells and the induction of antigen-specific killer cells. IL-2 has been reported to exert an antiviral effect (Siegel et al., 1985), and therefore has been examined for therapeutic efficacy in viral myocarditis models. A beneficial effect could not be demonstrated in the EMCV model (Matsumori & Kawai,
1982; Matsumori et al., 1987; Matsumori et al., 1988). IL-2 has been shown to worsen myocarditis when administered along with a variant of CB3 that normally produces minimal inflammation (Huber et al., 1994). Whilst IL-2 shows antiviral properties, its role in the Th1 immune response and the immunopathological nature of viral myocarditis render it an unsuitable therapeutic tool.

Mice treated with LPS and infected with CB3 or MCMV show significantly higher levels of myocarditis and elevated levels of TNF (Lane et al., 1992; Lenzo et al., 2001b). Indeed, exogenous administration of TNF together with CB3 in susceptible mice causes exacerbation of myocarditis (Lane et al., 1993). This evidence suggests that blocking TNF may be of therapeutic value. In the EMCV model of myocarditis, when monoclonal antibodies to TNF are administered one day before infection there is a significant reduction in myocardium inflammation and death rates, however, no therapeutic effect was observed when treatment commenced with viral infection (Yamada et al., 1994).

Several other cytokines have been examined for therapeutic efficacy, including IL-6, IL-10, IL-18 and IFNα. Treatment of EMCV infected mice with IL-6 reduced myocardial inflammation only when administration occurred at the time of virus infection (Kanda et al., 1996). Exogenous administration of IL-10 improves survival and reduces myocardial lesions in the EMCV model (Nishio et al., 1999). IL-18 treatment in the same model of disease again showed that a reduction in myocarditis was only observed when the cytokine was administered at the time of virus infection. IL-18 administered at any time after infection had no effect (Kanda et al., 2000). IFNα has also been proven effective in the EMCV model when given in large amounts (Kishimoto et al., 1987). Recent work has shown that low dose oral IFN (α/β mix) when treatment commences one week before MCMV infection in BALB/c mice, significantly reduces the level of myocarditis in the acute stage (Bosio et al., 1999). The individual subtypes of IFNα are also proving to be therapeutic when administered using naked DNA delivery. Lawson and colleagues have developed a mouse experimental model in which the tibialis anterior muscles are transfected in situ with naked DNA plasmids encoding for an IFN transgene (Lawson et al., 1997). Each subtype reduces MCMV-induced myocarditis, with IFN α6 subtype being the most effective when
treatment commences two weeks before infection (Cull V., Bartlett E. & Lawson C.M., unpublished observations).

Drugs that affect the physiology of the heart have been extensively studied for their therapeutic value. For example, alpha-adrenergic blockers such as bunazosin have vasodilating properties and have been shown to have a protective effect when administered at the time of infection and continued for 2 weeks (Yamada et al., 1992). Calcium antagonists such as verapamil have also been studied showing a beneficial effect in EMCV infected mice (Dong et al., 1992). Captopril is an angiotensin-converting enzyme inhibitor which has been shown to be beneficial in treating viral-induced myocarditis. When mice infected with CB3 were exposed to excessive exertion, such as being forced to swim, they had worsened prognosis. Captopril reduces oxygen demand both preload and afterload. Mice infected with CB3 showed significantly less inflammation and necrosis when treated with captopril early in infection (days 1-6), but captopril had no effect on inflammation and necrosis when treatment commenced later (Rezkalla et al., 1990).

Antiviral therapy has shown some promise in the treatment of myocarditis, given that a virus is responsible for triggering the disease. The antiviral agent ribavirin has been trialed in the CB3 myocarditis model. Early administration resulted in inhibition of viral replication, a reduction in myocardial damage during the acute phase of disease and increased survival. However, when treatment commenced on day 4 of the infection, myocardial damage was reduced, but there was no survival benefit (Kishimoto et al., 1988).

Clearly, timing of administration is critical in antiviral therapy. If virus replication can be halted at a very early stage, myocardial damage can be minimised. Antiviral therapy may be most beneficial when patients are at risk of exposure to myocarditis causing viruses, such as CMV from organ transplantation.
1.5 INTRODUCTION TO THESIS

Studies in the MCMV-induced model of myocarditis have implicated several mechanisms by which myocardial damage may occur. Virus is the trigger for disease with the viral strain being important for myocarditis development. Given the importance of virus, antiviral drugs were trialed in this thesis for therapeutic efficacy. The immune system also plays an important role, with T cells and NK cells being critical for disease development. Autoantibodies against cardiac myosin are produced following infection and most likely contribute to tissue damage. The inflammatory infiltrate has been well characterised in other models of myocarditis, but cell types present in MCMV-induced myocarditis remain unknown. Thus, cell types present in the inflammatory infiltrate were identified, together with attempts to identify the cause of myocardial damage. Non specific immune stimulation (via LPS) was investigated in order to further define the role of individual immune mechanisms in disease pathogenesis. Finally, the role of cytokine response was examined in the hearts of resistant and susceptible mice in order to identify differences and thus the importance of any given cytokine. Therefore, the aim of this thesis was to further characterise MCMV-induced myocarditis, to examine the efficacy of antiviral therapy and to further elucidate the role of the immune system in cardiac disease progression. The hypothesis addressed in this thesis is that MCMV-induced myocarditis is triggered by virus and as such, can be effectively treated with antiviral drugs, however, the subsequent development of chronic myocarditis is mediated by immune factors such as autoantibodies, T cells, phagocytic cells and cytokines.
CHAPTER 2

Materials and Methods
2.1 Mice
Six to eight week old specific pathogen-free female and male inbred BALB/cJ and C57BL/6J mice were supplied by the Animal Resources Centre (Murdoch, Western Australia). Mice were maintained under minimal disease conditions at the Department of Microbiology at the University of Western Australia (UWA), or at the Division of Veterinary and Biomedical Science, Murdoch University. Mice were known to be free of MCMV and other infections by routine serological screening. Such serological monitoring continued for long term experiments to ensure that mice remained free from pathogens other than those introduced. All animal experimentation was given prior approval by the Animal Ethics Committee at respective Universities which complies with the guidelines from the National Health and Medical Research Council, Australia.

2.2 Immunisation of mice
Mice were inoculated by the intraperitoneal (i.p.) route with 0.1 millilitre (ml) of either virus, anti-viral drug, LPS (Salmonella minnesota, Sigma RC595, St. Louis MO, USA) or TNF peptide (amino acids 144-130, ICN Biochemicals, Costa Mesa, CA, USA) diluted in sterile non-pyrogenic saline. K181 and the wild isolate G4 of MCMV were diluted in saline to obtain a concentration of 1x10^4 PFU of virus per 0.1 ml before inoculation of a mouse (approximately 0.3 LD50).

2.3 Virus
The K181 (Perth) laboratory strain of MCMV used in these studies was originally obtained from Dr. D.J. Lang (Duke University, Durham NC, USA). Although the virus was designated as the Smith strain of MCMV, it was subsequently found to have an identical digestion profile by restriction enzyme analysis to that of K181 strain (Hudson et al., 1988). The K181 (Perth) strain of MCMV used in the experiments described in this thesis had been passaged several times in the salivary gland of weanling female BALB/c mice since its arrival in 1977 at the Department of Microbiology (UWA) (Chalmer et al., 1977).

A further six different replication-competent strains of MCMV were obtained from the salivary glands of MCMV-seropositive wild mice trapped in various locations of Australia, as described in Booth et al. (1993). The MCMV strains from wild mice were designated Nannup (N), Kerguelen (K) and Geraldton (G) according to their trapping
site. Several strains were examined in a previous study for their ability to induce myocarditis (Fairweather et al., 1998). Strain G4, showing the highest levels of myocarditis in BALB/c mice, was prepared from brain homogenates (10-12 days p.i.) of newborn BALB/c mice inoculated intracerebrally with wild mouse salivary gland homogenates. The brain homogenates were plaque-purified twice on MEFs under conditions of limiting dilution to avoid multicapsid virion formation. The G4 strain of MCMV was used as a genetically different virus strain from K181 in these studies.

Virulent MCMV used for mouse inoculations and in vitro studies was prepared by salivary gland passage of laboratory and wild isolate MCMV in 3 week old female BALB/c mice, as previously described (Allan & Shellam, 1984; Lawson et al., 1987). Briefly, salivary glands were harvested 17 days p.i. and homogenised (Ultra Turrax electric homogeniser, Crown Scientific, Australia) in Eagles Minimal Essential Medium (MEM) to yield a 20% weight per volume (w/v) homogenate. The suspension was clarified by centrifugation at 1800 g for 20 minutes at 4°C and the supernatant stored in the gas phase of liquid nitrogen. Each batch was titrated by plaque assay on MEFs for quantitative determination of virus titre, PFU/ml.

To provide a source of viral antigen for enzyme linked immunosorbant assays (ELISA), K181 MCMV was propagated in MEFs as described by Lawson et al. (1988). Briefly, salivary gland virus stocks were used to infect confluent MEFs for one hour before removal of inoculum and replacement with fresh MEM plus 2% foetal calf serum (FCS). Supernatant, containing virus, was collected after the observed cytopathic effect was very advanced (3-4 days p.i.) and cell debris was removed by ultracentrifugation at 10 000 g for 35 minutes at 4°C. The supernatant containing the viral antigen was stored at −70°C until used in the ELISA.

2.4 Antiviral drugs

Ganciclovir (9-[1,3-dihydroxy-2-propoxymethyl]guanine), GCV; Roche Products, NSW, Australia (pharmaceutical grade), Cidofovir (1-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine dihydrate); Pharmacia & UpJohn, NSW, Australia (pharmaceutical grade) and Foscarnet (phosphonoformic acid), PFA; Sigma, St. Louis MO, USA (analytical grade) were trialed. Administration of these drugs dissolved in pyrogen-free saline was given to mice via the i.p. route.
2.5 Cell culture media and buffers

MEM (Gibco BRL, Life Technologies Inc., Grand Island NY, USA) was prepared according to the manufacturer’s instructions using double distilled water (ddH2O) and supplemented with gentamycin (160 micrograms (µg)/ml; Delta West Pty. Ltd. Bentley, Western Australia). The medium was adjusted to pH 7.2 and sterilised by membrane filtration. All media were checked for sterility by adding a sample of filtered media to tryptone soya broth and incubating at 37°C overnight. Rosewell Park Memorial Institute medium 1640 (RPMI; Gibco BRL, Life Technologies Inc., Grand Island NY, USA) and Dulbecco’s Modified Eagles Medium (DMEM; Gibco BRL, Life Technologies Inc., Grand Island NY, USA) were prepared according to the manufacturer’s instructions and adjusted to pH 7.2, as described above.

FCS (CSL, Melbourne Australia) was selected in batches that had minimum toxicity against lymphocyte cultures. Normal goat serum (NGS) was obtained from the Animal Resources Centre (Murdoch, Western Australia) for use in immunohistochemistry.

Phosphate buffered saline (PBS) was prepared with 0.6 M Na2HPO4, 0.4 M NaH2PO4, 0.15 M NaCl (pH 7.2).

Trypsin/EDTA solution was prepared by dissolving trypsin/EDTA (Gibco BRL) in PBS to give a final concentration of 5% (w/v). A working concentration of 2.5 mg/ml was used for routine tissue culture.

Methyl cellulose (3.5%) contained equal parts of autoclaved methyl cellulose (4000 centipoises, Fisher Scientific Co., Fairlawn HJ, USA) and double strength MEM/RPMI. The final mixture was supplemented with 2% FCS.

Methylene blue staining solution contained 1% methylene blue and 10% formalin made in ddH2O.

TBE (tris/boric acid/EDTA) buffer (10x) stock used for RT-PCR gel electrophoresis consisted of 0.9 M Tris Base, 0.89 M boric acid and 40 ml of 0.5 M EDTA pH 8.0 made up to 1 L with ddH2O.
Bouin's fixative contained 1500 ml saturated aqueous picric acid, 500 ml formaldehyde and 100 ml glacial acetic acid. Tissues were left overnight in Bouin's fixative, rinsed with 70% ethanol and remained in 70% ethanol until processed.

Carbonate-bicarbonate buffer was prepared with 0.015 M Na₂CO₃ and 0.035 M NaHCO₃ (pH 9.5).

Diethanolamine buffer (10%) was prepared by adding 9.7 ml of diethanolamine to 78 ml ddH₂O with the addition of 0.3 mM NaN₃ and 0.05 mM MgCl₂.6H₂O (pH 9.8).

2 x HEPES buffered saline was prepared for in vitro culture of murine myocytes with 0.03 M NaCl, 0.01 mM KCl, 0.16 mM Na₂HPO₄.2H₂O, 1.1 mM glucose and 4.2 mM HEPES. Solution was adjusted to pH 7.05 with NaOH.

2.6 Determination of viable cells by trypan blue exclusion
The viability of cells in culture was determined by diluting cells in a 0.5% (w/v) solution of trypan blue in PBS. Viable cells were counted in an improved Neubauer counting chamber by trypan blue exclusion and cell concentrations calculated.

2.7 Mycoplasma
A sample of media and cell cultures, grown in media without gentamycin, were routinely screened for mycoplasma contamination by PCR (PathCentre, Western Australia).

2.8 Tissue homogenisation
Organs used for plaque assays were first weighed and then homogenised with MEM or RPMI containing 2% FCS, to yield a 20% (w/v) homogenate. Homogenates were clarified by centrifugation at 1800 g for 20 minutes and stored at -70°C until used for virus titre determination by plaque assay.
2.9 Plaque assay

A plaque assay was performed in order to detect the titre of infectious MCMV in the organs of infected mice, as previously described (Chalmer et al., 1977). Initially, MEFs were used for the plaque assay, prepared by trypsin digestion of 15 to 17 day old BALB/c embryos. The single cell suspension was seeded into Wheaton roller bottles (USA) with MEM and 10% FCS and incubated for 24 hours at 37°C in the presence of 5% CO₂. Fresh medium was added the following day and confluent monolayers were trypsinized to yield a single cell suspension and stored in the gas phase of liquid nitrogen in MEM with 20% FCS and 5% dimethyl sulphoxide (DMSO). All propagation and titration of MCMV was later performed in the continuous cell line M2-10B4 (ATCC Number CRL-1972), a clone derived from bone marrow stromal cells from a (C57BL/6J x C3H/HeJ)F1 mouse. This cell line was reported to be superior for virus propagation and titration of MCMV (Lutarewych et al., 1997). The cells were grown in RPMI 1640 medium with 10% FCS and stored in the gas phase of liquid nitrogen in RPMI with 20% FCS and 5% DMSO.

Cells were seeded into 24-well tissue culture trays (Falcon) at 2 x 10⁵ cells per well and grown until 90% confluent in order to perform plaque assays. Monolayers were washed with fresh media and then serial four-fold dilutions of organ homogenates (200 µl/well) were added. After incubation for 1 hour at 37°C in 5% CO₂, virus homogenates were removed and replaced with 1 ml of methyl cellulose overlay. Infected cultures were subsequently incubated for 4 days at 37°C in 5% CO₂ and then stained with methylene blue staining solution. As positive controls, a standard MCMV stock of known titre as well as spleen and liver homogenates from mice infected for 3 days with MCMV were processed in parallel with test samples. Negative controls used in the plaque assay consisted of salivary gland homogenates from uninfected mice.

2.10 Plaque reduction assay

The sensitivity of the K181 and G4 strains of MCMV to anti-viral drugs was assessed by a plaque reduction assay using MEF monolayers as described in Li et al. (1995). Monolayers were washed with fresh media and 100 PFU of virus (200 µl) was applied to each well. After incubation for 1 hour at 37°C in 5% CO₂, virus inoculum was removed and overlayed with medium containing 1% methyl cellulose and various
concentrations of an antiviral drug, and were further incubated for three days. Monolayers were then stained with methylene blue staining solution and plaques counted under light microscopy. The 50% inhibitory concentration (IC50) corresponded to the dose of drug achieving 50% plaque reduction.

2.11 In vitro culture of murine myocytes
Myocytes were isolated and cultured from 2 day old neonatal BALB/c mice. Hearts were removed and minced with scissors in HEPES buffered saline containing 0.5 mg/ml collagenase II (Gibco BRL) and 0.1% trypsin (Gibco BRL). The tissue suspension was then incubated for 10 minutes at 37°C with gentle shaking. The tissue was pelleted by centrifugation (50 g for 5 minutes) and the supernatant put into an equal volume of DMEM (Gibco BRL) containing 20% FCS. This process was repeated three times. The collected medium was spun at 200 g for 10 minutes at 4°C, and the pellet resuspended in DMEM containing 10% FCS. Adherent cells were removed by plating cell suspension in a culture flask for two hours at 37°C and collecting the nonadherent cells. Cells were counted and plated onto laminin coated plasticware at a concentration of 1 x 10^5 cells/cm^2 in DMEM containing 0.1 mM bromodeoxyuridine (BrDU; Sigma).

2.12 Isolation of heart inflammatory cells
Inflammatory cells were obtained from the hearts of ten BALB/c mice 7 days after treatment with MCMV and LPS, or MCMV alone. The hearts were removed and placed into RPMI 1640 (Gibco BRL, Life Technologies Inc., Grand Island NY, USA), containing 0.5 mg/ml Collagenase Type II (Gibco BRL) and 0.1% trypsin (Gibco BRL) and minced with scissors under sterile conditions. The tissue was then incubated for 1 hour at 37°C with gentle rocking. The tissue suspension was centrifuged (50 g for 5 min), and the supernatant then layered onto Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Sweden) for inflammatory cell recovery. Inflammatory cells were placed into 24-well tissue culture plates (2x10^5/well) resuspended in 200 µl of RPMI 1640 containing 5% FCS and cultured for 48 hours at 37°C, 5% CO2. The contents of the tissue culture wells were centrifuged and the resultant supernatant was tested in the ELISA for cytokine quantitation. For comparison, tissue culture supernatants of splenic mononuclear cells from the same mice were similarly processed and examined.
2.13 **MTT assay**

Cells to be tested were cultured in round bottomed 96 well plates at $2 \times 10^3$ cells/well in medium containing 10% FCS. Cells were overlayed with media containing $1 \times 10^2$ PFU of MCMV per cell (MOI = 100) and incubated at 37°C for 1 hour. Virus inoculum was removed and replaced with culture medium. At the given time point 10 μl of 5 mg/ml MTT (Sigma) in PBS was added per well and the cells incubated at 37°C for 4 hours. 200 μl of acidified isopropanol was added per well to dissolve MTT precipitation and the absorbance read at 590 nm.

2.14 **Histology**

Whole hearts were fixed overnight in Bouin's fixative, transected along the midline and transferred to 70% ethanol before being processed into paraffin blocks by the Department of Pathology (UWA) and Histology (Murdoch University). Hearts used for staining immune cell subsets were first sectioned along the midline, embedded in O.C.T. Compound (Sakura Finetek, Torrance CA, USA) and then snap frozen in liquid nitrogen. Sections were stained with haematoxylin and eosin (H&E) for histopathological studies and/or cut for immunohistochemistry by the Department of Pathology (UWA) or Histology (Murdoch University).

A classification of myocarditis, which includes pericarditis and endocarditis, was based upon the Dallas Criteria definition of inflammation associated with necrosis of myocytes (Aretz, 1987). Myocarditis was scored as the average number of foci ± standard error (SE) from the hearts of individual mice for each time point. The number of foci was determined from examination of eight fields of view at x160 magnification per mouse (representing the average size of a mouse heart section), and included focal and dispersed inflammation. In order to ensure impartiality, sections were evaluated blindly.

2.15 **Immunohistochemistry**

Frozen sections of mouse heart were stained for immune cell subsets. Briefly, frozen sections were dessicated for 1 hour at 37°C and fixed with cold (-20°C) acetone for 10 minutes. Endogenous peroxidase was blocked with 1% hydrogen peroxide and 10% normal goat serum (NGS). Sections were then incubated with rat IgG or hamster IgG
monoclonal antibodies to various immune cell surface markers (Pharmingen, USA) (Table 2.1). Horseradish peroxidase conjugated anti-rat IgG (Santa Cruz) or Extavidin Peroxidase (Sigma) was applied to the sections before development with AEC (3-amino-9-ethylcarbazole) (Sigma). Frozen sections were counter stained with Mayer’s haematoxylin (Lillie’s modification) (Dako, Botany, Australia), rendering the cellular morphology and nuclei blue. Positive staining is visualised as a red staining. Slides were mounted with Faramount aqueous mounting medium (Dako, Botany, Australia) and viewed under light microscopy.

2.16 Detection of apoptosis

Apoptotic cells were visualised in frozen heart sections using the CardioTACS in situ apoptosis detection kit (R&D Systems, Minneapolis, USA). The kit utilises the TdT-mediated dUTP nick end labelling (TUNEL) assay and is optimised for cardiac derived samples. Samples were processed as per the manufacturer’s instructions. Briefly, frozen sections of cardiac tissue were fixed to prevent the loss of low molecular weight DNA fragments. DNA was made accessible to the labelling enzyme by permeabilizing the cell membrane with Cytonin reagent. Endogenous peroxidase activity was quenched using hydrogen peroxide. Biotinylated nucleotides are added to the 3’-ends of the DNA fragments by Terminal deoxynucleotidyl Transferase (TdT). A steptavidin conjugated horseradish peroxidase specifically binds to the biotinylated DNA fragments and generates a blue precipitation in the presence of substrate. To discriminate apoptotic cells from necrotic cells, the samples were counterstained to assist in the morphological verification of apoptosis. Heavy condensation of nuclear chromatin is indicative of apoptotic nuclei as compared with the dispersed chromatin of normal cells. Positive blue staining of the cytoplasm as well as the nucleus observed in enlarged or swollen cells in indicative of necrosis.

2.17 Sera

Blood samples of approximately 0.5-1.0 ml were obtained by bleeding from the retro-orbital venus plexus of anaesthetised mice. Blood was allowed to clot for 1 hour at 4°C and then spun at 2500 g for 10 minutes. Individual serum was collected and stored at −20°C until used. Normal mouse serum (NMS) was pooled from ten uninfected adult BALB/c mice.
### Table 2.1 Antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cell Type</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8b.2 (Ly-3.2) Rat anti-mouse Monoclonal</td>
<td>CD8^+ T cell</td>
<td>1:200</td>
<td>Anti rat horseradish peroxidase (1:300)</td>
<td>Ledbetter &amp; Herzenberg, (1979)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ledbetter et al., (1980)</td>
</tr>
<tr>
<td>CD4 (L3T4) Rat anti-mouse Monoclonal</td>
<td>CD4^+ T cell</td>
<td>1:300</td>
<td>Anti rat horseradish peroxidase (1:300)</td>
<td>Nitta et al., (1997)</td>
</tr>
<tr>
<td>CD45R/220 Rat anti-mouse Monoclonal</td>
<td>B cell</td>
<td>1:300</td>
<td>Anti rat horseradish peroxidase (1:300)</td>
<td>Hathcock et al., (1992)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Coffman, (1982)</td>
</tr>
<tr>
<td>Mac-3 Rat anti-mouse Monoclonal</td>
<td>Macrophage</td>
<td>1:300</td>
<td>Anti rat horseradish peroxidase (1:300)</td>
<td>Flotte et al., (1983)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ho &amp; Springer, (1983)</td>
</tr>
<tr>
<td>Hamster anti-mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat anti-mouse</td>
<td></td>
<td></td>
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<tr>
<td>Ly-6G (Gr-1) Biotin-conjugated Rat anti-mouse</td>
<td>Neutrophil</td>
<td>1:300</td>
<td>Extravidin-peroxidase (Sigma) (1:300)</td>
<td>Lewinsohn et al., (1987)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Lagasse &amp; Weissman, (1996)</td>
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</tbody>
</table>
MCMV hyperimmune serum (HIS) was pooled from BALB/c mice following multiple MCMV infections i.p. as described previously (Bartholomaeus et al., 1988). Briefly, ten adult BALB/c mice were infected with $1 \times 10^3$ PFU of MCMV. Mice received an additional $1 \times 10^4$ PFU of MCMV 2 weeks and 3 weeks after the initial infection. Sera were collected ten days following the final virus infection.

2.18 Absorption of sera
Myosin (cardiac and skeletal) protein (see 2.19 for details of preparation) and bovine serum albumin (BSA) preparations were coupled to CNBr-activated Sepharose 4B (Pharmacia, Sweden) at a concentration of 1.5 mg protein/ml. Absorption of sera was performed as previously described (O'Donoghue et al., 1990). Briefly, the pooled sera (5 mice/group) were incubated with 5 volumes of the protein-coupled Sepharose. After continuous mixing for 18 hours at room temperature, the beads were centrifuged at 100 g for 5 minutes and the absorbed sera saved. Myosin-reactive antibodies were eluted from the beads by incubation for 15 minutes at 4°C with one resin volume of 0.05 M acetate buffer, pH 2.8. The eluted antibodies were dialysed against PBS at 4°C overnight.

2.19 Myosin
Purified cardiac myosin used as antigen source in the ELISA and also used for absorption studies (2.18) was isolated from thirty uninfected BALB/c hearts according to the method of Kodama et al. (1990). All steps of myosin preparation were conducted at 4°C. Briefly, minced heart tissue was washed in 4 volumes (w/v) of 0.05 M potassium phosphate buffer (pH 6.8) containing 10 mM pyrophosphate and 2 mM 2-β-mercaptoethanol and gently stirred for 2 hours in 0.3 M KCl, 0.1 M KH₂PO₄, 0.05 M K₂HPO₄, 10 mM pyrophosphate and 2 mM 2-β-mercaptoethanol. The mixture was centrifuged for 30 minutes at 10 000 g. Lipids were removed by filtering the supernatant through 4 layers of gauze and precipitated with cold 1 mM EDTA, with vigorous stirring. After centrifugation for 10 minutes at 10 000 g, the pellet was dissolved in 0.025 M Tris-HCl buffer (pH 7.5) containing 0.3 M KCl and 1 mM MgCl₂. 2 mM adenine triphosphate (ATP) was used to separate actin from myosin and the mixture centrifuged at 80 000 g for 3 hours to pellet the actin. The supernatant was further purified by precipitating actin and other proteins in a 36% fraction of saturated
ammonium sulphate solution. After centrifugation at 10 000 g, the supernatant containing the myosin was precipitated with 45% saturated ammonium sulphate solution. Skeletal myosin was similarly isolated from BALB/c mice using the same method described above for cardiac myosin.

The concentration of myosin was calculated by protein assay using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond CA, USA), based on a method developed by Bradford (1976) where protein-dye binding is measured by a shift in absorbance at 595 nm. BSA (Fraction V, Sigma, St. Louis MO, USA) protein preparation (100 μg/ml) was used as a standard.

2.20 Detection of antibody and cytokine by ELISA

Reaction of serum antibodies with either MCMV antigen or purified cardiac myosin was quantitated by ELISA, as described previously (Lawson et al., 1988; O'Donoghue et al., 1990). ELISA microtitration plates (Nalge Nunc International, Denmark) were coated with MCMV antigen diluted in carbonate-bicarbonate buffer, or myosin diluted in carbonate-bicarbonate buffer with the addition of 50 mM tetrasodium pyrophosphate in order to solubilise the myosin. MCMV or myosin coated plates were left overnight at 4°C. Plates were then washed with PBS containing 0.1% BSA (Fraction V, Sigma, St. Louis MO, USA) and 0.05% Tween 20 (Sigma, St. Louis MO, USA). Primary antibodies were diluted in PBS with 1% BSA and 0.05% Tween 20 and incubated for 2 hours at 37°C. Total IgM and IgG antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse γ-chain specific immunoglobulins (Tago Inc., Burlingame CA, USA), diluted as for primary antibodies. Sera samples underwent a series of two-fold dilutions in order to obtain an average antibody titre ± SE. Positive reactions were read at an OD of 405 nm after the addition of phosphatase substrate buffer tablets (Sigma 104, St. Louis MO, USA) dissolved in 10% diethanolamine buffer.

Antibody titre endpoints are expressed as the reciprocal of the highest serum dilution having an OD greater than that of the negative control serum plus three standard errors. A commercial pan-myosin mAb (RPN 1169, Amersham, UK) diluted 1/50, reacting with the 200 kDa heavy chain of cardiac myosin was used as a positive control for myosin; whereas HIS diluted 1/200 served as the positive control for MCMV.
Cytokines were measured in sera or tissue culture supernatant using commercially available ELISA kits (Pharmingen, San Diego, USA).

2.21 RNA extraction
Total cellular RNA was extracted from snap frozen hearts. Frozen hearts were placed in TRIzol Reagent (GibcoBRL) (10 mg of tissue/500 µl reagent) and homogenised. Samples were treated with chloroform and the RNA precipitated with isopropanol and washed twice in 70% ethanol before resuspension. The RNA pellet was dissolved in 20 µl of H2O (irrigation H2O, pH 7; Baxter Healthcare Pty. Ltd., NSW, Australia), electrophoresed in 2% agarose/TBE gels and stained with ethidium bromide to check for integrity of RNA. RNA concentration was determined by optical density readings at 260 nm.

2.22 DNase treatment of RNA
RNA samples were treated with DNase in order to remove any DNA contamination. 12.5 µg of RNA was treated with DNase enzyme (Promega, Madison, WI, USA) followed by two phenol-chloroform extractions followed by a single chloroform extraction. RNA was precipitated with ethanol/sodium acetate, washed with 70% ethanol and resuspended in 20 µl of H2O.

2.23 RT-PCR
2.5 µg of DNase-treated heart RNA was transcribed into cDNA with AMV-RT (Promega) for 45 minutes at 42°C. MCMV gB gene fragments were amplified 28 cycles using forward (for) and reverse (rev) primers gBfor and gBrev, and nested primers gBNfor and gBNrev (Table 2.2). The PCR programme consisted of one cycle at 94°C for 5 minutes, 52°C for 1.5 minutes and 72°C for 2 minutes, followed by 28 cycles at 94°C for 1 minute, 52°C for 1.5 minutes and 72°C for 2 minutes and concluded with one cycle at 94°C for 1 minute, 52°C for 1.5 minutes and a 10 minute minute final extension at 72°C. Every RT-PCR and nested RT-PCR amplification included MCMV infected salivary gland supernatant as a positive control and negative water controls.
Table 2.2  Sequence of primers used in RT-PCR

<table>
<thead>
<tr>
<th>Primer(^a)</th>
<th>Sequence(^b)</th>
<th>Product size (bp)(^c)</th>
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</thead>
<tbody>
<tr>
<td><strong>MCMV gB direct</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gB(_{\text{for}})(^d)</td>
<td>5'-ACACTGGATCCCTGAACAACGCCTCG-3'</td>
<td>913</td>
</tr>
<tr>
<td>gB(_{\text{rev}})(^e)</td>
<td>5'-CGGTGATATCTCGACGAC-3'</td>
<td></td>
</tr>
<tr>
<td><strong>MCMV gB nested</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gBN(_{\text{for}})</td>
<td>5'-GCCGACTCAGGCCCTCGA-3'</td>
<td>437</td>
</tr>
<tr>
<td>gBN(_{\text{rev}})</td>
<td>5'-CTATAGCTATCTTGTTG-3'</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Primers were used in either direct PCR (28 cycles) or nested PCR (further 28 cycles), as indicated.
\(^b\) Nucleotide sequence of primers designed for the K181 MCMV strain.
\(^c\) Expected product size by electrophoresis (bp).
\(^d\) for, forward.
\(^e\) rev, reverse.
2.24 Statistics

Similarities in data were established by linear correlation. A Student’s $t$ test was used to assess differences between groups of data assuming unequal variance between the means. In all tests, $p$ values of 0.05 or less are considered to indicate statistical significance. Statistical analysis was performed using Microsoft Excel software.
CHAPTER 3

Characterisation of MCMV-induced Myocarditis
3.1 INTRODUCTION

Previous research has shown that MCMV infection causes myocarditis in BALB/c mice (Lawson et al., 1989; 1992). MCMV-induced myocarditis is evident in two phases, the acute stage, approximately 5 to 14 days p.i. and a second, chronic phase, after day 21 p.i. and persisting out at least to 100 days p.i. Inflammation in the murine cardiac tissue ranges from a focal lymphocyte infiltration to an intense, diffuse infiltration and necrosis (Bartholomaeus et al., 1988). Infectious virus can only be detected in the heart early in infection, however, during the chronic phase of myocarditis there is an absence of replicating virus (Lawson et al., 1990). These observations question the role of virus in myocarditis and suggest that the extensive necrosis and myocyte loss may be mediated by the immune system itself rather than by direct effects of MCMV on these cells.

The studies described in this chapter were designed to further elucidate the role of MCMV in myocarditis by examining the direct effect that the virus has on myocytes as well as determining the presence of virus in the heart throughout the acute and chronic phases of disease. Apoptosis has been implicated as a cause of myocyte death and subsequent myocardial damage in some animal models, and shown not to be a contributing factor in others (DeBiasi et al., 2001; Colston et al., 1998). Clearly apoptosis of cardiac myocytes has the potential to cause severe myocardial damage in MCMV-induced myocarditis, a concept that is explored in this chapter. The inflammatory infiltrate has been identified in other animal models of myocarditis. The most extensively studied model, Coxsackievirus myocarditis shows inflammatory cells comprised primarily of macrophages and include CD4+ and CD8+ T cells, B cells and NK cells. The number of both T cell subsets increases during the course of this disease, but the proportion of CD8+ cells increased predominantly (Rose et al., 1992). The composition of inflammatory foci has not been clearly defined in MCMV-induced myocarditis. In the studies which are described in this chapter, a panel of cell surface markers was used in immunostaining of heart sections in order to not only identify the inflammatory cells, but to also highlight differences between acute and chronic myocarditis in the susceptible and resistant mouse strains.
3.2 RESULTS

3.2.1 Kinetics of MCMV-induced myocarditis.

Previous studies have shown that infection with the K181 strain of MCMV induces acute and chronic myocarditis in adult BALB/c mice that persists out to day 100 p.i. (Lawson et al., 1990). It has been reported that C57BL/6 mice do not develop chronic myocarditis and exhibit very low levels of acute myocarditis (Fairweather et al., 1998). In order to confirm these findings and relate the presence of virus and the development of myocarditis with the virus stock prepared for the experiments which are described here, a detailed kinetic study of the levels of myocarditis induced by MCMV infection of BALB/c and C57BL/6 mice was performed (Fig. 3.1). Mice were infected with $1 \times 10^4$ PFU of MCMV i.p. and groups of five mice per time point were assessed for histological evidence of myocarditis on days 0, 3, 5, 7, 10, 14, 21, 28, 35, 42, 49 and 56 p.i.

Infection of adult BALB/c mice with the stock of MCMV, which was prepared for use in the present study, produced myocarditis which followed the kinetics of the established model (Fig. 3.1a). BALB/c mice developed few inflammatory foci in the heart on days 3 and 5 p.i., and no myocyte necrosis was observed at these early time points. On days 7 to 14 p.i., mice developed an acute focal infiltrate associated with necrosis of myocytes. This inflammation decreased by day 21 p.i. The chronic phase of myocarditis was evident by day 28 p.i., with a more dispersed inflammatory infiltrate observed together with larger areas of necrosis, which remained until at least day 56 p.i. In contrast, the same dose of virus inoculated into C57BL/6 mice resulted in minimal focal inflammation at days 5 to 7 p.i., with complete resolution of myocarditis from day 10 p.i. (Fig. 3.1b).
Figure 3.1  MCMV-induced myocarditis in BALB/c and C57BL/6 mice.
BALB/c (A) and C57BL/6 (B) mice were infected with $1 \times 10^4$ PFU of MCMV by the i.p. route. Myocarditis score represents the average number of foci ±SE from 5 individual mice per time point. Day 0 represents 5 uninfected control mice.
3.2.2 Detection of MCMV gB transcripts in the heart.

Previous research has established that very low levels of MCMV are detectable by plaque assay in the hearts of MCMV-infected BALB/c mice during the acute phase of disease. During the chronic phase there is an absence of detectable infectious virus by plaque assay (Lawson et al., 1990). To determine whether MCMV remains in the heart of BALB/c mice at a level undetectable by plaque assay, RT-PCR was performed in order to detect viral RNA transcripts. A late gene, encoding for the structural glycoprotein B (gB) was chosen to reflect active viral replication (Rapp et al., 1992). gB transcripts were detected by RT-PCR in the hearts obtained from MCMV-infected BALB/c mice at days 3, 5, 7, 10 and 35 p.i. (Fig. 3.2a). This would suggest that there is active virus replication occurring in the heart during the acute stage of myocarditis and extending into the chronic phase. A Southern blot of the gB PCR product from individual heart samples using a gB MCMV probe confirmed virus specificity (Fig. 3.2b). gB transcript was not found in the hearts of MCMV infected BALB/c mice beyond day 35 p.i. However, iel transcript, an early gene in the replicative cycle, was found out to day 100 p.i. in MCMV infected BALB/c mice (Lenzo J.C., Fairweather D., Cull V., Shellam G. & Lawson C.M., unpublished observation). Reactions containing no reverse transcriptase were negative (data not shown).

3.2.3 MCMV infection of murine myocytes in vitro.

Research in various other animal models of virus-induced myocarditis have shown that myocytes can be killed directly by virus (Herzum et al., 1994). Viral killing of myocytes is one method by which necrosis may appear in the myocardium. Whilst MCMV levels in the heart are extremely low following infection when compared to other models, MCMV may cause direct cell death of myocytes. An MTT proliferation assay was conducted on murine myocytes infected with MCMV in vitro. Myocytes were cultured and then MCMV added at 10 PFU per cell. A decrease in MTT turnover was taken as an indication of cell death. A significant decrease in OD was observed in the MCMV infected myocytes after approximately 12 hours compared to the uninfected myocytes, indicating cell death in the infected cultures. Measurements taken thereafter showed a continuing and statistically significant decrease in MTT turnover from the infected myocytes (Fig. 3.3a). M2-1OB4 cells showed a significant decrease in OD compared to uninfected controls after 48 hours (Fig. 3.3b). It should be noted that approximately 90% of myocytes ceased beating 4-8 hours post infection, whereas beating was observed in the control myocytes for the duration of the experiment.
Figure 3.2 MCMV gB transcript in hearts from infected mice.

The percentage of hearts positive for MCMV gB transcript in BALB/c mice (A) infected with $1 \times 10^4$ PFU of MCMV i.p., as determined by nested PCR (5 mice/group). Agarose gel electrophoresis of gB DNA (B) and Southern hybridisation of PCR products with gB MCMV probe.
Figure 3.3 MTT proliferation of MCMV-infected myocytes and M2-10B4.

In vitro cultures of murine myocytes (A) and control M2-10B4 cells (B) were infected with 10 PFU MCMV per cell. MTT turnover was measured as an increase in OD reading. Cell death is observed as a decrease in OD (performed in triplicate).

* Statistically significant difference compared to uninfected cells (p<0.05).
3.2.4 Apoptosis in cardiac myocytes and inflammatory cells.

Whilst MCMV was able to directly lyse myocytes in vitro at a high multiplicity of infection (3.2.3), the low levels of virus in the heart suggest that it is unlikely that viral killing of cells is the major cause of myocyte necrosis and loss. Apoptosis may be a mechanism of cell death in MCMV-induced myocarditis. Apoptosis was identified by the TUNEL assay. Heart sections from various time points across the acute and chronic phases of disease contained apoptotic bodies. Low numbers of myocytes clearly displayed apoptotic nuclei in the hearts from animals with myocarditis (Fig. 3.4a). However, the majority of cells in hearts with myocarditis which stained for apoptosis appeared to be lymphocytes (Fig. 3.4b). Susceptible BALB/c mice showed an increase in apoptotic lymphocytes and myocytes during the acute phase of disease, which decreased by day 21 p.i. A moderate increase was observed during the chronic phase of myocarditis (Fig. 3.5a). Resistant C57BL/6 mice displayed lower numbers of apoptotic bodies compared to the BALB/c strain. Apoptosis increased in both lymphocytes and myocytes during the acute phase of disease. Low levels of apoptosis were detected in myocytes during the chronic phase of disease (Fig. 3.5b). No apoptosis was detected in the hearts of uninfected C57BL/6 mice. Uninfected BALB/c mice exhibited a very low level of myocyte apoptosis.

3.2.5 CD8+ T lymphocyte identification in heart infiltrate.

CD8+ T lymphocytes are the effector cells that mount the classical cytotoxic T cell response. Such cells are considered to be important in virus infections, killing cells that express MHC class I in association with antigenic peptide. CD8+ T lymphocytes may also be primed by other antigen presenting cells present in the heart such as dendritic cells. These cells may infiltrate the MCMV infected heart in order to kill virus infected myocytes, thus leading to the necrosis observed in myocarditis. Sections of heart from various time points across the acute and chronic phases of disease were taken from susceptible BALB/c and resistant C57BL/6 mice and were subjected to immunohistochemistry to detect CD8+ cells. Since a subset of dendritic cells can also express CD8, cell morphology was examined. CD8+ positive cells exhibited lymphocyte morphology and were therefore assumed to be CD8+ T lymphocytes and are referred to as such hereafter.
Figure 3.4  Apoptotic bodies in the myocardium of MCMV infected mice.

Apoptosis was identified in myocytes (A) and lymphocytes (B) by TdT-mediated dUTP nick end labelling (TUNEL). Dark blue staining nuclei indicate apoptotic cells from MCMV infected BALB/c mice, day 7 p.i.
Figure 3.5  Total number of apoptotic cells in heart sections from MCMV infected BALB/c and C57BL/6 mice.

BALB/c (A) and C57BL/6 (B) mice were infected with 1x10⁴ PFU of MCMV. Heart sections from various time points were stained for apoptotic bodies using the TUNEL assay. Apoptotic score represents the average number of positive cells ± SE from 5 individual mice per time point.

*Statistically significant difference compared to uninfected mice (p<0.05).
Foci of inflammation in BALB/c mice detected during the acute phase of disease consisted of predominantly CD8+ T lymphocytes (Fig. 3.6). CD8+ cells were also observed scattered throughout the myocardium. The more diffuse foci indicative of the chronic phase of disease also consisted of predominantly CD8+ T lymphocytes (Fig. 3.7). C57BL/6 mice develop mild acute myocarditis with the foci of inflammation staining positive for CD8+ cells (Fig. 3.8). The total number of CD8+ staining cells in each heart section was counted at various time points p.i. (Fig. 3.9). In both mouse strains CD8+ T lymphocytes began to enter the myocardium by day 3 p.i. Levels peaked in both strains on day 7 p.i. however, the number of these cells was more than double in the BALB/c hearts compared to the C57BL/6. Kinetics of CD8+ cells in the myocardium mimicked the kinetics of myocarditis (3.2.1). Positive cells decreased after the acute phase in both strains by day 21 p.i. A second increase in CD8+ cells was observed in the susceptible BALB/c strain only, indicative of the chronic phase of MCMV-induced myocarditis. No CD8+ cells were detected in the myocardium of uninfected BALB/c or C57BL/6 mice.

3.2.6 Identification of CD4+ T lymphocytes in MCMV-induced myocarditis

CD4+ T lymphocytes are the subpopulation of T cells that have been termed T helper cells. These cells are primed to a particular antigen and recognise and bind to a combination of antigenic peptide and MHC class II on antigen presenting cells. Such activation of T helper cells signals transcription and production of a variety of cytokines which in turn modulate the immune response. CD4+ cells would be expected to be present in the myocardium of an MCMV-infected heart, recognising virus antigen with MHC Class II and releasing cytokines to induce the immune response. Heart sections were taken from BALB/c and C57BL/6 mice at various time points p.i. and stained to detect CD4+ cells. CD4+ cells showed lymphocyte morphology and were therefore assumed to be CD4+ T lymphocytes and are referred to as such hereafter.

CD4+ cells were observed scattered throughout the myocardium during the acute phase of myocarditis in both the BALB/c (Fig. 3.10) and C57BL/6 strain. Foci of inflammation typically contained several CD4+ cells, though the majority stained positive for CD8. Chronic foci examined in BALB/c mice generally lacked CD4+ cell, with only the occasional positive cell seen in the myocardium. The total number of positive cells per heart section was counted (Fig. 3.11). CD4+ numbers peaked during the acute phase of disease on day 7 p.i. in both mouse strains. BALB/c mice exhibited a
Figure 3.6  CD8+ T cell identification in BALB/c acute myocarditis.

BALB/c mice were infected with 1x10⁴ PFU of MCMV and sections of heart stained for CD8+ T cells. Foci of inflammation in BALB/c mice at day 7 p.i. (acute myocarditis) stain primarily for CD8+ cells (red staining cells) with positive cells scattered throughout the myocardium.
Figure 3.7  CD8+ T cell identification in BALB/c chronic myocarditis.

BALB/c mice were infected with 1x10^4 PFU of MCMV and sections of heart stained for CD8+ cells. Foci of inflammation in BALB/c mice at day 49 p.i. (chronic myocarditis) stain primarily for CD8+ cells (red staining cells) with positive cells scattered throughout the myocardium.
Figure 3.8  CD8+ T cell identification in C57BL/6 acute myocarditis.

C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV and sections of heart stained for CD8+ cells. Foci of inflammation in C57BL/6 mice at day 7 p.i. (acute myocarditis) stained primarily for CD8+ cells (red staining cells) with positive cells scattered throughout the myocardium.
Figure 3.9  Kinetics of the CD8+ T cell response in BALB/c and C57BL/6 mice. BALB/c and C57BL/6 mice were infected i.p. with $1 \times 10^4$ PFU of MCMV. Hearts were removed and sectioned at various times points p.i. and stained for CD8+ T cells. Number of cells/heart section represents the average number of positive staining cells ±SE from 5 individual mice per time point.

* Statistically significant difference compared to uninfected control (Day 0) mice ($p < 0.05$).
Figure 3.10  CD4+ T cell identification in MCMV-induced myocarditis.

BALB/c and C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV and sections of heart stained for CD4+ cells. Red staining positive cells were observed scattered throughout the myocardium, the above a representation from a BALB/c heart at day 7 p.i.
Figure 3.11  Kinetics of the CD4+ T cell response in BALB/c and C57BL/6 mice.
BALB/c and C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV. Hearts were removed and sectioned at various time points p.i. and stained for CD4+ T cells. Number of cells/heart section represents the average number of positive staining cells ±SE from 5 individual mice per time point.
* Statistically significant difference compared to uninfected control (Day 0) mice ($p<0.05$).
higher number of CD4+ cells compared to C57BL/6, though this difference was minimal when compared to the marked difference seen for CD8+ cells. The number of positive cells had decreased by day 14 p.i. and in contrast to CD8+ cells, a subsequent increase in the number of CD4+ T cells corresponding to the chronic phase of disease was not observed for either strain. Uninfected BALB/c and C57BL/6 mice had occasional CD4+ T lymphocytes in the myocardium.

3.2.7 Identification of B cells in MCMV-induced myocarditis.

B cell activation and subsequent antibody production is an integral part of the immune response. Previous research in the MCMV model of myocarditis has shown that pathogenic autoantibodies to cardiac myosin are produced in mice susceptible to the disease (O'Donoghue et al., 1990). B cells may therefore play an important role in disease pathogenesis in the heart. Heart sections were taken from BALB/c and C57BL/6 mice at various time points p.i. and stained for CD45R/220. Cells which stained positive here are assumed to be B lymphocytes and are described hereafter as B cells.

B cells were observed scattered throughout the myocardium during the acute and chronic phase of myocarditis in both mouse strains (Fig. 3.12). B cells were not associated with inflammatory foci. The total number of B cells was counted per heart section (Fig. 3.13). The number of positive cells significantly increased in BALB/c hearts on days 5 and 7 p.i. Low numbers of B cells persisted into the chronic phase of disease. A moderate increase was observed on day 7 p.i. in the hearts of C57BL/6 mice. Low levels of positive cells remained throughout the chronic phase. Several positive cells were observed in heart sections from uninfected BALB/c and C57BL/6 mice.

3.2.8 Identification of macrophages in MCMV-induced myocarditis.

Macrophages have been associated with both acute and chronic inflammation in many disease models. These cells play an important role in destroying pathogens and cells. Macrophages have been identified in the inflammatory infiltrate of other animal models of myocarditis (Toyozaki et al., 2001; Rose et al., 1992). Heart sections were taken from BALB/c and C57BL/6 mice at various time points and stained for macrophages using anti-MAC3 antibody.
Figure 3.12  B cell identification in MCMV-induced myocarditis.

BALB/c and C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV and sections of heart stained for B cells. Red staining positive cells were observed scattered throughout the myocardium, the above a representation from a BALB/c heart at day 7 p.i.
Figure 3.13  Kinetics of the CD45R/220+ B cell response in BALB/c and C57BL/6 mice.

BALB/c and C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV. Hearts were removed and sectioned at various time points p.i. and stained for CD45R/220+ B cells. Number of cells/heart section represents the average number of positive staining cells ±SE from 5 individual mice per time point.

* Statistically significant difference compared to uninfected control (Day 0) mice ($p<0.05$).
Macrophages were found scattered throughout the myocardium of both infected mouse strains (Fig. 3.14). Positive cells were occasionally associated with inflammatory foci. The total number of stained macrophages was counted per heart section (Fig. 3.15). A statistically significant increase in macrophage numbers occurred in the BALB/c heart on day 10 p.i. and this increase persisted out until at least day 56 p.i. In contrast, very low numbers of positive staining macrophages were observed in the hearts of infected C57BL/6 mice, with the positive cells being found scattered throughout the myocardium. Levels did not significantly increase at any time point following infection.

3.2.9 Identification of neutrophils in MCMV-induced myocarditis.
Polymorphonuclear neutrophils constitute the majority of the blood leukocytes and their ability to migrate into tissues in order to phagocytose material has been well established. Following phagocytosis the neutrophil produces acid hydrolases, myeloperoxidase and muramidase. Neutrophils have been implicated in the dissemination of CMV throughout the body and CMV infection increases production of neutrophil chemoattractants (Craigen et al., 1997). Heart sections from BALB/c and C57BL/6 mice were stained for Ly-6G positive neutrophils at various time points post-infection.

Neutrophils were observed in the myocardium of infected BALB/c mice (Fig. 3.16). Positive cells were rarely contained within inflammatory foci, but were often observed around foci. The totally number of positive cells were counted per heart section (Fig. 3.17). No neutrophils were found in the myocardium of uninfected BALB/c or C57BL/6 mice. Occasional positive cells were observed following infection in the resistant C57BL/6 strain, however, numbers were extremely low. No neutrophils were seen in the myocardium of the susceptible BALB/c strain on days 0, 3 and 5 p.i. A significant increase in positive cells occurred on day 7 p.i., peaking on day 10 p.i. Levels had decreased by day 14 p.i., however, significant levels persisted into the chronic phase of myocarditis.
Figure 3.14  Macrophage identification in MCMV-induced myocarditis.

BALB/c and C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV and sections of heart from various time points p.i. stained for macrophages. Red staining positive cells were observed scattered throughout the myocardium, the above a representation from a BALB/c heart at day 7 p.i.
Figure 3.15  Kinetics of the MAC3+ macrophage response in BALB/c and C57BL/6 mice.

BALB/c and C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV. Hearts were removed and sectioned at various time points p.i. and stained for Macrophages. Number of cells/heart section represents the average number of positive staining cells ±SE from 5 individual mice per time point.

* Statistically significant difference compared to uninfected control (Day 0) mice ($p<0.05$).
Figure 3.16 Neutrophil identification in MCMV-induced myocarditis.

BALB/c and C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV and sections of heart from various time point p.i. stained for neutrophils. Red staining positive cells were observed scattered throughout the myocardium, the above a representation from a BALB/c heart at day 7 p.i.
Figure 3.17 Kinetics of the Ly-6G+ neutrophil response in BALB/c and C57BL/6 mice.

BALB/c and C57BL/6 mice were infected with $1 \times 10^4$ PFU of MCMV. Hearts were removed and sectioned at various time points p.i. and stained for Neutrophils. Number of cells/heart section represents the average number of positive staining cells ±SE from 5 individual mice per time point.

* Statistically significant difference compared to uninfected control (Day 0) mice ($p<0.05$).
3.2.10 Dendritic cell identification in MCMV-induced myocarditis.

Dendritic cells are a heterogenous family of cells which function as sentinels of the immune system. They traffic from the blood to tissues, where, while immature they capture antigens. Following inflammatory stimuli, they move to the draining lymphoid organs where they are converted to mature dendritic cells and prime naïve T cells. Dendritic cells have been found in the heart and are considered to play an important role in the immunoresponsiveness of the heart (Yokoama et al., 2000). In order to identify dendritic cells in the myocardium of MCMV-infected BALB/c and C57BL/6 mice, heart sections were taken at various time points and stained for CD11c+ cells. CD11c was chosen as a marker for dendritic cells as CD11c+ blood immature dendritic cells respond to various chemokines expressed during inflammation. CD11c- dendritic cell precursors do not respond to these chemokines (reviewed in Caux et al., 2000).

No CD11c+ cells were observed in the myocardium of BALB/c or C57BL/6 mice at any time point examined during the acute and chronic phase of myocarditis (data not shown). The antibody was tested using spleen sections from MCMV-infected BALB/c mice (Fig. 3.18), with positive staining dendritic cells being observed throughout the spleen.
Figure 3.18  Dendritic cell identification in BALB/c spleen.

Spleen sections from MCMV-infected BALB/c mice were used as a positive control for antibody staining of CD11c+ dendritic cells.
3.3 DISCUSSION

Inflammation and necrosis of the myocardium are the histologically visible effects of MCMV-induced myocarditis. Investigations in other animal models attribute this damage to both direct cell death caused by the virus and to immune-mediated cell lysis. The research described in this chapter examined possible causes of myocyte necrosis and characterised the cellular infiltrate observed in MCMV-induced myocarditis. Differences were observed between susceptible and resistant mouse strains as well as between the acute and chronic phase of disease.

Previous research has described ongoing, chronic inflammation in the myocardium of BALB/c mice in the absence of detectable infectious MCMV (Lawson et al., 1990). In the research described here, MCMV was not detectable by plaque assay beyond day 5 p.i. (data not shown) however, the presence of viral gB RNA transcript in the heart out to day 35 p.i. in BALB/c mice indicates active viral replication in the heart. However, the lack of viral gB RNA transcript in the presence of iel RNA (Lenzo J.C., Fairweather D., Cull V., Shellam G.R & Lawson C.M., unpublished results) in the BALB/c heart during the late stages of chronic myocarditis (beyond day 35 to 100 p.i.) suggests that the heart harbours latent MCMV infection. These data suggest that MCMV persists through the acute phase of myocarditis at low levels and then establishes a latent infection. These results are consistent with other studies showing that MCMV establishes latent rather than persistent infections in organs (Klotman et al., 1990; Bevan et al., 1996; Kurz et al., 1997).

Having established that MCMV is in the heart during acute and chronic myocarditis, both as replicating virus and possibly latent virus respectively, it was then of interest to determine whether MCMV by itself is able to lyse cardiomyocytes or whether immune responses are essential for myocyte necrosis. Thus, the effect of virus on cardiac myocytes was studied in vitro, without any influence of the immune system. An MTT proliferation/cytotoxic assay was developed. MTT has been shown to produce comparable results to the commonly used chromium release assay in determining cell viability (Mossmann, 1983). Whilst the results presented in this chapter clearly show that MCMV was able to kill the cardiac myocytes rapidly in vitro at a high MOI, it remains unclear from this study how the virus actually lyses the cells.
Given the extremely low level of virus present in the heart, it would seem unlikely that
the massive levels of necrosis observed during myocarditis could be attributed to direct
MCMV killing of myocytes. Another means of cell death is apoptosis, which has been
explored in other animal models with mixed results. Inhibiting apoptosis in reovirus-
induced myocarditis resulted in a dramatic decrease in histopathologic evidence of
myocardial injury, suggesting that a large proportion of damage was apoptotic in nature
in this model (DeBiasi et al., 2001). Research conducted in the coxsackievirus model
concluded that myocyte apoptosis was rare in acute myocarditis attributed to this agent
(Colston et al., 1998). Apoptosis in MCMV-induced myocarditis was mainly restricted
to the infiltrating inflammatory cells. Levels corresponded to increasing inflammation
and thus followed the established kinetics of this disease. Whilst myocyte apoptosis was
observed, levels were low and therefore unlikely to be a major contributory mechanism
to disease pathogenesis. This observation was also seen in the EMCV model of
myocarditis, the main source of apoptotic cells in the heart in mice with viral
myocarditis appearing to be infiltrating mononuclear cells (Yamada et al., 1999).

Characterisation of the inflammatory cells has been carried out in other animal models
of myocarditis revealing mixed infiltrates whose composition change during the course
of disease (Rose et al., 1992). A panel of immunologically important cells were chosen
to be examined in MCMV-induced myocarditis. The majority of cells in the
inflammatory foci were CD8+ T cells, in both BALB/c and C57BL/6 strains of mice,
through the acute and chronic phases of disease. CD8+ T cells have been shown to be
important cells in myocarditis, not only in the animal models but also in human patients.
Immunohistochemical staining identified the infiltrating cells in herpes simplex virus
and Epstein-Barr virus induced myocarditis in children to be CD8+ T cells (Koga et al.,
2001). Mice lacking CD8+ cells showed increased survival and reduced myocarditis
when infected with coxsackievirus (Henke et al., 1995). Depletion of CD8+ T cells in
the early stages of MCMV-induced myocarditis reduced myocardial inflammation.
Although this effect was not observed later in disease progression (Fairweather D.F.,
unpublished observations). CD8+ T cells are likely to play a critical role in antiviral
defence, entering the heart to eliminate virus-infected cells, however, a delicate balance
exists between the benefits and costs of this antiviral immune response.
Various other cells were identified in the inflammatory infiltrate, both scattered throughout the myocardium and associated with inflammatory foci following MCMV infection. CD4+ T cells comprised a small percentage of the cells in the foci and were present throughout the heart. CD4+ T cells have been identified in the heart of coxsackievirus infected mice (Rose et al., 1992), with depletion protecting against early death but greatly magnifying the severity of myocarditis (Henke et al., 1995). CD4+ cells play a crucial role in the immune response. These cells are present in the hearts of both BALB/c and C57BL/6 mice during acute myocarditis and may skew the immune response to appropriate virus clearance as seen in the resistant strain or an inappropriate response leading to chronic inflammation.

The presence of B cells in the myocardium of MCMV infected mice correlates with the observation of antibody production against cardiac myosin (O'Donoghue et al., 1990). Macrophages have been identified as the major cell type present in the heart of coxsackievirus infected mice (Rose et al., 1992). In the MCMV model of myocarditis, macrophages were identified scattered throughout the myocardium, occasionally associated with inflammatory foci. Unlike the coxsackievirus model, macrophages did not make up the majority of cells present during acute myocarditis. Indeed, significant numbers of the cells did not begin to enter the myocardium until day 7 p.i. in BALB/c mice. Interestingly, the resistant C57BL/6 mouse strain did not mount a significant macrophage response in the heart, suggesting that these cells may contribute to the ongoing chronic disease observed in the susceptible BALB/c strain.

Neutrophils were also observed in the myocardium of MCMV-infected BALB/c mice. Cells began to enter on day 7 p.i. and persisted in the heart until at least day 56 p.i. As was seen for macrophages, the resistant C57BL/6 mouse strain did not mount a neutrophil response in the heart in response to MCMV infection. Interestingly, these observations do not correlate to the traditional view that neutrophils are the first cell type to be seen in acute inflammation. Nevertheless, neutrophils may be recruited to the heart for phagocytic purposes, and this capacity carries with it an implicit capacity for cardiac tissue destruction, as observed in other inflammatory and autoimmune diseases (Burg & Pillinger, 2001).

The absence of CD11c+ dendritic cells in the heart is surprising as such dendritic cells are thought to be important antigen presenting cells. It is possible that CD11c- cells are
circulating through the myocardium and are therefore not observed in this study. It has recently been shown that dendritic cells are permissive to MCMV infection and such infection interferes with their function (Andrews et al., 2001). Such infection may occur in the heart and contribute to myocarditis pathology.

The pathogenesis of MCMV-induced myocarditis remains unclear. However, from the results presented in this chapter it is possible to conclude that MCMV is capable of killing cardiac myocytes at a high MOI in vitro, but extremely low levels of MCMV which persist in the heart make it an unlikely mechanism for the massive necrosis seen in the myocardium. Apoptosis, whilst deemed an important contributing factor in some animal models, does not appear to be of major importance in the MCMV-induced myocarditis. CD8+ T cells were the major cells in the inflammatory infiltrate in the susceptible BALB/c mouse strain, which also mounted both macrophage and neutrophil responses. Whilst CD8+ T cells were also the major cell type in the resistant C57BL/6 strain, no macrophage or neutrophil response was observed. The type of immune cells involved in the cardiac inflammation may ultimately lead to either resolution or progression of disease.
CHAPTER 3 SUMMARY

Several aspects of MCMV-induced myocarditis were examined in this chapter. A repeat kinetic study was performed to confirm the kinetics of myocarditis in susceptible BALB/c and resistant C57BL/6 mice using the virus stock prepared for all experiments reported in this thesis. Persistence of virus in the heart was examined using RT-PCR to detect \( gB \) transcripts. RNA transcript for the MCMV \( gB \) gene was detected in the hearts of BALB/c mice up to day 35 p.i. \( gB \) encodes for a late structural protein and its presence is indicative of active virus replication. Direct MCMV lysis of myocytes was confirmed \textit{in vitro} indicating that MCMV is capable of killing myocytes and impairing their function without any contribution from the immune system. Apoptosis was examined as a possible cause of myocyte necrosis seen in myocarditis. Apoptotic cells were observed in the myocardium of both mouse strains following MCMV infection, however, the majority were inflammatory cells with only low levels of myocytes undergoing apoptosis. The inflammatory infiltrate was characterised from both the resistant and susceptible strains of mice. CD8+ T cells were identified as the major cell type in inflammatory foci for both mouse strains during acute and chronic myocarditis. CD4+ T cells and B cells were seen in both strains, however, only the susceptible BALB/c strain mounted a macrophage and neutrophil response in the heart during MCMV infection.
CHAPTER 4

Antiviral Treatment of Murine Cytomegalovirus-induced Myocarditis
4.1 INTRODUCTION

A number of pathogenic mechanisms have been suggested to account for the inflammation and necrosis found in the myocarditic heart following virus infection. Direct viral infection of the heart may provide an early insult to cardiac tissue. Alternatively, the production of an antiviral immune response following viral infection may cause necrosis or apoptosis of infected cells in the heart (e.g., myocytes) through the release of inflammatory mediators (Neumann et al., 1993; Rose, 1996; Sato et al., 1994; Sherry et al., 1993). Furthermore, an antiviral immune response that cross reacts with self cardiac antigens, such as cardiac myosin, may lead to tissue destruction and autoimmune disease (Herzum et al., 1994; Huber, 1997; Kandolf et al., 1993; Lawson, 2000; Lawson et al., 1992; Von Herrath & Oldstone, 1995). Despite considerable advances in medicine and a greater understanding of the diagnosis of myocarditis, treatment for this disease remains primarily supportive. The therapy regime is focused on the myocardial dysfunction and includes such measures as oxygenation and the administration of inotropic agents to increase cardiac output.

The role of virus as a trigger for myocarditis is very clear, therefore the therapeutic effects of antiviral drugs on the subsequent course of myocarditis have been one focus of research. Studies in the coxsackievirus B3 myocarditis mouse model have shown some promise for antiviral drugs, with ribavirin being trialed as a possible therapeutic agent (Kishimoto et al., 1988). This study showed that early administration of the antiviral drug resulted in inhibition of viral replication, a reduction in myocardial damage during the acute phase of disease and increased survival. When treatment was delayed by 4 days, myocardial damage was reduced, however there was no survival benefit. As MCMV clearly induces myocarditis in susceptible mice (Lawson et al., 1990), and antiviral therapy appeared effective under appropriate regimes in other animal models, such antiviral treatment was examined in the MCMV model of myocarditis. It should be noted that direct comparisons of antiviral efficacy between CB3 and MCMV are difficult to make as these viruses differ in many ways and the antiviral drugs used have different modes of action.

Immunocompromised individuals are at increased risk of CMV infection developing into serious CMV disease. The introduction into clinical use of potent antiviral
compounds and of rapid detection assays for CMV during the past two decades has allowed development of strategies for the prevention and treatment of disease caused by CMV. At present, the antiviral drugs ganciclovir, foscarnet and cidofovir are commonly used in the treatment of CMV infection and disease (Reusser, 2001). These antiviral drugs were examined for therapeutic effects in our model of MCMV-induced myocarditis. Ganciclovir (GCV; 9-[1,3-dihydroxy-2-propoxymethyl]guanine; Fig. 4.1a) is used extensively in the treatment of CMV retinitis in immunocompromised patients, especially those with AIDS (DeArmond, 1991). CMV possesses a unique viral kinase capable of phosphorylating GCV to GCV monophosphate in infected cells (Littler et al., 1992). GCV monophosphate is readily converted by cellular enzymes to the active form of the drug; GCV triphosphate (Smee et al., 1985), which is the active form of the drug and inhibits viral DNA polymerase (Freitas et al., 1985). Cidofovir (CDV; 1-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl] cytosine dihydrate; Fig. 4.1b) is a monophosphate nucleotide analogue that undergoes cellular phosphorylation to produce an active diphosphate compound (Ho et al., 1992). CDV competitively inhibits the incorporation of deoxycytosine-5'-triphosphate by viral DNA polymerase into viral DNA (Xiong et al., 1996). Once incorporated into viral DNA it slows further DNA synthesis and causes DNA destabilisation. Foscarnet (PFA; trisodium phosphonoformate; Fig. 4.1c) is an analogue of pyrophosphate that inhibits DNA chain elongation catalyzed by the viral DNA polymerase by preventing pyrophosphate exchange (Crumpacker, 1992).

The antiviral potency of these drugs is well documented, however their therapeutic value for CMV-induced myocarditis is unknown. In this chapter, various treatment regimes have been trialed in the MCMV-induced myocarditis model, focusing on the effect of these drugs on the acute and chronic phase of infection and disease. CMV reinfection is a common problem leading to increased disease and prolonged recovery time (Bale et al., 1996). The effect that reinfection has on MCMV-induced myocarditis is also examined in this chapter with antiviral drugs trialed for their therapeutic efficacy following virus reinfection.
Figure 4.1. **Antiviral drug structure.**

Ganciclovir, 9-[1,3-dihydroxy-2-propoxymethyl]guanine (A); Cidofovir, 1-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine dihydrate (B); Foscarnet, trisodium phosphonoformate (C).
4.2 RESULTS

4.2.1 Antiviral activities of GCV, PFA and CDV for MCMV infection in vitro.
The antiviral activities of GCV, PFA and CDV were determined by plaque reduction assays using MEFs grown in vitro. The replication of the K181 laboratory strain and the G4 wild isolate of MCMV were inhibited by the antiviral drugs (Table 4.1). These 50% inhibitory concentrations confirm that GCV, PFA and CDV are effective against the K181 and a wild isolate of MCMV.

4.2.2 Synergistic activity of GCV, PFA and CDV in vitro.
In order to determine whether these drugs acted synergistically against the K181 laboratory strain of MCMV, combinational plaque reduction assays were performed. Dose isobolograms revealed that GCV and PFA when used in combination in vitro showed synergistic activity (Fig. 4.2a). Similar isobolograms were performed for GCV/CDV and PFA/CDV combinations with both showing some level of synergy (Fig. 4.2b; 4.2c). Interactions between the drugs were also evaluated by the fractional inhibitory concentration (FIC) method. The FIC for each evaluable drug concentration was calculated by the formula:

\[
\text{FIC} = \frac{\text{IC}_{50} \text{ of drug A in combination}}{\text{IC}_{50} \text{ of drug A alone}} + \frac{\text{IC}_{50} \text{ of drug B in combination}}{\text{IC}_{50} \text{ of drug B alone}}
\]

FIC values lower than 1.0 suggest synergistic effects (Elion et al., 1954). GCV and PFA produced a FIC value of 0.9, whereas GCV and CDV revealed a value of 0.91, confirming synergy. PFA and CDV had a FIC value of 0.99, suggesting that these two drugs may not have synergistic interactions against MCMV in vitro.

4.2.3 Antiviral treatment of acute MCMV infection.
The antiviral effect of GCV, CDV and PFA in vivo on virus replication in the major target organs, liver, spleen and salivary gland was next investigated. Adult BALB/c mice were infected with K181 MCMV then treated with varying doses of GCV and CDV, commencing day 1 p.i. and continuing daily for 7 days. As viral replication can recommence in the absence of PFA, this drug is most effective when administered by a slow infusion over at least 1 hour in humans, initially repeated every 8 hours (Oberg,
Table 4.1  Antiviral activity *in vitro*.

<table>
<thead>
<tr>
<th>Antiviral Drug</th>
<th>IC$_{50}$ K181 MCMV$^a$</th>
<th>IC$_{50}$ G4 MCMV$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganciclovir</td>
<td>8.9</td>
<td>5.6</td>
</tr>
<tr>
<td>Cidofovir</td>
<td>0.17</td>
<td>0.23</td>
</tr>
<tr>
<td>Foscarnet</td>
<td>142.3</td>
<td>131.5</td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ values (μM) for antiviral drugs determined by a 50% reduction in plaques formed in K181 MCMV-infected MEF monolayers. MEFs were infected with 100 PFU per well.

$^b$ IC$_{50}$ values (μM) for antiviral drugs determined by a 50% reduction in plaques formed in G4 MCMV-infected MEF monolayers. MEFs were infected with 100 PFU per well.
Figure 4.2. Dose isobolograms for evaluation of the activity of antiviral drugs in combination.

Concentrations of two drugs which, in combination, result in 50% inhibition of viral replication are plotted as fractions of the IC₅₀ of each drug when tested alone. The broken line represents an isobol which indicates no interaction. The solid line represents the isobol for the given drug combination, bowing of the isobol towards the origin is indicative of a synergistic interaction. Synergistic activity was detected between ganciclovir and foscarnet (A) and ganciclovir and cidofovir (B). No synergistic activity was observed between foscarnet and cidofovir (C).
1989). PFA was therefore used at a dose of 250mg/kg twice a day. Such an antiviral
dose had previously been shown to reduce mortality in mice after being infected with a
lethal dose of MCMV (Kern et al., 1978).

MCMV-infected mice treated with GCV showed significant ($p<0.05$) reductions in
virus titres in the spleen and salivary gland at day 7 p.i. with all doses of this drug (12,
25, 40 mg/kg/day) compared to untreated MCMV-infected mice (Fig. 4.3a). MCMV-
infected mice treated with CDV showed a significant ($p<0.05$) reduction in virus titres
in the salivary gland at day 7 p.i. with all doses compared to the untreated MCMV-
infected mice (Fig. 4.3b). Whilst the reductions in the spleen and liver were not
statistically significant, doses of 5 and 10 mg/kg/day of CDV reduced viral titres in
these organs to the limit of detection of the plaque assay (100 PFU/g) (Fig. 4.3b).
MCMV-infected mice treated with PFA showed a significant ($p<0.05$) decrease in virus
 titres in the spleen and salivary gland at day 7 p.i. (Fig. 4.3c). It should be noted that
whilst PFA reduced viral titres at day 3 p.i. it was less effective than either GCV or
CDV (data not shown).

4.2.4 Antiviral treatment of acute MCMV-induced myocarditis.
The therapeutic efficacy of the antiviral drugs on MCMV-induced myocarditis was also
examined. Myocarditis was significantly reduced (6.2-fold reduction; $p<0.05$) at day 7
in MCMV-infected mice treated with either 25 or 40 mg/kg/day GCV (Fig 4.4a). The
dose of 12 mg/kg/day of GCV had no observed effect on the severity of myocarditis
when compared to the untreated MCMV-infected mice. CDV-treated mice showed a
significant reduction (36-fold reduction; $p<0.05$) in myocarditis at day 7 for all doses of
CDV, with no detectable inflammation being observed in mice given doses of 5 and 10
mg/kg/day (Fig 4.4b). However, no therapeutic benefit was observed after treatment
with PFA at 250mg/kg twice a day (Fig 4.4c). As expected, myocarditis was not
observed in control uninfected BALB/c mice treated with the above mentioned defined
doses of GCV, CDV or PFA.
Figure 4.3. Antiviral therapy reduces MCMV titres in major target organs.

BALB/c mice were inoculated with 1x10^4 PFU of MCMV i.p. at day 0 and treated with varying doses of GCV (A), CDV (B), or 250 mg/kg/twice daily PFA (C) daily from day 1 to 7 p.i., or treated with saline as a placebo. The average number of PFU/g of liver, spleen and salivary gland at day 7 p.i. from groups of 5 mice per dose ± SE are shown. * Statistically significant difference compared to untreated MCMV-infected mice (p<0.05).
Figure 4.4. Early antiviral treatment reduces the severity of acute MCMV-induced myocarditis.

BALB/c mice were inoculated with $1 \times 10^4$ PFU of MCMV i.p. at day 0 and treated with either GCV (A), CDV (B), or 250 mg/kg/twice daily PFA daily from day 1 to 7 p.i., or treated with saline as a placebo. The average number of inflammatory foci/heart section at day 7 p.i. from groups of 5 mice per dose ± SE are shown.

*Statistically significant difference compared to untreated MCMV-infected mice ($p<0.05$).
4.2.5 Antiviral treatment of chronic MCMV-induced myocarditis.

The efficacy of antiviral treatment on the chronic phase of MCMV-induced myocarditis was next examined. Effective doses of GCV (40 mg/kg/day) and CDV (5 mg/kg/day) were administered to BALB/c mice, commencing on day 1 p.i. and continuing daily until day 7 p.i. Hearts were removed for histological examination at day 35 p.i., a time point well characterised in the chronic phase of disease. Control mice were either uninfected and drug treated or MCMV-infected without drug treatment. Viral titres were not examined in this study as infectious MCMV in BALB/c mice is resolved by this time. PFA was not included in this study as the ineffective dose of 250 mg/kg twice a day given to mice had marked side effects with weight loss, ruffling of fur and lethargy.

A significant reduction in chronic myocarditis was observed under both the GCV (Fig 4.5a) and CDV (Fig 4.5b) treatment regimes (1.8- and 2.8-fold reduction, respectively; \(p<0.05\)), however, the disease was not completely abolished.

In order to examine the efficacy of antiviral treatment once cardiac disease is present, GCV and CDV treatment commenced after myocarditis had been established (day 14 p.i.) and treatment then continued daily into the chronic phase of disease (day 35 p.i.) at which point hearts were examined for myocarditis. No significant decrease in myocarditis was observed in the chronic phase with either the GCV (Fig 4.6a) or CDV (Fig 4.6b) treatment regimes.

4.2.6 Exacerbation of MCMV-induced myocarditis by virus reinfection.

The effect of MCMV reinfection with the same or a genetically different strain of virus was examined in mice during the chronic phase of myocarditis. Mice were infected with the K181 laboratory strain of MCMV on day 0, then reinfected with either the K181 or G4 strain of MCMV on day 56 p.i. The G4 strain is a wild isolate of MCMV that has previously been shown to induce myocarditis in BALB/c mice (Fairweather et al., 1998). Viral titres and myocarditis were examined 7 days post-reinfection and compared against data from mice that were not reinfected.

No virus was detected by plaque assay in any of the target organs, namely the liver, spleen, and salivary gland, taken from previously infected mice challenged with either MCMV strain. Reinfection of BALB/c mice with K181 significantly exacerbated
Figure 4.5. Early antiviral therapy reduces the severity of chronic MCMV-induced myocarditis.

BALB/c mice were inoculated with $1 \times 10^4$ PFU of MCMV i.p. on day 0 and treated with either GCV at 40 mg/kg/day (A) or CDV at 5 mg/kg/day (B) from day 1 to 7 p.i., or treated with saline as a placebo. The average number of inflammatory foci/heart section at day 35 p.i. from groups of 5 mice ± SE are shown.

* Statistically significant difference compared to untreated MCMV-infected mice ($p<0.05$).
Figure 4.6. Late antiviral therapy has no therapeutic effect on chronic MCMV-induced myocarditis.

BALB/c mice were inoculated with \(1 \times 10^4\) PFU of MCMV i.p. on day 0 and treated with either GCV at 40 mg/kg/day (A) or CDV at 5 mg/kg/day (B) from day 14 to 35 p.i., or treated with saline as a placebo. The average number of inflammatory foci/heart section at day 35 p.i. from groups of 5 mice ± SE are shown.
myocarditis (1.3-fold increase; Fig. 4.7). An increase in cardiac inflammation was also observed after reinfection of mice with G4 MCMV (1.2-fold increase), however, this was not statistically significant. The ability of antiviral therapy to reduce exacerbated myocarditis upon reinfection was assessed. Treatment with either 50 mg/kg/day GCV or 5 mg/kg/day CDV commenced on day 57 post-initial infection and continued daily until day 63 post-initial infection (day 7 post-reinfection), at which point hearts were examined for myocarditis and major target organs assessed for infectious virus. Treatment with GCV or CDV resulted in a slight decrease in myocarditis in non-reinfected mice, but both drugs caused a significant decrease in disease in both the K.181 and G4 MCMV-reinfected animals (2.2- and 2.6-fold reduction, respectively; p<0.05; Fig 4.7).

4.2.7 Antibody response to MCMV reinfection.

The generation of cardiac myosin specific auto-antibodies in MCMV-induced myocarditis is a well established phenomenon (Lawson et al., 1991; 1992). As these autoantibodies may contribute to the inflammation and necrosis seen in the myocardium, it was of interest to see whether levels of such cardiac myosin antibodies were increased with MCMV reinfection. An ELISA was used to determine the titre of auto-antibodies to cardiac myosin 7 days post reinfection (63 days post initial infection). Sera was absorbed against skeletal myosin to obtain cardiac myosin specific antibodies. Reinfeciton with the same or genetically different strain of MCMV did not increase antibodies to cardiac myosin (Table 4.2). Treatment with the antiviral drugs GCV and CDV, whilst decreasing levels of myocarditis, did not significantly alter the levels of cardiac myosin specific auto-antibodies.
Figure 4.7. Exacerbation of MCMV-induced myocarditis by virus reinfection is prevented with antiviral therapy.

BALB/c mice were inoculated with $1 \times 10^4$ PFU of MCMV (K181 strain) i.p. on day 0. Mice were then reinjected with $1 \times 10^4$ PFU of either K181 (K181/K181) or G4 (K181/G4) MCMV i.p. on day 56 p.i. and then treated with either GCV at 40 mg/kg/day or CDV at 5 mg/kg/day from day 57 to 63 p.i., or treated with saline as a placebo. The average number of inflammatory foci/heart section at day 63 p.i. (day 7 post-reinfection) from groups of 5 mice ± SE are shown.

* Statistically significant difference compared to untreated MCMV-reinfected mice ($p<0.05$).
Table 4.2  MCMV reinfection – cardiac myosin specific antibodies.

<table>
<thead>
<tr>
<th>MCMV Strain</th>
<th>Saline&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GCV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CDV&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>K181&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>K181 + K181&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>K181 + G4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32</td>
<td>32</td>
<td>64</td>
</tr>
</tbody>
</table>

<sup>a</sup> BALB/c mice were inoculated with 1 x 10⁴ PFU of K181 MCMV, then reinfected with either saline (K181), 1 x 10⁴ PFU K181 MCMV (K181 + K181) or 1 x 10⁴ PFU G4 MCMV (K181 + G4) on day 56 p.i. Mice were treated with GCV (50 mg/kg/day) or CDV (5 mg/kg/day) commencing on day 57 p.i. and continued daily until day 63 p.i.

<sup>b</sup> Endpoint antibody titre (reciprocal dilution) in pooled sera (5 mice per group) determined by ELISA using cardiac myosin as the antigen. Sera was collected 7 days post reinfection and absorbed to skeletal myosin, eluted sera containing cardiac myosin specific antibodies.
The research described in this chapter examined the potential use of the antiviral drugs, GCV, CDV and PFA, as therapeutic agents in a MCMV-induced model of myocarditis using adult BALB/c mice. Antiviral therapy was trialed as the role of MCMV in the induction of cardiac inflammation is unquestionable. These results clearly support this vital role but additional mechanisms besides direct viral effects also contribute to the development of myocarditis. Early treatment (day 1 p.i.) with either GCV or CDV significantly reduced myocarditis in the acute phase of disease (day 7 p.i.). Similar treatment of susceptible mice with PFA reduced virus titres in the spleen and liver but was not effective at reducing myocarditis. This observation may reflect the inadequacy of systemic delivery of PFA in this animal model. These findings support the hypothesis that early myocardial inflammation is mediated either directly or indirectly by virus infection. Data from the CB3 model of myocarditis shows that virus alone can cause direct myocyte death without involving any immune mechanism of host defence (Herzum et al., 1994; McManus et al., 1993). During the early stage of CB3-induced cardiac disease, virus can be readily obtained from heart tissue (Kandolf et al., 1993), and in situ hybridisation studies demonstrated that viral infection, leading directly to myocyte necrosis and inflammatory foci, were consistently associated with virus-infected cells in the myocardium (Herzum et al., 1994; Kandolf et al., 1993; Klingel & Kandolf, 1993). In the MCMV model of myocarditis, virus titres in the heart are extremely low, indeed 100-fold lower than CB3 titres in the heart (Fairweather et al., 1998; Rose et al., 1986; Sato et al., 1994).

Although treatment of MCMV-infected mice with 12 mg/kg/day of GCV significantly reduced virus titres in the spleen, liver and salivary gland, this dose of GCV had no effect on myocarditis severity. However, higher doses of this drug further reduced viral titres and caused significant decreases in myocardial inflammation. This result suggests that MCMV-induced myocarditis cannot be treated in a dose-dependant manner with antiviral drugs, rather, viral titres must be reduced to a relatively low level before any therapeutic effect can be observed in the acute phase. Acute viral burdens in the liver have also been shown to be decreased with GCV treatment of weanling BALB/c mice infected intraperitoneally with MCMV (Bolger et al., 1999).
In this chapter it is reported that chronic MCMV-induced myocarditis cannot be prevented by early antiviral treatment with either GCV or CDV given during the acute stage of infection. Whilst the severity of chronic myocarditis was reduced, inflammation and necrosis were still observed. During the chronic phase of disease, infectious MCMV was not detected in the heart, spleen, liver or salivary glands of susceptible BALB/c mice, providing supporting evidence that this phase is not mediated by direct viral damage. Furthermore, antiviral treatment with either GCV or CDV after the acute phase of disease and extending into the chronic phase had no significant therapeutic effect. This finding supports an important role for other governing factors besides virus in the development of myocarditis following virus infection.

BALB/c mice reinfected with the same or a different strain of MCMV during the chronic phase of disease showed a significant increase in myocarditis. While a previous CMV infection resulted in rapid clearance of virus upon reinfection, with no infectious virus being detected at day 7 post-reinfection in major target organs, previous MCMV exposure did not protect mice against the development of myocarditis. Antiviral treatment with GCV or CDV reduced myocarditis severity in reinfected mice, however, inflammation and necrosis indicative of the pre-existing chronic phase were still evident. Clearly, further myocardial damage was caused by subsequent viral infections, with such damage able to be treated with antiviral drugs. However, such treatment was not effective for the existing chronic inflammation and necrosis, which is possibly autoimmune in nature, induced by the primary CMV infection. These results suggest that further damage is not mediated by the autoantibodies to cardiac myosin, but rather other mechanisms may be the driving force behind exacerbated disease.

Clinical trials of CDV for CMV retinitis in AIDS patients have demonstrated efficacy in slowing the progression of this disease and have provided insights into toxicity, an adverse side effect of this antiviral drug (Safrin et al., 1997). However, emergence of drug-resistant CMV mutants has been observed for CDV-treated and GCV-treated AIDS patients (Emery, 1998). The molecular basis of such de novo resistance is often described as mutations in the UL54 DNA polymerase gene (Bowen et al., 1999; Safrin et al., 1997; Wolfe et al., 1995). Perhaps combinational therapy using such antiviral agents, not necessarily administered simultaneously, may have desirable synergistic effects in vivo without potential problems associated with the development of drug
resistance. Furthermore, incorporation of new analogues of such antiviral compounds in therapeutic regimes may enhance the potency of these agents at affecting early virus replication (Bedard et al., 2000). Furthermore, the development of new antiviral compounds may offer new therapeutic hope in the treatment of CMV-induced myocarditis. Valganciclovir is a newly developed oral prodrug of GCV, with 10-fold greater bioavailability than oral GCV (Reusser, 2001). This drug offers greater potency than GCV with a more convenient method of delivery.

The results presented in this chapter show that antiviral therapy is an effective treatment for MCMV-induced myocarditis when initiated very early in infection. Such early treatment provides protection against severe acute myocarditis and reduced the level of chronic myocarditis. However, the studies of treatment during the chronic phase of disease showed no therapeutic effect in the murine model, providing further evidence that an immunopathogenic mechanism perpetuates the late phase of disease. Nonetheless, antiviral therapy is a viable option to prevent exacerbation of disease when reinfection is recognised as a high risk factor, especially in transplant patients. This study has highlighted possible clinical applications of antiviral drugs as therapeutic agents in CMV-induced myocarditis as well as providing further insights into the pathogenesis of cardiac disease.
CHAPTER 4 SUMMARY

The therapeutic efficacy of select antiviral drugs against MCMV-induced myocarditis was examined. Ganciclovir, foscarnet and cidofovir, commonly used in the treatment of CMV infection in humans, were trialed in the MCMV murine model. All three drugs showed potent antiviral activity against the K181 laboratory strain of MCMV and the wild isolate G4 MCMV in vitro. GCV in combination with PFA, and CDV showed synergistic activity in vitro. GCV and CDV significantly reduced acute and chronic myocarditis when treatment commenced early after virus infection. However, antiviral therapy was not effective for chronic myocarditis when treatment commenced after establishment of myocarditis. PFA was not effective against acute myocarditis even when treatment commenced early after virus infection. This was most likely due to an inadequate treatment regime in the mouse studies. Reinfection of mice with the same or genetically different MCMV strain during the chronic phase of myocarditis caused exacerbation of inflammation in the myocardium. Antiviral treatment with GCV or CDV successfully eliminated this exacerbation, but failed to treat the pre-existing inflammation and necrosis of the heart in virus-infected animals. Antibody titres to cardiac myosin were not elevated in reinfected mice suggesting that increased disease did not correlate with an increase in anti-myosin autoantibody production. Antiviral drugs are an effective therapy against CMV-induced myocarditis when treatment commences very early after the onset of disease in the murine model. Such antiviral treatment is also effective when the risk of re-exposure to virus is high.
CHAPTER 5

Immunomodulation of Murine Cytomegalovirus-induced Myocarditis in Mice Treated with Lipopolysaccharide and Tumour Necrosis Factor
5.1 INTRODUCTION
The aetiological factors involved in the development of myocarditis have become evident since the introduction of endomyocardial biopsy (Feldman & McNamara, 2000). The role of virus in myocarditis is now unquestionable, with a wealth of data from the various animal models and human biopsies. MCMV infection of susceptible mice induces myocarditis with a predominantly CD8+ T cell infiltrate associated with areas of myocyte necrosis. Such inflammation is successfully prevented with antiviral drugs if treatment is commenced early after infection (Lenzo et al., 2001), thus providing further evidence that virus is the trigger for the development of cardiac disease, as examined in Chapter 4. Host genetic factors influence susceptibility to MCMV-induced myocarditis, as inbred mouse strains show variation to both the severity and the duration of disease (Lawson et al., 1990). Genes linked to the H-2 complex influence the susceptibility of mice to the development of acute myocarditis. The H-2 haplotypes associated with increased resistance to MCMV-induced myocarditis, with mice of the b haplotype (C57BL/6) being more resistant than those of the d haplotype (BALB/c) (Lawson et al., 1990). In the BALB/c strain cardiac inflammation is observed during acute MCMV infection, with a chronic phase of disease gradually increasing in severity and persisting for at least 100 days p.i. However, MCMV-infection of C57BL/6 mice results in only mild acute myocarditis, which is then completely resolved (Lawson et al., 1990).

As examined in Chapter 3, infectious virus cannot be isolated from cardiac tissue after day 10 p.i. (Lawson et al., 1990). Importantly, susceptibility to myocarditis does not correlate with the presence of viral antigen, viral genome or viral RNA transcripts in the heart, as these are present in both susceptible and resistant mouse strains. As inflammation continues in the myocardium of susceptible mice well after MCMV infection has been resolved emerging data suggests there are immunological mechanisms involved in MCMV-induced myocarditis (Lawson et al., 1990; O’Donoghue et al., 1990; Lawson et al., 1989; Lawson et al., 1991; Lawson et al., 1992; Fairweather et al., 1998).

In addition to the role of virus and genetic factors, autoimmunity is prevalent following MCMV infection. BALB/c and C57BL/6 mouse strains develop autoantibodies to myosin in response to MCMV infection, however only susceptible BALB/c mice
develop antibodies specific for the cardiac isoform of myosin during chronic myocarditis (O'Donoghue et al., 1990; Lawson et al., 1992). These cardiac myosin-reactive antibodies cross-react with MCMV polypeptides and the S2 region of cardiac myosin suggesting the possibility of molecular mimicry as a pathogenic mechanism (Lawson et al., 1992; Fairweather et al., 1998; Lawson, 2000). Furthermore, a role for T cells in the pathogenesis of viral myocarditis is indicated by the finding that T cell-deficient nude mice do not develop myocarditis, and cyclosporin A treatment of BALB/c mice suppresses the severity and delays the onset of myocarditis (Lawson et al., 1989). NK1.1\(^+\) cells are also important in the innate immune response to MCMV infection, particularly by conferring resistance in C57BL/6 mice (Bancroft et al., 1981; Scalzo et al., 1992).

CB3 infection of the heart is associated with the local production of specific cytokines in the heart. The immunomodulator LPS stimulates TNF and IL-1 production and exacerbates myocarditis in CB3-infected resistant B10.A mice (Lane et al., 1991; 1992; Neumann et al., 1993). In this chapter, attempts are disclosed to further dissect the immunological nature of MCMV-induced myocarditis by treating MCMV-infected mice with LPS and TNF to assess the immunomodulatory effect on myocarditis. The effect that these immunomodulators have on MCMV titres, myocarditis, endogenous cytokine production and antibody response are examined.
5.2 RESULTS

5.2.1 LPS treatment increases myocarditis in MCMV-infected susceptible and resistant mice.

The effect of LPS on the pathogenesis of MCMV-induced myocarditis was investigated. Susceptible BALB/c and resistant C57BL/6 mice were injected with either LPS, MCMV (6x10^3 PFU/mouse) or co-injected with both LPS and MCMV (LPS/MCMV). Hearts were examined histologically for myocarditis at days 7, 11, 28 and 56 p.i. Control, uninfected BALB/c and C57BL/6 mice injected only with LPS had negligible levels of myocarditis (day 7 p.i., 1.6±0.5 and 0.0±0.0, respectively). LPS/MCMV-infected BALB/c mice showed statistically significant increases in the level of myocarditis at days 7 (p<0.008), 11 (p<0.0006), 28 (p<0.003) and 56 (p<0.002) p.i. compared to untreated MCMV-infected mice (Fig. 5.1a). The greatest increase in myocarditis was observed in the chronic phase of the disease (day 56 p.i., approximately 6-fold). Interestingly, LPS/MCMV-infected C57BL/6 mice exhibited a significantly greater severity of myocarditis at days 7 (p<0.03), 11 (p<0.02) and 28 (p<0.025) p.i. compared to mice infected with MCMV only (Fig. 5.1b). LPS/MCMV-infected C57BL/6 mice displayed acute myocarditis (peak at day 7 p.i.) at levels similar to those observed for susceptible MCMV-infected BALB/c mice. Furthermore, LPS treatment induced chronic myocarditis at days 28 and 56 p.i. in MCMV-infected C57BL/6 mice, which do not normally develop chronic myocarditis. It should be noted that although myocarditis levels were increased following LPS treatment in C57BL/6 mice, levels remained much lower than those observed for BALB/c mice.

5.2.2 Virus titres are not affected in LPS/MCMV-infected mice.

To assess whether LPS treatment increased the titre of replicating virus, infectious virus was quantitated at days 7, 11, 28 and 56 p.i. in the salivary glands, a major target organ for persistent MCMV replication. Viral titres in LPS/MCMV-infected BALB/c mice reached a peak at day 28 p.i., declined to undetectable levels by day 56 p.i. (400 PFU/g) (Fig. 5.2a), and were not statistically different from mice infected with MCMV only. Similarly, no statistical difference in viral titres was observed for MCMV-infected C57BL/6 mice treated with or without LPS (Fig. 5.2b). Although viral titres in C57BL/6 mice were approximately 1.5 log_{10} lower than those found in BALB/c animals.
Figure 5.1. LPS promotes myocarditis in MCMV-infected mice.
BALB/c mice (A) and C57BL/6 mice (B) were inoculated with $6 \times 10^3$ PFU of MCMV i.p. and LPS at days 0 (50 µg) and 4 (25 µg) p.i., or infected with MCMV only. The average numbers of inflammatory foci/heart section from groups of 5 mice per time point ± SE are shown.
*Statistically significant differences between groups ($p<0.05$).
Figure 5.2. LPS treatment has no effect on virus titre in the salivary glands of mice infected with MCMV.

BALB/c (A) and C57BL/6 (B) mice were inoculated with $6 \times 10^3$ PFU MCMV i.p and LPS at days 0 (50 μg) and 4 (25 μg) p.i., or infected with MCMV only. Viral titres are expressed as the average log$_{10}$ PFU/g of salivary glands from groups of 5 mice per time point ±SE.

*Statistically significant differences between groups ($p<0.05$)
from days 7 through 28 p.i., higher levels of infectious virus persisted in the salivary glands of C57BL/6 mice at day 56 p.i.

In order to determine the affect of LPS on the level of infectious virus in other organs, infectious viral titres were determined in the heart, spleen and liver of MCMV-infected BALB/c mice (day 7 p.i.) treated with or without LPS. Infectious virus was not detected in the heart at the above-mentioned timepoints. The mean viral titres in the spleen (LPS, 790 PFU/g; untreated, 650 PFU/g) and liver (LPS, 28 PFU/g; untreated, 39 PFU/g) were found to be similar for infected mice treated either with or without LPS, respectively.

5.2.3 LPS treatment increases serum TNF levels in MCMV-infected mice.
TNF levels in the sera were found to be elevated when B10.A mice were infected with CB3 and treated with LPS (Lane et al., 1991). A corresponding exacerbation in myocardial inflammation was also observed in the CB3 model. Therefore serum TNF levels were measured in MCMV-infected mice treated with LPS. LPS treatment without virus infection stimulated a low level of TNF production at day 7 (approximately 170 pg/ml for each mouse strain. Circulating serum TNF levels during the acute phase of myocarditis (day 7 p.i.), from both virus-infected mouse strains, increased significantly (p<0.05) with LPS treatment (Fig. 5.3). TNF levels peaked early after infection (day 7 p.i.), with higher levels (1.7-fold) observed in BALB/c mice compared to C57BL/6 mice. However, during the chronic phase of the disease (day 28 and 56 p.i.), there was no statistically significant difference between the TNF levels from LPS-treated and untreated MCMV-infected mice.

5.2.4 Cytokine expression in sera following MCMV-infection and LPS treatment.
The production of the following cytokines, IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF was examined in LPS/MCMV-infected BALB/c mice (Fig. 5.4). Circulating levels of TNF in sera samples at day 7 p.i. were significantly increased with LPS treatment (p<0.0001). However, circulating IL-6, IL-10 and IFN-γ titres were unchanged with LPS treatment. Both IL-2 and IL-4 were undetectable in all sera tested.
Figure 5.3. LPS treatment increases circulating TNF levels during acute MCMV-induced myocarditis.

BALB/c (A) and C57BL/6 (B) mice were inoculated with 6x10³ PFU MCMV i.p. and LPS at days 0 (50 µg) and 4 (25 µg) p.i., or infected with MCMV only. The average titres of TNF (pg/ml) ±SE from serum of individual mice (5 mice per time point) are shown.

*Statistically significant differences between groups (p<0.05).
Figure 5.4. Cytokine levels in sera from LPS/MCMV-infected BALB/c mice.

Mice were inoculated with 6x10³ PFU MCMV i.p. and LPS at days 0 (50 µg) and 4 (25 µg) p.i., or infected with MCMV only. Sera was collected from BALB/c mice during the acute phase of disease (day 7 p.i.). Cytokine levels (IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF) were determined in the sera by ELISA and are expressed as the average titre (pg/ml) ±SE for sera from individual mice (5 mice per group).

*Statistically significant differences between groups (p<0.05).
5.2.5 Cytokine expression from heart infiltrating cells and splenocytes.
The effect of LPS treatment on cytokine production in the heart infiltrating lymphocytes and splenocytes of virus-infected BALB/c mice (Fig. 5.5) was next examined. Heart lymphocytes produced IL-6 after MCMV infection, with significantly increased levels observed after LPS treatment (p<0.0001). IL-10 was not detected in heart infiltrating lymphocytes after infection alone, however, significant levels were observed after LPS treatment (p<0.0001). IL-2, IL-4 and IFN-γ were not detected in the supernatant from lymphocytes recovered from the heart or spleen after only virus infection, nor affected by LPS treatment. Interestingly, while a significant increase in TNF was observed in the sera of LPS/MCMV-infected mice, TNF was not detected in the supernatant from heart lymphocytes after infection and only a slight increase was seen after LPS treatment. A slight increase in IL-6 was found in the splenic lymphocyte populations of LPS treated and MCMV-infected mice. TNF was also found in the heart infiltrating cells but not in the splenocytes derived from LPS treated mice with acute viral myocarditis.

5.2.6 TNF peptide exacerbates myocarditis in MCMV-infected C57BL/6 mice.
To determine whether TNF is responsible for the exacerbation of myocarditis after LPS treatment, MCMV-infected BALB/c and C57BL/6 mice were treated with TNF peptide. The synthetic TNF peptide fragment (aa 114-130) has been shown to demonstrate biological activity comparable with the active site of human and mouse TNF (Beutler & Cerami, 1986) and has been examined in the CB3 model (Lane et al., 1993). Acute myocarditis levels at days 7 and 11 p.i. did not significantly increase in BALB/c mice given TNF peptide (Fig. 5.6a). However, acute myocarditis at both of these time points was significantly increased in C57BL/6 mice given the TNF peptide (Fig. 5.6b, p<0.0005, p<0.03, respectively), similar to LPS treatment (Fig. 5.1b).

5.2.7 Antiviral effect of TNF peptide in MCMV-infected mice.
The effect of the biologically active TNF peptide treatment of MCMV-infected mice was assessed in the salivary glands at days 7 and 11 p.i. Viral titres in the salivary glands of both strains of mice treated with TNF peptide were markedly reduced at days 7 and 11 p.i. of BALB/c mice (Fig. 5.7a; p<0.03, p<0.009, respectively) and at day 7 p.i. of C57BL/6 mice (Fig. 5.7b; p<0.03).
Figure 5.5. Cytokine protein levels from heart infiltrating cells and splenocytes derived from LPS/MCMV-infected BALB/c mice.

Mice were inoculated with $6 \times 10^3$ PFU MCMV i.p. and LPS at days 0 (50 μg) and 4 (25 μg) p.i., or infected with MCMV only. Heart infiltrating cells and splenocytes were isolated from BALB/c mice during the acute phase of disease (day 7 p.i.) and were cultured without stimulation for 48 hours in vitro. Cytokine levels (IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF) were determined in the tissue culture supernatant by ELISA and are expressed as the average titre (pg/ml) ±SE (triplicate) from pooled heart and spleens (10 mice per group).

* Statistically significant difference between groups ($p<0.05$).
Figure 5.6. TNF peptide exacerbates acute viral myocarditis in C57BL/6 mice. Myocarditis in BALB/c (A) and C57BL/6 (B) mice inoculated with $6 \times 10^3$ PFU MCMV and TNF peptide at days 0 (250 ng) and 4 (100 ng) p.i., or infected with MCMV only. Myocarditis is expressed as the average number of inflammatory foci/heart section from groups of 5 mice per time point ±SE.

*Statistically significant differences between groups ($p<0.05$).
Figure 5.7.  TNF peptide exerts an anti-viral effect in MCMV-infected mice.  
MCMV titres in BALB/c (A) and C57BL/6 (B) mice inoculated with $6 \times 10^3$ PFU MCMV and TNF peptide at days 0 (250 ng) and 4 (100 ng) p.i., or infected with MCMV only. Viral titres are expressed as the average $\log_{10}$ PFU/g salivary glands from groups of 5 mice per time point ±SE.  
*Statistically significant differences between groups ($p<0.05$).
5.2.8 Serum cytokine expression in response to TNF peptide treatment.

In order to characterise the production of cytokines stimulated by TNF peptide treatment during viral infection, IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF titres, were measured from sera of MCMV-infected mice with acute myocarditis (Fig. 5.8a, day 7 p.i.). Both IL-2 and IL-4 were undetected in BALB/c and C57BL/6 mice. No significant change in the titres of circulating IL-6 and IL-10 were observed in BALB/c mice treated with TNF (Fig. 5.8a). However, BALB/c mice showed significantly elevated serum levels of endogenous TNF \( (p<0.0001) \) and reduced levels of IFN-γ \( (p<0.0001) \) protein expression with TNF peptide administration. TNF peptide treatment produced a similar effect to LPS on cytokine production (Fig. 5.4a) except for IFN-γ, which was significantly reduced after TNF treatment. A distinctly different expression profile was found for C57BL/6 mice for the cytokines IL-10, IFN-γ and TNF (Fig. 5.8b). C57BL/6 mice produced markedly lower titres of circulating IFN-γ than BALB/c mice following virus infection. With TNF peptide administration, C57BL/6 mice did not show an increase in endogenous TNF levels in the sera but did show significantly higher titres of IL-10 \( (p<0.02) \). The level of IFN-γ production was not influenced by TNF peptide treatment of MCMV-infected C57BL/6 mice.

5.2.9 Antibody responses in LPS-treated and MCMV-infected mice.

Both BALB/c and C57BL/6 mouse strains develop autoantibodies to myosin in response to MCMV infection, however, only susceptible BALB/c mice develop antibodies specific for the cardiac isoform of myosin during chronic myocarditis (O'Donoghue et al., 1990; Lawson et al., 1992). Interestingly, passive transfer of affinity-isolated anti-cardiac myosin IgG antibodies, obtained from late immune sera of MCMV-infected BALB/c mice, induces cellular inflammation and myocardial necrosis in uninfected animals (O'Donoghue et al., 1990; Lawson et al., 1992). This evidence suggests that the autoantibodies to the cardiac isoform of myosin play a role in the pathogenesis of MCMV-induced myocarditis. It was therefore determined whether cardiac myosin-specific autoantibodies were induced during LPS exacerbation of myocarditis in MCMV-infected mice. In separate experiments, sera were either unabsorbed, absorbed or eluted using skeletal and cardiac isoforms of myosin and the residual reactivity to cardiac myosin determined by ELISA. LPS treatment induced higher titres of anti-cardiac myosin antibodies in BALB/c mice at days 11 and 56 p.i.
Figure 5.8. Cytokine expression in sera of TNF peptide treated and MCMV-infected mice.

BALB/c (A) and C57BL/6 (B) mice were inoculated with $6 \times 10^3$ PFU MCMV and TNF peptide at days 0 (250 ng) and 4 (100 ng) p.i., or infected with MCMV only. Cytokine levels (IL-2, IL-4, IL-6, IL-10, IFN-$\gamma$, TNF) were determined in the sera at day 7 p.i. by ELISA and are expressed as the average titre (pg/ml) $\pm$SE for sera from individual mice (5 mice per group).

*Statistically significant differences between groups ($p<0.05$).
(Table 5.1) than mice infected with virus alone. Higher anti-cardiac myosin antibodies were also found after LPS treatment of C57BL/6 mice at day 11 p.i. (Table 5.2), indicating a possible role for autoantibodies in the development of chronic myocarditis. LPS-treated C57BL/6 mice produced antibodies to cardiac myosin during the chronic phase of the disease (day 56 p.i.), which could be fully absorbed with cardiac myosin but only partially absorbed with skeletal myosin (data not shown). These data suggest the existence of a population of cardiac isoform-specific antibodies induced by LPS treatment of virus-infected C57BL/6 mice. LPS treatment did not influence the anti-MCMV antibody titres of either MCMV-infected mouse strains during the acute phase of the disease (day 11 p.i., 160, BALB/c; 2,560, C57BL/6).
Table 5.1. Influence of LPS on cardiac myosin isoform specific antibodies in MCMV-infected BALB/c mice.

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>MCMV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MCMV/LPS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>11</td>
<td>27</td>
<td>81</td>
</tr>
<tr>
<td>56</td>
<td>9</td>
<td>27</td>
</tr>
</tbody>
</table>

<sup>a</sup> Endpoint antibody titre (reciprocal dilution) in pooled sera (5 mice per group) determined by ELISA using cardiac myosin as the antigen. BALB/c mice were injected with either saline or LPS and infected with 6x10<sup>3</sup> PFU of MCMV i.p. Sera was collected on days 11 and 56 p.i. and absorbed against skeletal myosin. Eluted antibodies were specific for the cardiac isoform of myosin.
Table 5.2. Influence of LPS on cardiac myosin isoform specific antibodies in MCMV-infected C57BL/6 mice.

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>MCMV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MCMV/LPS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>9</td>
<td>2187</td>
</tr>
<tr>
<td>56</td>
<td>243</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

<sup>a</sup> Endpoint antibody titre (reciprocal dilution) in pooled sera (5 mice per group) determined by ELISA using cardiac myosin as the antigen. C57BL/6 mice were injected with either saline or LPS and infected with 6x10³ PFU MCMV i.p. Sera were collected on days 11 and 56 p.i. and absorbed against skeletal myosin. Eluted antibodies were specific for the cardiac isoform of myosin.
5.3 DISCUSSION

BALB/c mice are susceptible to the development of myocarditis following MCMV infection, whereas C57BL/6 mice similarly infected with virus are resistant (Lawson et al., 1990). The results in this chapter report that LPS treatment of mouse strains either susceptible or resistant to MCMV-induced myocarditis was capable of exacerbating inflammation in the heart. Thus, for the first time, it has been shown that resistant C57BL/6 mice develop chronic disease triggered by MCMV infection concurrent with LPS treatment. It has also been shown that the increased severity of myocarditis with LPS treatment was not due to an increase in viral replication in target organs, namely the heart, spleen, liver, and salivary glands. These results confirm the previous findings of the lack of correlation between replicative virus load and cardiac inflammation (Lawson, 2000). However, the increased severity of acute myocarditis stimulated by LPS treatment correlated with an increase in circulating TNF in the sera of both mouse strains. The production of the cytokines IL-6 and IL-10 were also elevated in cultures of heart infiltrating lymphocytes taken from LPS/MCMV-infected BALB/c mice, however, TNF production was not significantly increased in lymphocytes from the heart. These results provide insights into the role of TNF in partly regulating the development of disease. However, it is important to note that TNF levels cannot reliably be used as a diagnostic marker of disease since TNF titres in the sera did not accurately reflect the level of TNF production in affected tissues such as the heart and spleen. Thus it is important to measure cytokine levels at the site of disease, rather than a systemic serum measurement.

TNF peptide treatment of MCMV-infected mice suggests that TNF release after LPS treatment leads to increased myocarditis in C57BL/6 mice. However, separate mechanisms may be employed in the development of disease after LPS treatment in the BALB/c mouse strain, since myocarditis was not increased after TNF peptide treatment. Despite the above differences in disease outcome, there was a reduction of acute viral titres in the salivary glands after administration of the biologically active TNF peptide in both mouse strains. The reduction in circulating IFN-γ in BALB/c mice may also play a role in myocarditis development after TNF treatment. These differences in the progression of disease require further investigation, however, this study indicates that TNF has the ability to affect the development of myocarditis. The cytokine expression profiles at day 7 p.i. of BALB/c and C57BL/6 MCMV-infected mice suggest that
myocarditis is driven by distinct immunopathogenic mechanisms in these two mouse strains. Even with LPS treatment, C57BL/6 mice remained more resistant to MCMV-induced myocarditis than BALB/c mice.

The ability of LPS and TNF to produce disease in resistant mice is not a phenomenon found only for MCMV, but also occurs after CB3 infection of resistant B10.A mice (Lane et al., 1991). Indeed, the cytokines IL-1 and TNF have been shown to induce myocarditis when administered in conjunction with CB3 virus in B10.A mice which are also resistant to the development of disease (Lane et al., 1992; 1993). The similar results obtained with both the MCMV and CB3 myocarditis models suggests that the immune response to a viral infection is more important in determining the outcome of disease than the replication of the virus itself. Proinflammatory cytokines released in the heart or serum after viral infection may attract inflammatory cells to the heart (Penninger et al., 1996). Recent evidence has shown that LPS binds with high affinity to CD14, a glycosyl phosphatidylinositol-linked protein expressed on the surface of macrophages and to a Toll-like receptor 4 (TLR-4) protein (Lien et al., 2000). LPS binding activates the transmembrane TLR-4 protein, initiating an intracellular signalling cascade, that ultimately leads to the expression of immunomodulatory genes, including cytokines and co-stimulatory molecules. Rapid recognition of the LPS component of bacterial cell walls through Toll-like receptors is an important part of an effective innate immune response. In the MCMV-induced myocarditis model, increased levels of either TNF, IL-6 or IL-10 released from heart infiltrating cells after LPS treatment may result in exacerbated myocardial inflammation. Thus, LPS-induced cytokines may be capable of regulating the development of the cardiac inflammatory response triggered by MCMV infection.

Cardiac myosin-specific antibodies are normally found in MCMV-infected BALB/c mice during the chronic phase of disease (O'Donoghue et al., 1990; Lawson et al., 1992). Here it has been shown that LPS stimulated greater levels of anti-cardiac myosin specific antibody in both the susceptible and resistant strains during the chronic phase of disease. The heightened cardiac-specific antibody response stimulated by LPS may contribute to the exacerbation of the disease.
This study was designed to determine the role of LPS and the LPS-induced cytokine TNF in the development of myocarditis following MCMV infection. Early insult to cardiac tissue may involve direct viral infection of the heart. Myocardial damage may be further mediated by the innate immune responses such as activated NK cell response to viral infection. This may lead to the production of inflammatory cytokines by activated cells which may be pathogenic in the heart. TNF and IFN-γ are released after MCMV infection where they can influence the development of adaptive immunity (Orange & Biron, 1996). Post-viral mechanisms may involve the adaptive immune response, such as activated B cells associated with the production of autoantibodies directed at myofibre autoantigens, and/or the activation of cardiac antigen presenting cells, as well as CD4+ and CD8+ T cells, with associated release of the proinflammatory cytokines TNF and IL-6.

Together, these results have shown that LPS is capable of inducing chronic myocarditis in MCMV infected resistant mice. These factors, along with the cytokines TNF, IL-6 and IL-10, and anti-myosin antibodies may play important roles in the pathogenesis of viral myocarditis. Importantly, the results suggest that pathogenic epitopes of cardiac myosin can be effectively presented to the immune system in C57BL/6 mice with LPS treatment, resulting in both the exacerbation of the acute phase and the development of a chronic phase of disease. Hence, MCMV-infected C57BL/6 mice, in the absence of LPS, must regulate their immune response and prevent autoreactive responses to disease-inducing self epitopes, such as cardiac myosin. There needs to be an exquisite balance between mechanisms of viral clearing and injury to myocytes. Reasons for the apparent dysregulation of the immune response following MCMV infection in BALB/c mice need to be further investigated.
CHAPTER 5 SUMMARY

The results in this chapter show the effect of administration of the immunomodulator LPS on the development of post-viral myocarditis in both susceptible BALB/c and resistant C57BL/6 mice. LPS exacerbated heart inflammation in both strains of MCMV-infected mice, with normally resistant C57BL/6 mice developing chronic myocarditis. LPS treatment also increased the production of TNF in the sera without affecting virus titres in the salivary glands, a target organ most affected during persistent virus infection. In the LPS/MCMV-infected BALB/c mouse, TNF and IL-10 titres were detected in cultures of heart infiltrating lymphocytes but not in splenic lymphocytes. Similarly, IL-6 titres were increased in heart infiltrating lymphocytes compared to splenocytes in LPS/MCMV-infected BALB/c mice. Importantly, administration of the biologically active TNF peptide increased myocarditis in C57BL/6 mice but not in BALB/c mice although an antiviral affect was observed in the salivary glands of both mouse strains. TNF peptide/MCMV-infected BALB/c and C57BL/6 mice showed distinct differences in the serum expression pattern of IFN-γ, IL-10 and TNF. Anti-myosin autoantibodies were markedly increased in LPS/MCMV-infected mice with a population of autoantibodies being specific for the cardiac isoform of myosin. These data show that factors such as the production of the cytokines TNF, IL-6 and IL-10 play important roles in the pathogenesis of viral myocarditis and highlight the complex immunopathogenic nature of MCMV-induced myocarditis.
CHAPTER 6

Cytokine Production in Response to MCMV Infection and Myocarditis
6.1 INTRODUCTION

The results presented in this thesis thus far clearly show two distinct mechanisms operating in MCMV-induced myocarditis. Firstly, MCMV infection is critical for the development of myocarditis in this model. Whilst MCMV has the capacity to directly kill myocytes and is clearly the trigger for myocardial inflammation and necrosis as examined in Chapter 3, infectious virus levels are extremely low in the heart of infected mice. Indeed, infectious virus can only be detected in the heart of MCMV-infected BALB/c mice by plaque assay during the acute phase of disease. RT-PCR for the viral transcript gB, a late structural protein indicative of virus replication, showed that virus is indeed present in the heart until at least day 35 p.i. However, given the low level of virus detected in the heart and the fact that antiviral therapy is ineffective during the chronic phase of disease, it appears unlikely that direct viral damage of the myocytes is the major pathological mechanism.

The second mechanism operating during MCMV-induced myocarditis is clearly immunological in nature. Previous research has shown a role for autoantibodies directed against cardiac myosin (Lawson et al., 1992), as well as a role for T lymphocytes in the development of myocarditis (Lawson et al., 1989). The consequences of administering the immunomodulator LPS on myocarditis in both susceptible and resistant mouse strains were explored in chapter 5. Once again the immunopathological nature of this disease was highlighted with the susceptible BALB/c mouse strain exhibiting exacerbated myocardial inflammation and the resistant C57BL/6 strain developing chronic disease. This exacerbation of disease occurred independent of virus titres in any of the target organs and was also associated with a change in cytokine expression patterns. LPS treatment caused an increase in systemic TNF as well as increased production of IL-6 and IL-10 in the heart infiltrating cells.

Clearly cytokines play an important role in the development of myocarditis. Previous research in other animal models highlights this importance. The development of autoimmune myocarditis in CB3-infected mice is associated with infiltration of the heart by inflammatory cells that secrete TNF and IL-1 (Lane et al., 1993). Indeed, administration of TNF or IL-1 together with CB3 can promote myocarditis in normally resistant B10.A mice (Lane et al., 1992). Very recent studies have shown TNF to have a protective role in acute encephalomyocarditis virus-induced myocarditis (Wada et al.,
2001), a conclusion drawn from investigation of mice lacking TNF. IL-6, another proinflammatory cytokine has also been shown to paradoxically suppress inflammation in various animal models (Ulich et al., 1991; Barton & Jackson, 1993). The time of administration of IL-6 was critical, with no beneficial effect being observed when cytokine treatment began later in infection. In addition to the above mentioned cytokines, several others were examined as they have properties that may play important roles in cardiac disease. IL-2 is predominantly associated with T cell activation which in turn is thought to play a crucial role in many viral infections and the subsequent inflammation that may follow. IL-4 is the prototypic Th2-type cytokine that recruits Th2 cells to the site of inflammation (Nelms et al., 1999). IL-18 has been shown to be an important regulator of both innate and acquired immune responses (McInnes et al., 2000). The ability of IL-18 to modulate the activity of Th1/Th2 cells renders it an important cytokine to study in autoimmune inflammatory diseases. IFN-γ is the major cytokine product of the Th1 cell population. The ability of IFN-γ to induce the cell surface expression of class II MHC molecules on myocytes in human myocarditis (Herskowitz et al., 1988) makes it a possible contributor to myocardial damage. There is clearly a delicate balance between maintaining the innate immune response, thereby controlling virus infection and over expression of proinflammatory cytokines that lead to long-term detrimental effects.

The research described in this chapter examines the production of various cytokines in response to MCMV infection in an attempt to determine which cytokines play an important role in the development of both acute and chronic myocarditis. Expression patterns are compared between susceptible and resistant mouse strains as well as between the acute and chronic phases of disease. The disparity of expression profiles between the local site of inflammation and systemic levels is also addressed.
6.2 RESULTS

6.2.1 IL-2 protein levels in the heart and sera of BALB/c and C57BL/6 mice following MCMV infection.

In order to investigate the possible role of IL-2 in MCMV-induced myocarditis, levels were measured in the sera and hearts of susceptible BALB/c and resistant C57BL/6 mice across the acute and chronic phases of disease. Mice were infected with $1 \times 10^4$ PFU MCMV i.p. and at various time points p.i. sera were collected and hearts removed. Hearts were blotted on absorbent paper to remove excess blood and then homogenised in sterile PBS to form a 20% homogenate which was clarified by centrifugation at 1000 $g$ for 20 minutes. IL-2 was undetectable by ELISA in the hearts of uninfected BALB/c mice, with very low levels (< 5 pg/ml) being detected in the hearts of uninfected C57BL/6 mice. After MCMV infection of BALB/c mice, levels increased moderately, peaking at day 5 p.i. No IL-2 was detected at days 35 or 42 p.i. however, low levels were observed on day 49 and 59 p.i. Following MCMV infection of C57BL/6 mice, increased levels were observed on all days p.i. except for days 5, 7 and 56 p.i. (Fig. 6.1a). C57BL/6 mice produced more IL-2 in the heart on days 3, 10, 21, 28, 35, 42 and 49 p.i. compared to BALB/c mice. Following MCMV infection, serum IL-2 was only detected on days 3 and 7 p.i. in both mouse strains (Fig 6.1b). It should be noted that the levels of IL-2 observed were extremely low, both in the heart and serum, with peak concentrations of approximately 35 pg/ml.

6.2.2 IL-4 proteins levels in the heart and sera of BALB/c and C57BL/6 mice following MCMV infection.

IL-4 was examined in MCMV-induced myocarditis in a similar fashion to IL-2 (6.2.1). Both uninfected BALB/c and C57BL/6 mice produced IL-4 in the heart. After MCMV infection, the BALB/c strain produced significantly more IL-4 on day 5 p.i. IL-4 levels in the hearts of C57BL/6 mice did not increase after MCMV infection. BALB/c mice had significantly higher levels of IL-4 in the heart on days 3, 21, 49 and 56 p.i. when compared to the C57BL/6 strain, suggesting a Th2-like phenotype in BALB/c mice (Fig. 6.2a). IL-4 was detected in the sera from both mouse strains with significant levels being produced when compared to uninfected control mice on day 3 p.i. only (Fig. 6.2b), levels then declined to baseline by day 10 p.i.
Figure 6.1. IL-2 levels following MCMV infection.
BALB/c and C57BL/6 mice were inoculated with 1x10⁴ PFU MCMV i.p. on day 0. On various days p.i. hearts were removed and homogenised in PBS and serum was collected. IL-2 levels were determined in the heart homogenates (A) and sera (B) by ELISA and are expressed as the average titre (pg/ml) ± SE for individual mice (5 mice per group).

*Statistically significant difference compared to uninfected mice (p<0.05).
Figure 6.2.  IL-4 levels following MCMV infection.  
BALB/c and C57BL/6 mice were inoculated with $1 \times 10^4$ PFU MCMV i.p. on day 0. On various days p.i. hearts were removed and homogenised in PBS and serum was collected. IL-4 levels were determined in the heart homogenates (A) and sera (B) by ELISA and are expressed as the average titre (pg/ml) ± SE for individual mice (5 mice per group).

*Statistically significant difference compared to uninfected mice ($p<0.05$).
6.2.3 IL-6 protein levels in the heart and sera of BALB/c and C57BL/6 mice following MCMV infection.

As IL-6 is an important cytokine involved in the acute phase of the immune response, its levels in the heart and sera were examined in MCMV-infected BALB/c and C57BL/6 mice. Again, various time points were examined across the acute and chronic phases of MCMV-induced myocarditis. Very low levels of IL-6 were detected in the hearts of uninfected BALB/c and C57BL/6 mice (< 5 pg/ml). Very little IL-6 was observed in the heart following MCMV infection in both strains of mice. Increased levels were only statistically significant on day 5 p.i. in the BALB/c strain (Fig. 6.3a). Interestingly, high levels of IL-6 were measured in the sera from both strains of mice following MCMV infection (Fig. 6.3b). Significant levels of IL-6 were observed on days 3, 5, 7, 10 and 14 p.i. in BALB/c mice and on days 3, 5, and 7 in the C57BL/6 strain. The resistant C57BL/6 strain produce significantly higher serum levels of IL-6 early in infection (day 3 p.i.) compared to the susceptible BALB/c strain.

6.2.4 IL-10 protein levels in the heart and sera of BALB/c and C57BL/6 mice following MCMV infection.

The anti-inflammatory role of IL-10 has been examined in various animal models of myocarditis, primarily as a therapeutic agent. The role of IL-10 in MCMV-induced myocarditis has not previously been examined, therefore levels in the heart and sera following MCMV-infection were measured through the acute and chronic phases of disease. IL-10 was observed in the hearts of uninfected BALB/c and C57BL/6 mice. A large increase in the production of IL-10 in the heart was measured following MCMV infection (Fig. 6.4a). BALB/c mice exhibited significantly higher levels of IL-10 on days 3, 5 and 7 p.i. compared to control mice, whereas, C57BL/6 mice had significantly higher levels of IL-10 on days 3, 5, 7, 10, 14 and 21 p.i. compared to control mice. Whilst the susceptible BALB/c strain initially produced higher levels of IL-10 in the heart, the resistant C57BL/6 strain continued to produce significant levels of IL-10 in the heart when levels had subsided back to baseline in the BALB/c strain.

IL-10 was detected in the sera of uninfected mice from both strains. Following MCMV infection IL-10 levels increased significantly in the BALB/c strain on days 3, 5, 7 and 10 p.i. Significantly higher levels were observed on days 3 and 7 p.i. in the C57BL/6 strain. As seen for IL-6, resistant C57BL/6 mice produce significantly higher levels of
Figure 6.3. IL-6 levels following MCMV infection. BALB/c and C57BL/6 mice were inoculated with $1 \times 10^4$ PFU MCMV i.p. on day 0. On various days p.i. hearts were removed and homogenised in PBS and serum was collected. IL-6 levels were determined in the heart homogenate (A) and sera (B) by ELISA and are expressed as the average titre (pg/ml) ±SE for individual mice (5 mice per group).

*Statistically significant difference compared to uninfected mice ($p<0.05$).
Figure 6.4. IL-10 levels following MCMV infection.
BALB/c and C57BL/6 mice were inoculated with $1 \times 10^4$ PFU MCMV i.p. on day 0. On various days p.i. hearts were removed and homogenised in PBS and serum was collected. IL-10 levels were determined in the heart homogenates (A) and sera (B) by ELISA and are expressed as the average titre (pg/ml) ± SE for individual mice (5 mice per group).
*Statistically significant difference compared to uninfected mice ($p<0.05$).
serum IL-10 early in infection (day 3 p.i.) compared to the susceptible BALB/c mice (Fig. 6.4b).

6.2.5 IL-18 protein levels in the heart and sera of BALB/c and C57BL/6 mice following MCMV infection.

IL-18 has been implicated in various autoimmune inflammatory diseases and has been examined for therapeutic efficacy in EMCV-myocarditis. The ability of IL-18 to modulate the early immune response proved therapeutic in the EMCV model when administered very early in infection. Whilst exogenous administration has been examined in this disease, the endogenous production of this cytokine during infection has not been examined. Using the MCMV-induced model of myocarditis, IL-18 levels were examined in the resistant and susceptible mouse strains in both the acute and chronic phases of disease.

IL-18 was detected in the hearts of uninfected BALB/c and C57BL/6 mice. Levels increased following MCMV infection peaking at day 3 p.i. for C57BL/6 mice and day 5 p.i. for BALB/c mice. Significantly higher levels were maintained in BALB/c mice out to day 21 p.i. whereas in the C57BL/6 strain, levels were significantly higher compared to the uninfected control on only days 3 and 5 p.i. (Fig. 6.5a). Low levels of IL-18 were detected in the sera of BALB/c mice on days 3 and 7 p.i. only. No IL-18 was detected in the sera from C57BL/6 mice (Fig. 6.5b).

6.2.6 IFN-γ protein levels in the heart and sera of BALB/c and C57BL/6 mice following MCMV infection.

The anti-viral effect of IFN-γ may afford protection from MCMV infection, however the subsequent immune cell activation may contribute to inflammation in the myocardium. IFN-γ levels were examined in the heart and serum of C57BL/6 and BALB/c mice infected with MCMV. Both strains produced low levels of the cytokine in the heart before infection. Levels of IFN-γ significantly rose in both strains following MCMV infection (Fig. 6.6a). Susceptible BALB/c mice had significantly higher levels compared to the uninfected control on days 3, 5 and 7 p.i whereas the resistant C57BL/6 mice continued to have significantly higher levels in the heart from day 3 to day 35 p.i. Very low levels of IFN-γ were observed in the sera of uninfected mice. A dramatic
Figure 6.5. IL-18 levels following MCMV infection.
BALB/c and C57BL/6 mice were inoculated with $1 \times 10^4$ PFU MCMV i.p. on day 0. On various days p.i. hearts were removed and homogenised in PBS and serum was collected. IL-18 levels were determined in the heart homogenates (A) and sera (B) by ELISA and are expressed as the average titre (pg/ml) ±SE for individual mice (5 mice per group).

*Statistically significant difference compared to uninfected mice ($p<0.05$).
Figure 6.6. IFN-γ levels following MCMV infection.
BALB/c and C57BL/6 mice were inoculated with 1x10⁴ PFU MCMV i.p. on day 0. On various days p.i. hearts were removed and homogenised in PBS and serum was collected. IFN-γ levels were determined in the heart homogenate (A) and sera (B) by ELISA and are expressed as the average titre (pg/ml) ±SE for individual mice (5 mice per group).
*Statistically significant difference compared to uninfected mice (p<0.05).
increase was observed on day 3 p.i. in the BALB/c strain, but levels decline to uninfected levels by day 10 p.i. A moderate but still significant increase was measured in the C57BL/6 strain on day 3 p.i., however levels decreased to uninfected levels by day 7 p.i. (Fig. 6.6b).

### 6.2.7 TNF protein levels in the heart and sera of BALB/c and C57BL/6 mice following MCMV infection.

The proinflammatory effects of TNF are well documented. The role of this cytokine in myocarditis has been the focus of intense research in several of the animal models. Chapter 5 reported increased levels of this cytokine in LPS treated, MCMV infected mice, and that exogenous administration exacerbated acute myocarditis in normally resistant C57BL/6 mice. Here, the levels of TNF are examined in the hearts and sera of susceptible and resistant mice across the acute and chronic phases of MCMV-induced myocarditis.

TNF was present in the hearts of uninfected mice of both strains. A significant increase was observed following MCMV infection in both strains (Fig. 6.7a). TNF levels increased in the hearts of susceptible BALB/c mice, peaking at day 7 p.i. and subsiding to uninfected levels by day 10 p.i. Levels TNF in the C57BL/6 strain peaked at day 3 p.i., returning to uninfected levels by day 7 p.i. No significant TNF was measured in the sera of either mouse strain (Fig. 6.7b).

### 6.2.8 Cytokine response to MCMV reinfection.

The effect of MCMV reinfection with the same or genetically different strain of virus was examined in Chapter 5. Mice were infected with the K181 laboratory strain of MCMV on day 0, then reinfeclted with either the K181 or G4 strain of MCMV on day 56 p.i. Such reinfection causes exacerbation of myocarditis with both strains of virus, even though no infectious virus was detected in the any of the target organs, namely the spleen, liver, or salivary gland.

Clearly the cytokine response following repeated exposure to virus is an important event in the immune response, therefore, levels of the cytokines IL-2, IL-4, IL-6, IL-10, IL-18, IFN-γ and TNF were examined in BALB/c mice upon reinfection with the same strain (K181 MCMV) or a genetically different strain (G4 MCMV) of MCMV. Serum
Figure 6.7. TNF levels following MCMV infection.
BALB/c and C57BL/6 mice were inoculated with $1 \times 10^4$ PFU MCMV i.p. on day 0. On various days p.i. hearts were removed and homogenised in PBS and serum was collected. TNF levels were determined in the heart homogenate (A) and sera (B) by ELISA and are expressed as the average titre (pg/ml) ±SE for individual mice (5 mice per group).

*Statistically significant difference compared to uninfected mice ($p<0.05$).
cytokines levels were measured by ELISA on day 56 post-initial infection (day 0) and day 7 post-reinfection (day 63 post initial infection). No IL-2, IL-18 or TNF was measured on day 0 or day 7. No IL-4 was measured at day 56 post-initial infection, however, low amounts of IL-4 were observed following reinfection with either strain of virus at day 7 post reinfection (Fig. 6.8a). IL-6 was also absent on day 56 post-initial infection, with very low levels being produced after reinfection with either strain of MCMV by day 7 post-reinfection (Fig. 6.8b). Low levels of IL-10 were observed at day 56 post-initial infection, with a significant increase measured following reinfection (Fig. 6.8c). As seen with IL-10, IFN-γ levels were low at day 56 post-initial infection. Following reinfection with either strain of MCMV significant increases were observed (Fig. 6.8d) for IFN-γ.
Figure 6.8. Cytokine production following MCMV reinfection. 

BALB/c mice were inoculated with $1 \times 10^4$ PFU MCMV (K181 strain) i.p. on day 0. Mice were then reinjected with $1 \times 10^4$ PFU of either K181 or G4 MCMV i.p. on day 56 p.i. Sera were collected on day 7 post reinfection and cytokine levels measured by ELISA. IL-4 (A), IL-6 (B), IL-10 (C) and IFN-γ (D) levels are expressed as the average titre (pg/ml) ± SE for individual mice (5 mice per group).

*Statistically significant difference after reinfection ($p<0.05$).
6.3 DISCUSSION

Several pathogenic mechanisms have been implicated in the inflammation and necrosis found in virus-induced myocarditis. This chapter has focused on the cytokine response to infection, both systemically and in the heart. MCMV infection of mice is known to induce cytokines which can directly inhibit virus replication and promote defence by activating immune cells (Orange & Biron, 1996). Production of cytokines in response to infection may play an important role in the subsequent myocardial inflammation. Previous research has demonstrated that exogenous cytokine administration can affect the course of myocarditis, indicating that the type of cytokine response modulates the subsequent immune response and thus directly affects disease severity. The cytokines IL-2, IL-4, IL-6, IL-10, IL-18, IFN-γ and TNF are examined in this chapter based on their known roles in autoimmune disease, inflammation and Th1/Th2 type responses.

MCMV is a virus that targets various organs in the body, such as the spleen, liver, salivary gland, lungs and heart. Cytokine levels measured in the sera would therefore reflect this systemic infection and not be an adequate reflection of what is occurring in the heart during inflammation. The research described in this chapter, therefore, examines cytokine levels both systemically and in the heart. Marked differences were observed between the sera and the heart homogenates. For example, IFN-γ in the heart and sera of BALB/c mice both peak on day 3 p.i. at 350 pg/ml and 3000 pg/ml respectively. Alternatively, TNF levels peaked at 550 pg/ml in the heart, but only at 25 pg/ml in the sera. The results presented in this chapter clearly demonstrate the disparity observed when measuring cytokines from different tissue sites. Any conclusions drawn from such measurements must take into account the issue of systemic levels versus local production.

IL-2 levels observed in the heart of MCMV-infected mice peak at 30 pg/ml, a level that may induce responses in the local site of the heart. Though not statistically significant, the C57BL/6 strain demonstrated higher levels of IL-2 in the heart in the chronic phase of disease compared to the BALB/c strain. This result questions the role of IL-2 in MCMV-induced myocarditis given that C57BL/6 mice do not develop chronic myocarditis and previous research has shown IL-2 administration later in disease exacerbates CB3-induced myocarditis (Kishimoto et al., 1994). Further research shows
that IL-2 gene knockout C57BL/6 mice do not develop chronic myocarditis (Grassl et al., 1997), suggesting that the levels of IL-2 observed in the hearts of C57BL/6 MCMV-infected mice are not protective.

Th1/Th2 phenotypes have been implicated in susceptibility versus resistance theories with autoimmune diseases believed to be mediated by predominantly Th1-like responses (Liblau et al., 1995; Mosmann & Sad, 1996). However, recent research has shown that experimental autoimmune myocarditis in A/J mice has a Th2-like phenotype (Afanasyeva et al., 2001). Levels of IL-4, the prototypic Th2-type cytokine, were significantly higher in the hearts of BALB/c mice compared to C57BL/6 mice on several time points across the acute and chronic phases of myocarditis. This result agrees with the experimental autoimmune model of disease, in that a Th2 response is being mounted in the hearts of susceptible BALB/c mice. IL-4 production in the heart could stimulate Th2 cell development, activate B cells, induce MHC class II expression as well as recruiting Th2 cells to the site of inflammation.

The varied biological activities of IL-6 have made it an important candidate for research in cardiac disease. Clinical studies have shown that circulating levels of IL-6 are increased in human patients with congestive heart failure (Wollert & Drexter, 2001). Investigations into the role of this cytokine in myocarditis have produced mixed results. Exogenous administration of IL-6 to mice infected with EMCV reduced myocardial damage if administration occurred with virus inoculation. Treatment after virus infection had been initialised had no effect on myocardial inflammation (Kanda et al., 1996). MCMV infection of BALB/c mice induced low levels of IL-6 in the heart on day 5 p.i. compared to the high levels of IL-6 measured systemically after infection out to day 14 p.i. These data reflect work carried out in the CB3 model of myocarditis. IL-6 mRNA transcripts were only detected in the heart at low levels early in the acute phase of CB3-induced cardiac disease (Schmidtke et al., 2000). Systemic levels of IL-6 in MCMV-infected mice show significant increases in both susceptible and resistant mouse strains following infection. Collectively, the results presented in this chapter show that any role IL-6 is playing in MCMV-induced myocarditis is restricted to the early acute phase of disease. IL-6 may promote early adaptive immune responses in order to attenuate MCMV replication.
The anti-inflammatory properties of IL-10 suggest that it may play an important role in limiting the inflammatory response in myocarditis. Administration of IL-10 with EMCV suppressed myocardial inflammation without altering virus replication, however, treatment after virus infection has no effect (Nishio et al., 1999). IL-10 has been reported to play an important role in CMV infection (Redpath et al., 1999). CMV can down regulate MHC class II expression on macrophages by the induction of IL-10, indeed, CMV itself encodes an IL-10 homologue (Kotenko et al., 2000). MCMV infection of susceptible BALB/c and resistant C57BL/6 mice caused increased IL-10 levels in the heart of both strains. In BALB/c mice, IL-10 levels decline to uninfected levels by day 10 p.i. However, in C57BL/6 mice, significantly increased levels persist in the heart until day 21 p.i. This raises the interesting possibility that the resistant strain is able to keep IL-10 production elevated in the heart in order to suppress inflammation, whereas the susceptible strain, with low levels of IL-10 develops chronic myocardial inflammation. Early IL-10 production may be beneficial for MCMV, in that it can interfere with antigen presentation and hence allow for more virus replication. Clearly a delicate balance must be achieved between reducing the inflammatory response in the heart and reducing virus replication.

MCMV-infected BALB/c mice exhibited statistically significant levels of IL-18 in the heart across the acute and leading into the chronic phase of disease. C57BL/6 mice produced significant levels on day 3 p.i. only, indicating that this cytokine is important in the early stage of disease. The implications of the susceptible strain producing more IL-18 for an extended period of time in the heart are many. IL-18 is known to promote the expression of the pro-inflammatory cytokine TNF as well as upregulating iNOS (reviewed in McInnes et al., 2000). Treatment with IL-18 early in EMCV infection reduced myocarditis, whereas treatment commencing later in infection had no effect (Kanda et al., 2000). The many functions of IL-18 make it impossible to draw definitive conclusions from this data, however, it can be speculated that the poor reduction of cardiac inflammation in susceptible BALB/c until after day 21 p.i. may be a contributing factor in the development of chronic myocarditis.

IFN-γ has been implicated in myocarditis with extensive research being conducted in several animal models. IFN-γ is a strong activation signal for NK cells and is therefore
an important cytokine in antiviral defence, as shown in the CB3 model of myocarditis (Horwitz et al., 2000). C57BL/6 mice produce significant levels of IFN-γ in the heart until day 28 following MCMV infection. IFN-γ levels in the heart of BALB/c mice return to uninfected levels by day 10 p.i. The consequences of maintained IFN-γ levels in the resistant strain may be the maintenance of a Th1-type response, hence a highly antiviral state and the avoidance of a Th2-type response that is now seen as detrimental in myocarditis (Afanasyeva et al., 2001).

Clearly a delicate balance between viral clearance and inflammation is needed for the successful resolution of infection without damaging the host. This is most eloquently observed in the TNF response to virus infection and myocarditis. TNF plays a critical role in activating host defence mechanisms, however, over expression produces negative consequences. The exogenous administration of TNF aggravates myocarditis and the neutralisation of TNF by antibodies or soluble receptors attenuates viral myocarditis (Yamada et al., 1994). Alternatively, TNF gene deficient mice infected with EMCV have a decreased survival rate, which is subsequently improved by exogenous administration of TNF (Wada et al., 2001). TNF levels peaked in MCMV-infected C57BL/6 mice on day 3 p.i. whereas levels peaked on day 5 p.i. in BALB/c mice. Collectively, these data suggest that an early TNF response is critical to the immune response against virus. A delayed or extended response results in myocardial inflammation.

Chapter 4 examined the effect that MCMV reinfection has on myocarditis. Reinfection is a common problem associated with the immunosuppressed, those that have multiple sexual partners and in children who attend childcare centres (Bale et al., 1996). The results presented in chapter 4 showed exacerbation of chronic myocarditis by MCMV reinfection with the same or genetically different strain. This chapter examined the effect that reinfection has on cytokine production at the systemic level. The cytokines examined showed modest increases, if any, 7 days post-reinfection. Such increases in IFN-γ, IL-10, IL-6, and IL-4 in the BALB/c strain may contribute to the very rapid clearance of virus by the primed immune system, as well as contributing to further inflammation by the various mechanisms previously alluded to.
Cytokine activities occur in complex balanced networks dependent on variables such as the type of antigen and the genetic predisposition of the organism. The results presented in this chapter form the basis of continuing work into the cytokine response to MCMV and subsequent effect on myocarditis. This large kinetic study attempted to define differences between the acute and chronic phase of disease as well as to determine the differences in cytokine response between susceptible and resistant strains of mice.
CHAPTER 6 SUMMARY

A large kinetic study examining the cytokine response to MCMV infection was performed. This study examined several cytokines previously shown to be important in myocarditis in humans and various animal models. Levels were examined in the sera and the hearts of susceptible BALB/c and resistant C57BL/6 mice during the acute and chronic phases of disease. These two mouse strains mounted different cytokine responses to MCMV, both systemically and in the heart. BALB/c mice produced higher levels of IL-4 in the heart suggesting a Th2-type response. These susceptible mice lacked a significant IL-2 response as well as a substantial IFN-γ response in the heart following virus infection, however, IL-6, IL-10, IL-18 and TNF were present in the heart during acute infection. C57BL/6 mice produce significant levels of IFN-γ in the heart, with no IL-4 or IL-6 response indicating a Th1-type response to MCMV infection. A significant IL-10 response was also observed in the C57BL/6 mice, extending into the chronic phase of disease. IL-18 and TNF are produced early in infection, with modest levels of IL-2 observed into the chronic phase. MCMV reinfection causes exacerbation of chronic myocarditis together with significant increases in systemic IL-4, IL-6, IL-10 and IFN-γ in BALB/c mice.
CHAPTER 7

General Discussion
The cause of myocarditis in any given patient often remains unknown, however, a large variety of infections, systemic diseases, drugs and toxins have been associated with the development of this disease. Viruses are an important cause of myocarditis (Feldman & McNamara, 2000), with increased understanding of the pathophysiology resulting from animal studies. In this thesis, the MCMV-induced model of myocarditis was used to examine multiple aspects of cardiac disease. Virus infection of the heart was examined, together with the role of the immune system. The apparent dysregulation of the immune response to cardiac infection was further investigated in susceptible and resistant mice with various mechanisms highlighted.

Myocyte necrosis is a predominant feature of myocarditis leading to reduced cardiac function. The cause of myocyte death was examined in Chapter 3. MCMV was shown to be able to directly cause myocyte death without immune system involvement, indicating that the necrosis observed may be caused by direct virus infection of myocytes. However, very low levels of replicative MCMV are found in the heart during infection, indeed, virus transcripts are only detectable by RT-PCR following the acute phase of disease. The extremely low levels of MCMV in the heart render the direct virus model of myocyte death unlikely. This is in contrast to the CB3 model of myocarditis, in which high levels of virus capable of directly killing myocytes are harbouredin the heart (Herzum et al., 1993). Another mechanism by which the observed myocyte necrosis may occur is apoptosis, a mechanism implicated in the reovirus-induced model of myocarditis (DeBiasi et al., 2001). While apoptotic myocytes were observed during the acute and chronic phase of MCMV-induced myocarditis, the levels were low and would not account for the high levels of necrosis.

Given that MCMV triggers myocardial inflammation and necrosis, a possible treatment would be to prevent MCMV infection of the heart with antiviral therapy. Drugs known for their potency against CMV successfully reduced myocarditis when treatment commenced very early in infection. This finding is similar to other virus-induced models of myocarditis (Kishimoto et al., 1988). Antiviral treatment commencing after the establishment of acute myocarditis had no beneficial effect. Reinfection with MCMV leads to exacerbation of myocarditis which can be prevented if antiviral treatment commences immediately after reinfection, however, the pre-existing myocarditis remains.
The results discussed thus far highlight several important features of MCMV-induced myocarditis. Virus is undoubtedly the trigger for disease, however, there is a virus level threshold that must be reached to break tolerance and allow the development of myocarditis in the susceptible BALB/c mouse. Antiviral drug studies revealed that myocarditis developed despite significant decreases in MCMV titres in major target organs such as the spleen, liver and salivary gland. Higher doses of antiviral drugs had more therapeutic effect in the heart despite subtle decreases in virus titre in other organs. Given that low levels of infectious MCMV are harboured in the heart, the therapeutic efficacy of antiviral drugs is most likely due to an early reduction in systemic MCMV leading to less virus in the heart. These results also indicate an important role for the immune system in the development of chronic myocarditis.

A major finding from this thesis was the identification of the cell types contained within the inflammatory infiltrate. Foci of inflammation were composed of predominantly CD8+ T cells during the acute and chronic phase of myocarditis in the susceptible BALB/c mouse. The resistant C57BL/6 strain develops low levels of acute myocarditis also consisting of predominantly CD8+ T cells. A mixed infiltrate of B cells, CD4+ T cells, macrophages and neutrophils was observed scattered throughout the BALB/c myocardium post-infection. Interestingly, the C57BL/6 mice do not mount a significant macrophage or neutrophil response in the heart following MCMV infection. This observation suggests that phagocytic cells entering the heart may be, in part, responsible for the development of chronic MCMV-induced myocarditis. Depletion of such cell types in the BALB/c strain may further clarify this issue.

The influx of immune cells into the myocardium may be explained in several ways. Firstly, infection of myocytes by MCMV is detected by macrophage or dendritic cells, which then cross-prime T cells, thus initiating a CD8+ cytolytic T cell response to virus infected cells. Alternatively, MCMV infection of myocytes may cause the release of cardiac myosin, which the immune system deems "foreign", thus causing an immune response against cardiac myosin. This theory is supported by the experimental autoimmune model of myocarditis in which mice inoculated with cardiac myosin develop myocarditis (Neu et al., 1987b). Finally, there is the concept of molecular mimicry in which the immune system mounts a response to a viral epitope that is
similar to a self antigen, in this case, cardiac myosin. Mounting evidence suggests that MCMV may mimic cardiac myosin, thus causing the immune response against cardiac tissue (Lawson, 2000). Further research into this area is required before the antigenic determinant is classified and shown to be a proven mimic.

The dramatic differences observed in the cellular infiltration of the heart between susceptible BALB/c and resistant C57BL/6 mice must also be investigated further. The lack of a macrophage and neutrophil response in the C57BL/6 heart may be further elucidated by examining the chemokine response to MCMV infection. Chemokines such as macrophage inflammatory protein 2 (MIP-2) and monocyte chemotactic protein (MCP)-1 and 1α, which have chemotactic activity for neutrophils and macrophages respectively, may be over-expressed in susceptible mouse strains, thus leading to an exacerbated response. Similarly, chemokines that cause trafficking of T and B cells to the heart may be over-expressed. Such concepts have been examined in the EMCV-induced model of myocarditis, with MIP-2 expression enhanced following virus infection (Kishimoto et al., 2001), clearly indicating an important role for chemokines in cardiac disease. Indeed, the exacerbation of myocarditis following administration of LPS may be partly due to the ability of LPS to induce the expression of chemokines such as MCP-1 (Shyy et al., 1993), thus increasing the trafficking of leukocytes to the myocardium.

An appropriate response must be mounted by the immune system in order to effectively clear virus infection without causing myocardial inflammation and necrosis. This response can be manipulated, as explored in chapter 5 of this thesis. The immunomodulator, LPS, was able to exacerbate acute myocarditis and induce chronic myocarditis in resistant C57BL/6 mice. Further evidence has emerged from this study that the immune system is the driving force behind ongoing disease rather than virus infection, with virus titres being unaffected by LPS even though myocarditis was exacerbated. Potential mechanisms by which LPS acts in this model were investigated by examining cytokine responses in the sera and in heart infiltrating cells following MCMV infection and LPS treatment. A predictable increase in TNF levels was observed, as seen in the CB3/LPS model (Lane et al., 1991). TNF has been implicated in many inflammatory diseases such as rheumatoid arthritis (Richard-Miceli & Bougados, 2001), asthma (Thomas, 2001) and chronic heart failure (Bolgar & Anker,
2000), however the role of TNF in MCMV-induced myocarditis remains unclear. TNF administered to resistant C57BL/6 mice increased myocarditis, whereas, no increase was observed in the susceptible strain. This observation suggests that multiple immune interactions lead to myocarditis, not just over expression of TNF. Interestingly, TNF stimulates neutrophil activity (Klebanoff et al., 1986), a cell type present in the myocardium of BALB/c mice during myocarditis but absent in C57BL/6. It could be speculated that exacerbated myocarditis in the resistant strain following administration of TNF or LPS is caused by neutrophil activation in the heart induced by TNF. However, TNF interacts with many other immune responses that may be responsible for the observed increase in myocardial inflammation, such as the induction of iNOS, which induces the expression of NO, a molecule capable of exacerbating inflammation.

Controversy exists over the role for cardiac myosin specific autoantibodies in the pathogenesis of myocarditis. Myosin specific antibodies produced following CB3 infection did not cross-react with the virus (Neu et al., 1987b), while in another study, autoantibodies induced disease (Gauntt et al., 1991). The pathogenic mechanism of disease induction or exacerbation by autoreactive antibodies could involve ADCC which in turn would inhibit cardiac function (Rose & Hill, 1996; Huber, 1997). The precise role of autoantibodies in disease induction or myocyte damage was not investigated in this study, however, exacerbation of MCMV-induced myocarditis by LPS was accompanied by an increase in anti-cardiac myosin specific antibodies in both susceptible and resistant strains during the chronic phase of disease. This observed increase in autoantibodies to cardiac myosin may have contributed to the increased inflammation and damage of the myocardium. Clearly, more research is needed in order to fully elucidate the role of autoantibodies in cardiac disease.

The complex interactions of cytokines play an important role in myocarditis. The need to clear virus infection without causing chronic inflammation and necrosis of the myocardium requires a delicate balance to be maintained by the immune system’s cytokine response. In this thesis, several cytokines were measured both in the serum and in the heart of MCMV-infected susceptible and resistant mice. An interesting finding of this study was that serum levels of cytokines are not always indicative of levels at the local site of disease. It is therefore important to be cautious when drawing conclusions based upon serum cytokine data. Differences in cytokine levels emerged between the
acute and chronic phases of disease and also between the resistant and susceptible strains of mice. The results clearly show that the timing, level and duration of any given cytokine response is critical in order to effectively clear a virus infection without causing myocardial damage.

Resistant C57BL/6 mice have an early TNF response in the heart (peaking at day 3 p.i.), whereas the susceptible BALB/c mice have a later TNF response (peaking at day 5 p.i.). A TNF response is critical for virus clearance, mice with targeted disruption of the TNF gene have increased mortality after infection with EMCV (Wada et al., 2001). However, administration of TNF exacerbates viral myocarditis. These conflicting observations can be reconciled if TNF signalling is considered within the context of innate immunity. Clearly, TNF must be expressed in order to mount an effective innate immune response to aid in virus clearance, however, the pro-inflammatory properties of TNF make later, prolonged expression of this cytokine detrimental to the heart.

C57BL/6 mice have prolonged expression of IL-10 in the heart following MCMV infection compared to BALB/c mice. IL-10 has been recognised as a potent anti-inflammatory cytokine as it down regulates production of potentially detrimental mediators such as TNF, IL-1, IL-6, IFN-γ and GM-CSF (Fiorentino et al., 1991; de Waal Malefyt et al., 1991; Moore et al., 1993; Casatella et al., 1993; Tiegs et al., 1994). Prolonged expression of this cytokine in resistant mice would indicate an immune response designed to suppress inflammation thus preventing damage to the heart. Increased IL-10 production was observed from the inflammatory infiltrating cells of the heart in BALB/c mice following MCMV-infection and LPS treatment. Such an increase would occur in order to reduce levels of the proinflammatory cytokines, TNF and IL-6, which were also increased following LPS treatment. Indeed, IL-10 treatment has been shown to protect mice against lethal doses of LPS (Howard et al., 1993; Gerard et al., 1993). However, the timing of an IL-10 response is critical, as the anti-inflammatory effects may impair an immune response against virus infection.

Positive and negative effects of cytokines based upon timing, duration and level of expression are important with regard to myocarditis. IL-6 is another cytokine which can have therapeutic as well as deleterious affects. Transgenic mice with targeted over expression of IL-6 have accelerated myocardial tissue injury following infection with
EMCV (Tanaka et al., 2001). However, acute administration of IL-6 at the time of EMCV infection results in a reduction of myocarditis (Kanda et al., 1996). These observations suggest that short term administration of IL-6 is sufficient to avert myocardial injury following infection, however, sustained expression of IL-6 enhances tissue destruction. Results from this thesis are in agreement with this theory. Resistant mice have levels of IL-6 peaking systemically on day 3 p.i. with a rapid decrease evident by day 5 p.i. Susceptible mice have systemic IL-6 levels peaking at day 3 p.i. with significant levels being detected until day 14 p.i. In addition, exacerbated myocarditis following LPS treatment of MCMV infected BALB/c mice is accompanied by large increases in IL-6 production by inflammatory cells in the heart. The examples discussed above show the fine balance that must be achieved by a cytokine response. The ability of the resistant C57BL/6 mouse to effectively clear MCMV infection without the development of chronic myocarditis is in part due to its cytokine response. The inability of the susceptible BALB/c mouse to control appropriate cytokine expression is an area of ongoing research and may reveal further insights into the clinical progression of this disease.

Collectively, the data presented in this thesis combine to further elucidate the MCMV-induced model of myocarditis. Possible mechanisms involved in the pathogenesis of this disease are depicted in Fig. 7.1. In summary, MCMV infection results in viral replication in organs such as the spleen, liver, salivary gland and the heart. While virus replication in organs other than the heart appear not to affect the development of myocarditis, antiviral drug studies suggest that MCMV titres in these organs must initially reach a threshold level before myocarditis develops. Although susceptible mice have higher levels of virus replication in the heart early after infection than resistant mice, replication occurs at low levels without associated necrosis of myocytes. MCMV infection of the heart most likely results in the release of cardiac myosin. Antigen presenting cells then process and present MCMV and/or cardiac myosin antigens. These APC may release cytokines which can cause NK cell activation and subsequent myocardial damage. Activated NK cells may release other cytokines such as IFN-γ which can then direct the adaptive immune response by influencing the cytokine environment during antigen presentation and the development of a Th1 or Th2-type response. MCMV and/or cardiac myosin reactive CD8+ T cells infiltrate the heart and induce myocyte damage. Cardiac myosin specific autoreactive CD4+ T cells may
MCMV infection of the heart/myocytes
Threshold level of MCMV to induce myocarditis (Chapter 3 & 4)

Antigen processing and presentation by APC

Virus mediated myocyte damage and cardiac myosin release

Cytokine release (Chapter 5 & 6)

NK Cell activation (innate antiviral response)

B cell response IgG (Chapter 3)

Antigen presentation in lymph node and activation of T cells

Continued cytokine expression TNF/IL-6/IL-10/IFNy/IL-18 (Chapter 6)

CD8+ T cell response in the heart (Chapter 3)

Autoreactive/directed against virus antigen/molecular mimicry?

Inflammation and myocyte damage

Figure 7.1 Possible mechanisms of MCMV-induced myocarditis.
stimulate B cells to produce pathogenic autoantibodies that may exacerbate myocarditis. Neutrophil and macrophage infiltration may also contribute to myocardial damage given the phagocytic potential of such cells. The continued expression of pro-inflammatory cytokines influences the ongoing immune response and can contribute to the development of chronic myocarditis.

These findings have important implications for the treatment of clinical myocarditis. Given the importance of the immune system in myocarditis, immunosuppressive treatment was thought to have therapeutic potential, however, the Myocarditis Treatment Trial failed to show significant differences in treatment groups receiving immunosuppressive therapy (Hahn et al., 1995; Mason et al., 1995). Antiviral drug therapy appears to be restricted to those patients at risk of reinfection or reactivation from latency. Results from this thesis suggest that antiviral drug treatment coupled together with immunomodulatory treatment may provide effective therapy for CMV-induced myocarditis. Research in various animal models has shown some promise for immunomodulatory treatment with IFN-α, IFN-γ, IL-6, IL-10, IL-12 and IL-18 (Kanda et al., 1996; Shioi et al., 1997; Yamamoto et al., 1998; Nishio et al., 1999; Lawson & Beilharz, 1999; Kanda et al., 2000). Therapeutic strategies such as anti-TNF administration are being considered in the clinical treatment of viral myocarditis and dilated cardiomyopathy (Ferrari, 1999), however, the critical role that TNF plays in the immune system may place patients at risk during infection. This highlights the importance of fully understanding the complex cytokine networks and the effect that each cytokine plays in the immune response.

Cytokines are also important in controlling T cell responses to self antigens and are critical in shifting the immune response towards a Th1 or Th2 pattern (Liblau et al., 1995; Falcone & Sarvetnick, 1999). Experimental autoimmune myocarditis in A/J mice has recently been shown to have a Th2-type phenotype (Afanasyeva et al., 2001). Evidence presented in this thesis suggests that susceptible BALB/c mice may also have a Th2 phenotype in response to CMV infection. Cytokine administration that is capable of regulating whether a Th1 or Th2 type response develops may be therapeutic and prevent harmful autoimmune responses. However, the Th1/Th2 type response issue remains unclear, particularly in the MCMV model. Further research is required to
further delineate the role of cytokines and their complex interactions before more effective immunomodulatory therapies can be developed.

The overall findings of this thesis indicate that virus is necessary for disease induction and as such can be successfully treated with antiviral drugs if administered very early in infection. The presence of MCMV in the heart induces inflammation in the myocardium consisting of a mixed cellular infiltrate of predominantly CD8+ T cells. Immune mechanisms are also necessary for the development of myocarditis. The lack of a macrophage or neutrophil response in the heart may prevent the development of chronic myocarditis. Immune modulation with LPS and cytokines can exacerbate and induce disease. The complex cytokine interactions occurring within the heart following infection can lead to resolution or progression of disease. Future studies into the cellular and humoral responses in MCMV-induced myocarditis may provide important information for developing new and effective clinical treatments for myocarditis.
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