In Vivo REGULATION OF MURINE CYTOMEGALOVIRUS INFECTIONS: THE ROLE OF CELL SURFACE MOLECULES AND MECHANISMS OF CONTROL BY NATURAL KILLER CELLS

A Dissertation Presented

By

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ABSTRACT

The overall aim of this thesis was to determine how natural killer (NK) cells regulate virus infections *in vivo*. Anti-viral mechanisms by which NK cells control murine cytomegalovirus (MCMV) infection in the spleens and livers of adult C57BL/6 mice were first studied, revealing different mechanisms of control in different organs. Three days post-infection, MCMV titers in the spleens of perforin-deficient (perforin 0/0) mice were higher than in wild type controls, but no elevation of liver titers was found in perforin 0/0 mice. NK cell depletion in MCMV-infected perforin 0/0 mice resulted only in an increase in liver viral titers but not in spleen titers. Depletion of IFN-γ in adult C57BL/6 mice by injections with mAbs to IFN-γ resulted in an increase in viral titers in the liver but not in the spleen. Analyses using IFN-γ-receptor-deficient (IFN-γR$^{0/0}$) mice, rendered chimeric with C57BL/6 bone marrow cells, indicated that even though the donor spleen cells could respond to IFN-γ, the depletion of NK cells in a recipient environment where the host cells could not respond to IFN-γ caused an increase in MCMV titers in the spleens but had little effect in the liver. IFN-γ has the ability to induce a variety of cells to produce nitric oxide (NO), and administrating the nitric oxide synthase (NOS) inhibitor N$^{\omega}$-monomethyl-$_{L}$-arginine (L-NMA) into MCMV-infected adult C57BL/6 mice resulted in MCMV titer increases in the liver but not in the spleen. These data indicate that in adult C57BL/6 mice, there is a dichotomy in the mechanisms utilized by NK cells in the regulation of MCMV in

different organs. In the spleen NK cells exert their effects in a perforin-dependent manner, suggesting a cytotoxic mechanism, whereas in the liver the production of IFN-γ by NK cells may be a predominant mechanism in the regulation of MCMV synthesis. These results may explain why the *Cmv-1*^{r} (*Cmv-1*-resistant) locus, which maps closely to genes regulating NK cell cytotoxic function, confers an NK cell-dependent resistance to MCMV infection in the spleen but not in the liver.

The ability of adoptively transferred cells to protect suckling mice from MCMV was another model used to study the mechanisms utilized by NK cells in the regulation of MCMV. Adoptive transfers of 129, C57BL/6 and perforin 0/0 spleen cells or lymphokine-activated killer (LAK) cells into 4 - 6 day old MCMV-infected C57BL/6 suckling mice significantly lowered the splenic MCMV titers in these mice compared to the infected controls. Adoptive transfers of C57BL/6 spleen cells into MCMV-infected 129 suckling mice also decreased the amount of MCMV in the 129 suckling mice, but C57BL/6 spleen cells could not regulate MCMV synthesis when adoptively transferred into 129/IFN-γR$^{0/0}$ suckling mice. These results suggest that, in the suckling mouse model, the regulation of MCMV by the adoptively transferred NK cells is via an IFN-γ-dependent, perforin-independent, *Cmv-1*-independent mechanism.

The *Cmv-1* gene locus resides within the NK gene complex, in close proximity to the Ly49 NK cell receptor family. Analyses were carried out to determine if any of the 4 known Ly49 NK cell receptors (Ly49A, C, D and G2) played a role in the control of MCMV synthesis by NK cells. Studies comparing the expression of the different Ly49 NK cell subsets in the

spleen and the peritoneal cavity revealed that there were differences in the distribution of the Ly49 receptors on NK1.1$^+$ cells. Three days post-MCMV infection, the percentage of NK1.1$^+$-Ly49$^+$ NK cells in the spleen and the peritoneal cavity were different than in naive controls. Within the splenic NK1.1$^+$ population, increases in NK1.1$^+$-Ly49A$^+$ and NK1.1$^+$-Ly49G2$^+$ cells but decreases in NK1.1$^+$-Ly49C$^+$ and NK1.1$^+$-Ly49D$^+$ cells were observed. These changes in the spleen were accompanied by a concomitant decrease in NK1.1$^+$-Ly49A$^+$ cells and increases in NK1.1$^+$-Ly49C$^+$, NK1.1$^+$-Ly49D$^+$ and NK1.1$^+$-Ly49G2$^+$ cells within the NK1.1$^+$ population in the peritoneal cavity. These data suggest that 3 days post-MCMV infection, there may be movement of NK cells between the different organs. The role of Ly49 NK cell receptors in the regulation of MCMV was tested using adult C57BL/6 mice depleted of single or multiple Ly49 NK cell subsets. These *in vivo* depletions did not affect the ability of the residual NK cells to regulate MCMV synthesis. LAK cells sorted into the different Ly49 NK cell subsets and adoptively transferred into C57BL/6 suckling mice lowered the splenic MCMV titers in these mice. Together, these results indicate that even though there is a redistribution of the Ly49 NK cell subsets during MCMV infection, the presence or absence of any one of the 4 tested Ly49 NK cell receptors does not affect the regulation of MCMV by NK cells. However, there remain a possibility that one of the undefined Ly49 receptors or an untested NK cell receptor may be important in the control of MCMV.

Most of the cloned NK cell receptors have been shown to bind to MHC class I molecules, and MHC class I antigens have been implicated as modulators of target cell

sensitivity to NK cell-mediated lysis. The regulation of virus infections and the fate of NK cells and their natural targets was examined in β_2-microglobulin-deficient mice [β_2m (-/-)], which have defective MHC class I expression. Infections with either the NK cell-sensitive MCMV or the NK cell-resistant lymphocytic choriomeningitis virus (LCMV) significantly augmented NK cell activity in either C57BL/6 or β_2m (-/-) mice. Depletion of NK cells *in vivo* with antiserum to asialo GM_1 markedly enhanced the synthesis of MCMV but had no effect on the synthesis of LCMV in either strain of mouse. Adoptively transferred β_2m (-/-) spleen cells lowered splenic MCMV titers in C57BL/6 suckling mice, not unlike adoptively transferred C57BL/6 spleen cells. Analysis of naturally NK cell-sensitive thymocyte targets from these virus-infected β_2m (-/-) mice revealed no cell surface expression of class I MHC detectable by conformation-dependent or -independent antibodies, but the virus infections enhanced class I expression on thymocytes from C57BL/6 mice. The sensitivity of C57BL/6 thymocytes to NK cell-mediated lysis was markedly reduced after *in vivo* poly inosinic:cytidylic (poly I:C) treatment or viral infection; in contrast, the sensitivity of the β_2m (-/-) thymocytes was significantly less affected by poly I:C or viral infection. These data indicate that the normal expression of MHC class I antigens on NK cells or their targets is not required for the anti-viral functions of NK cells against an NK-sensitive virus (MCMV) nor do they protect an NK-resistant virus (LCMV) from the anti-viral activity of NK cells.

Together, the data presented in this thesis help to further our understanding of the mechanisms utilized by NK cells in the control of MCMV in both adult and suckling mice, and

also help clarify the roles played by Ly49 NK cell receptors and MHC class I molecules in the regulation of MCMV.

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ABBREVIATIONS

β_2m	β_2-microglobulin
β_2m (-/-)	β_2-microglobulin-deficient
aGM_1	asialo GM_1
Cmv-1^r	*Cmv-1* resistant
Cmv-1^s	*Cmv-1* susceptible
Con A	concanavalin A
CTL	cytotoxic T lymphocytes
D-NMA	N^{ω}-monomethyl-$_D$-arginine
EBV	Epstein-Barr virus
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
gm-csf	granulocyte-macrophage colony stimulating factor
HCMV	human cytomegalovirus
HIV-1	human immunodeficiency virus type 1
HSV-1	herpes simplex virus type 1
i.p.	intraperitoneal
i.v.	intravenous

IFN-γ	interferon gamma
IFN-α/β	interferon alpha/beta
IFN-γR$^{0/0}$	interferon gamma receptor-deficient
IL	interleukin
ITIM	immunoreceptor tyrosine-based inhibitory motif
L-NMA	N^{ω}-monomethyl-$_L$-arginine
LAK	lymphokine-activated killer
LCMV	lymphocytic choriomeningitis virus
MCMV	murine cytomegalovirus
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
NK	natural killer
NO	nitric oxide
NOS	nitric oxide synthase
PE	phycoerythrin
PEC	peritoneal exudate cells
perforin 0/0	perforin-deficient
PFU	plaque forming units
poly I:C	poly inosinic:cytidylic acid
SCID	severe combined immunodeficiency

SHP	SH2 domain-containing tyrosine phosphatase
TAP	transporter associated with antigen processing
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
VV	vaccinia virus

CHAPTER I

INTRODUCTION

The natural immune system consists of pre-existing or rapidly inducible effector components as a first line of defense against pathogens to which the host has not previously been exposed. Such a system is necessary, as the B and T cell-specific responses to any pathogen require the selective expansion of high affinity antigen-specific clones which take several days to develop. An unimpeded replication of a virus producing 10^5 progeny per cell could potentially infect all of the cells in the host after only 3 - 4 replication cycles. Without the natural immune system holding infections in check, pathogens could overwhelm the host before the T and B cell responses become effective.

In the mid 1970s, experiments studying cell-mediated immune responses to tumor cells found that tumorigenic cells induced by Moloney leukemia virus or other retroviruses could be lysed by leukocytes from unimmunized mice (Herberman et al., 1975; Kiessling et al., 1975b). These effector cells represented a new kind of cytotoxic lymphoid cell and were named as 'natural killer' (NK) cells by Kiessling et al. (1975a). NK cells have a large granular lymphocyte morphology (Monten et al., 1979) with their granules containing the membrane pore-forming molecule, perforin, and a group of serine proteases known as granzymes (O'Shea and Ortaldo, 1992). These cells are bone-marrow derived (Haller and Wigzell, 1977; Welsh, 1978), do not express the T cell receptor/CD3 complex or

immunoglobulins on their cell surfaces and do not rearrange their B and T cell antigen receptor genes (Lanier et al., 1986; Biron et al., 1987). NK cells express receptors for the Fc portion of IgG (Herberman et al., 1977) and activated NK cells also express the T cell-specific antigen, Thy 1 (Herberman et al., 1978). NK cells can be further distinguished from other lymphoid cells by the expression of asialo GM_1 (aGM_1 (Kasai et al., 1980) and NK1.1 (Glimcher et al., 1977) in mice, and CD56 (Lanier et al., 1989) in humans. In recent years, NK cells have been shown to express a variety of NK cell-specific receptors encoded by genes found within the distal portion of mouse chromosome 6 and on human chromosome 12p13.2, in a region now known as the NK gene complex (Yokoyama, 1993; Yokoyama, 1995a). Many of these NK cell receptors have MHC class I molecules as their ligands (Gumperz and Parham, 1995; Raulet and Held, 1995; Colonna, 1996). NK cells can secrete a number of cytokines, including interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), granulocyte/macrophage colony stimulating factors (gm-csf) and interleukin 1 (IL-1) (Trinchieri, 1989).

A. NK CELLS AND VIRUS INFECTIONS

NK cells, which provide an early host response to viral, parasitic and bacterial infections, are important components of the natural immune system. NK cells have now been shown to provide resistance to some of these infections, as well as playing roles in tumor surveillance and in the regulation of hematopoiesis (Storkus and Dawson, 1991;

Welsh and Vargas-Cortes, 1992; Bellone et al., 1993; Scott and Trinchieri, 1995). The *in vivo* role of NK cells is most definitively shown by the regulation of murine cytomegalovirus (MCMV) infection in mice. Genetic resistance of mice to MCMV maps to a single gene within the NK gene complex (Scalzo et al., 1990; Scalzo et al., 1992). Intraperitoneally (i.p.)-inoculated adult C57BL/6 mice depleted of NK cells with antisera to aGM_1 or with mAbs to NK1.1 have enhanced virus replication in the spleen, lung, and liver (Bukowski et al., 1984; Welsh et al., 1991; Welsh et al., 1994). Suckling mice are very sensitive to MCMV until the third week of life, at which time the NK cell response develops to maturity (Boos and Wheelock, 1971; Kiessling et al., 1975a). Adoptive transfer experiments using adult splenocyte populations or purified culture-derived NK cells showed that NK cells protected suckling mice from MCMV (Bukowski et al., 1985). NK cells were also shown to inhibit MCMV replication in mice with severe combined immunodeficiency (SCID), demonstrating the function of these cells in an environment devoid of T and B cells (Welsh et al., 1991; Welsh et al., 1994). However, NK cells in SCID mice were incapable of completely eradicating MCMV, even at limiting dilutions of the virus, indicating that T and/or B cells were ultimately necessary for clearance (Brubaker, 1993). This is consistent with the concept that NK cells inhibit the spread of infection, allowing time for specific immune responses to develop. In humans, the importance of NK cells was highlighted in our institution by a patient who had a complete and selective NK cell immunodeficiency; this patient had unusually severe cases of human cytomegalovirus (HCMV) infection and other herpes virus infections (Biron et al., 1989).

How NK cells control MCMV and other viral infections *in vivo* has until recently been a poorly understood phenomenon. Potential mechanisms would include the secretion of anti-viral cytokines, such as IFN-γ and TNF-α, or alternatively, by the direct lysis of virus-infected cells prior to the release of infectious virus progeny.

ACTIVATION OF NK CELLS DURING VIRUS INFECTIONS

When a virus infects a host, virus-infected cells are stimulated to produce IFN-α and IFN-β, which will induce the NK cells to proliferate and increase their cytotoxic potential (Welsh, 1978; Biron and Welsh, 1982; Biron et al., 1984). In mice, the levels of NK cell activation and blastogenesis parallel the IFN-alpha/beta (IFN-α/β) levels (Welsh, 1978; Biron and Welsh, 1982), and direct injections of purified IFN-β into mice induce NK cell activation and proliferation (Biron et al., 1984). Mice treated with antibodies to IFN-α/β (Gidlund et al., 1978) or mice whose IFN-α/β receptors have been inactivated by genetic recombination (Muller et al., 1994) generate a poor NK cell response to viral infections (Biron, 1994). These activated NK cells respond to chemotactic factors and migrate into areas of virus-infected tissue, such as the liver (McIntyre and Welsh, 1986; Natuk and Welsh, 1987b; Natuk et al., 1989), where they are presumed to mediate their anti-viral activities.

NK cells from virus-infected C57BL/6 mice have both high cytolytic activity and the potential to produce anti-viral cytokines. They express transcripts for IFN-γ, but the translation of these transcripts into the IFN-γ protein is dependent on additional factors. Orange and Biron studied the differences in the cytokine responses in mice infected with the NK-sensitive virus, MCMV, and in mice infected with lymphocytic choriomeningitis virus (LCMV), which is very resistant to NK cells (Orange and Biron, 1996a; Orange and Biron, 1996b). Both infections induced Type I IFNs, the cytolytic activation of NK cells, and the expression of IFN-γ mRNA in NK cells. However, only in the MCMV infection did the NK cells synthesize and secrete the IFN-γ protein. The IFN-γ production during MCMV infection was linked to the fact that MCMV but not LCMV was a good inducer of IL-12 *in vivo* (Orange and Biron, 1996a). IL-12 is a pleiotropic cytokine produced by macrophages and was shown in these studies to be required for the production of IFN-γ by NK cells early in the infection. Antibodies to IFN-γ or IL-12 did not affect NK cell cytolytic activity, a result consistent with the concept that IFN-α/β rather than IFN-γ activates NK cells at the early stages of infection (Orange et al., 1995).

Another cytokine that plays a role in the activation of NK cells is TNF-α. Data generated using anti-TNF-α-treated, MCMV-infected normal mice suggest that TNF-α can synergize with IL-12 to induce the production of IFN-γ by NK cells (Orange and Biron, 1996b). TNF-α can also inhibit the effects of IFN-α/β on NK cells by negatively influencing NK cell blastogenesis and the induction of NK cell cytotoxicity (Orange and Biron, 1996b).

ANTI-VIRAL FUNCTIONS OF NK CELL-PRODUCED CYTOKINES

A suggestion that NK cell-produced cytokines are important in controlling virus infections came initially from studies using athymic nude mice infected with a vaccinia virus (VV)-IL-2 recombinant (Karupiah et al., 1990). IL-2 is a strong activator (Kuribayashi et al., 1981) as well as a chemotactic factor (Natuk and Welsh, 1987a) for NK cells. This cytokine also has the ability to induce NK cells to produce IFN-γ (Young and Ortaldo, 1987). Administration of either anti-IFN-γ Abs or anti-aGM_1 antiserum into the VV-IL-2 recombinant-infected nude mice exacerbated the infection, suggesting that the production of IFN-γ by the NK cells in the nude mice was controlling the infection (Karupiah et al., 1990). A role for NK cell-produced IFN-γ in an unmodified system has since been established with MCMV (Orange et al., 1995). Depletion of IFN-γ or IL-12 with mAbs increased the incidence of MCMV-induced hepatitis and virus replication in the liver (Orange et al., 1995; Orange and Biron, 1996a; Tay and Welsh, 1997).

The anti-viral functions of IFN-γ have been studied extensively, with the results indicating that the cytokine can impair various stages of viral replication, including viral penetration and uncoating, transcription, translation and assembly of progeny viruses (Vilcek and Sen, 1995). In the inhibition of positive stranded RNA viruses, Type I and Type II interferons can induce the expression of an enzyme called oligoadenylate synthetase. Oligoadenylate synthetase is thought to be activated by the protein-free, naked

replicative intermediates of positive stranded RNA viruses to synthesize a family of oligonucleotides, collectively known as 2,5-Oligo-A. 2,5 Oligo-A in turn will activate a cytoplasmic ribonuclease called RNase L, which will cleave the viral RNA (Vilcek and Sen, 1995). During virus infections, a dsRNA-dependent protein kinase PKR is also induced by IFNs. PKR is activated by the viral dsRNA to autophosphorylate and also to phosphorylate the α subunit of the translation initiation factor eIF-2. Phosphorylated eIF-2 cannot be used again in the translation process, thus effectively inhibiting the cellular processes of the invading viruses in the cell (Vilcek and Sen, 1995). Another way IFN-γ can exert its anti-viral effects is by inducing the expression of the gene for inducible nitric oxide synthase (iNOS) in cells such as macrophages, Kupffer cells and hepatocytes (Nathan, 1992). NOS in turn catalyzes the production of a free radical gas, nitric oxide (NO), from the guanidino nitrogen of L-arginine. NO production by NOS has been shown both *in vivo* and *in vitro* to inhibit the replication of VV, ectromelia virus, and herpes simplex virus type 1 (HSV-1) (Karupiah et al., 1993a; Harris et al., 1995). Although the molecular basis of NO action in the inhibition of virus replication is not known, NO production has been shown *in vitro* to inhibit VV replication by blocking the replication of viral DNA and late protein synthesis, but this molecule has no effect on the expression of early VV proteins (Harris et al., 1995; Melkova and Esteban, 1995).

Another anti-viral cytokine that NK cells can secrete to control viral infections is TNF-α. An early report provided some evidence that TNF-α and lymphotoxin produced by cloned human NK cell lines can have anti-viral cytotoxicity and that human

recombinant TNF-α can selectively induce the lysis of vesicular stomatitis virus-, HCMV-, Theiler's murine encephalomyelitis virus-, and HSV-1-infected cells (Paya et al., 1988). *In vivo*, $CD4^+$ T cell-dependent TNF-α production has been shown to participate in the inhibition of MCMV replication (Pavic et al., 1993), and *in vitro* this cytokine can synergize with IFN-γ to inhibit MCMV late protein production (Lucin et al., 1994). The role of NK cell-produced TNF-α in the regulation of MCMV is not clear, as E26 mice, which do not have any T or NK cells, produce normal levels of TNF-α 2 to 3 days after MCMV infection (Orange and Biron, 1996b). This means that NK cells are not a primary or required source for the production of TNF-α, which can be made at high levels by macrophages and other cell types. Nevertheless, it is possible the NK cell-produced TNF-α feeds back onto its own regulatory pathway and synergizes with IL-12 to stimulate the production of more IFN-γ.

Role of NK Cell Cytotoxicity in the Regulation of Virus Infections

In the cytoplasm of activated cytotoxic T lymphocytes (CTL) and NK cells are azurophilic granules that contain the pore forming molecule perforin (Henkart et al., 1986; Podack and Konigsberg, 1984). Perforin is structurally similar to the C9 component of complement (Tschopp et al., 1986; Young et al., 1986a). When an NK cell comes in contact with its target cell, microtubule and cytoskeleton reorganization within the NK cell allows the cell to position its granules near the area of contact (Kupfer et al., 1983). In the

presence of Ca^{2+}, the contents of the granules are released into the intracellular space. Monomers of perforin then bind to the target cell membrane and aggregate to form polymers of perforin known as polyperforin, which creates holes or 'punctures' in the target cell membrane (Henkart et al., 1984; Young et al., 1986b). These polyperforin channels disrupt the resting membrane potential of the target cell and allow the indiscriminate exchange of water and cytoplasmic contents, ultimately leading to the lysis of the target cell. The only evidence prior to the work presented in this thesis that suggest a cytotoxic mechanism may be involved in the control of MCMV comes from studies with beige mice. Beige mice, whose NK cells are deficient in cytotoxic function, control MCMV infection poorly (Shellam et al., 1981; Bukowski et al., 1984). However, the beige mutation confers a lysosomal defect affecting many types of leukocytes, and it is possible that these other defects may influence the ability of the beige mouse to control the infection. NK cells have also been shown *in vitro* to be able to kill via Fas/Fas ligand interaction, but the role of Fas in the ability of NK cells to control virus infections has not been tested (Murphy et al., 1995).

CMV-1 AND THE GENETIC RESISTANCE OF MICE TO MCMV

Scalzo et al have shown that there is a non-MHC linked resistance gene to MCMV that maps very closely to the *NK1.1* and the Ly49 loci within the NK gene complex (Scalzo et al., 1990; Scalzo et al., 1992; Scalzo et al., 1995b). The gene *Cmv-1* confers resistance to MCMV in the spleen but not in the liver (Scalzo et al., 1990). The effects of

Cmv-1 are mediated through NK1.1$^+$ cells, and mice that have the gene (*Cmv-1*^{r}) have lower splenic MCMV titers than strains of mice that do not have the gene (*Cmv-1*^{s}) (Scalzo et al., 1992). However, the viral titers in the livers of the different strains of mice are similar. Since *Cmv*-1 is found within the NK locus, it is possible that the gene may be differentially expressed in the various subsets of NK cells and that different subsets of NK cells control MCMV in different organs. Alternatively, NK cells may utilize a *Cmv-1*-dependent mechanism to control MCMV in the spleen but another mechanism to control the virus in the liver. *Cmv-1* in the context of NK cell receptors will be discussed further in the next section.

NK Cell Receptors and Virus Infections

The mapping of the *Cmv-1* locus within the NK gene complex raises the possibility that the NK cell receptor molecules may interact with virus-infected targets. In both the human and mouse systems, most of the cloned NK cell receptors interact with MHC class I molecules and such an interaction can be either 'activatory' or inhibitory. In the mouse most of the NK cell receptors cloned to date share a common feature: dimeric type II transmembrane proteins whose extracellular domains have structural features of calcium-dependent (C-type) lectins (Yokoyama, 1993; Brown et al, 1997; Ryan and Seaman, 1997). In humans, two classes of NK cell receptors have been cloned. One group of receptor complex is composed of Type II proteins with a C-type lectin domain, not unlike the mouse NK cell receptors, and the second group belong to the Ig superfamily,

with 2 or 3 Ig-like domains in their extracellular portion (Moretta et al, 1997; Ryan and Seaman, 1997). Recently, there is a report that mouse NK cells may also express NK cell receptors belonging to the Ig superfamily, but this has yet to be confirmed by other investigators (Wang et al., 1997).

Different NK cell receptors have been shown to inhibit or activate NK cell function, and within the same NK cell receptor family, different members can have opposing functions (Mason et al., 1996; Raziuddin et al., 1996; Yu et al., 1996; Moretta et al., 1997). This suggests that the molecular pathways by which NK cells are activated and the inhibitory signals sent to prevent such an activation are different. In humans, two NK cell receptors of the same family (both of the receptors are recognized by the same antibody and both bind to HLA-C "group 2" antigens) are virtually identical in their extracellular domains but differ in the length of their cytoplasmic tails (Moretta et al., 1997). Inhibitory NK cell receptors have in their cytoplasmic domain a motif known as the immunoreceptor tyrosine-based inhibitory motif (ITIM). The short cytoplasmic tails of activatory NK cell receptors lack the ITIM sequence. ITIM is characterized by the amino acid sequence (V/I) XYXXL, which is involved in the recruitment of SH2 domain-containing tyrosine phosphatases (SHP) (Thomas, 1995). SHP has been implicated to play an important role in the negative regulation of many immunoreceptors (Thomas, 1995). This negative regulation of immunoreceptors by SHP can be appreciated in the motheaten mice, which harbor a mutation in the SHP gene. Motheaten mice have numerous

immunological disorders, including chronic macrophage and neutrophil disorders, lymphopenia and circulating autoantibodies (Schultz, 1991).

In the mouse, the ITIM sequence is also found in the Ly49 receptor family. Recently, Nakamura et al showed that the inhibitory signal sent through the Ly49A NK cell receptor utilized the cytoplasmic phosphatase, SHP-1, which binds to ITIM (Nakamura et al., 1997). In SHP-1 mutant viable motheaten mice and SHP-1-deficient motheaten mice the Ly49A function in NK cells is impaired. It is also demonstrated that the mutation of the tyrosine site within the proposed SHP-1 binding motif of Ly49A abrogates the inhibition of NK cell cytotoxicity through this receptor (Nakamura et al., 1997). These results allow us to arbitrarily divide the NK cell receptors into 2 groups, activatory or inhibitory, based on the presence or absence of the ITIM sequence. Table I-1 is a summary of some of the known mouse and human NK cell receptors.

Investigators working on the identification of the *Cmv-1* gene product have indicated that *Cmv-1* is closely linked to the Ly49 receptor family, suggesting that *Cmv-1* may be an existing or undefined member of the Ly49 multigene family (Brown et al., 1997). Figure I-1 depicts the relative position of *Cmv-1* in the mouse NK gene complex. Members of the Ly49 NK cell receptor family have been to shown to interact with MHC class I molecules (Leibson, 1995; Yokoyama, 1995a). The interactions between Ly49A, C or G2 with their respective MHC class I ligands have an inhibitory effect on the NK cells, whereas the Ly49D NK cell receptor activates the NK cells (Yokoyama, 1995a; Mason et al, 1996; Raziuddin et al., 1996; Table I-1).

Table I-1. Human and Mouse NK Cell Receptors*

Species	Molecule	Structure	# in Family	Ligand	ITIM	Effect on NK
Mouse	NKRP-1	Type II homodimer	>3	Carbohydrate (?)	-/?	Activates
Mouse	Ly49	Type II homodimer	8, possibly more	Ly49A/H-2D^d, D^k Ly49C/H-2K^b Ly49D/? Ly49G2/H-2D^d, L^d	+ + - +	Inhibits Inhibits Activates Inhibits
Human	p58.1	2 Ig domains	2	HLA-C 'group 1'	+	Inhibits
Human	p50.1	2 Ig domains	2	HLA-C 'group 1'	-	Activates
Human	p58.2	2 Ig domains	2	HLA-C 'group 2'	+	Inhibits
Human	p50.2	2 Ig domains	1	HLA-C 'group 2'	-	Activates
Human	p50.3	2 Ig domains	1	?	-	Activates
Human	p70	3 Ig domains	2	HLA-Bw4	+	Inhibits
Human	p140	3 Ig domains	3	HLA-A3/-A11	+	Inhibits
Human	CD69	Type II homodimer	1	Carbohydrates (?)	?	Activates

Human	CD94/NKG2	Type II heterodimer	1, possibly more	CD94/kp43/HLA-A, -B, -C	+	Inhibits
				CD94/p39/ ?	-/?	Activates

*Table is adapted from Brown, M.G. et al and Moretta, A. et al, Immunological Reviews 1997, Vol. 155, pg 53-65 and pg105-117.

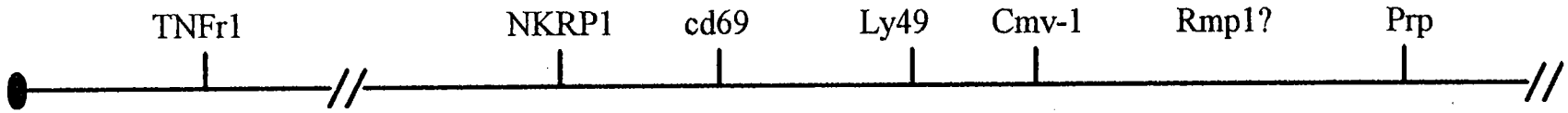

Figure I-1: Mouse chromosome 6 encompassing the NK gene complex. The centromere is located on the left. The NK gene complex is between the 2 break marks (//). Depicted are the relative positions of the NKRP1 gene family (NK1.1 is one of the members), the Ly49 multigene family and *Cmv-1*. The other genes found in the same vicinity are TNFr1 (tumor necrosis factor receptor 1), cd69 (an early activation marker found on all hematopoietic cells), Rmp1 (ectromelia virus resistance gene) and Prp (a chromosome marker).

Members of the Ly49 family have been shown to have physiological roles in bone marrow transplants, especially in the phenomenon of F1 hybrid resistance (Sentman et al., 1989; Yokoyama, 1995b; Yu et al., 1996). F1 hybrid resistance is the ability of H-2 heterozygous mice to reject parental bone marrow grafts by NK cells. In F1 hybrids, the Ly49C$^+$ NK cells (recognized by the Ab SW5E6), which bind to H-2^{b+} cells have been demonstrated to mediate the specific rejection of H-2^d parental bone marrow cells (Raziuddin et al, 1996; Yu et al., 1996). *In vivo* depletion of 5E6$^+$ NK cell subset abrogates this rejection (Raziuddin et al, 1996; Yu et al., 1996). On the other hand, Ly49G2$^+$ NK cells, which bind to H-2^{d+} cells, are able to complement the Ly49C$^+$ NK cells by specifically mediating the rejection of H-2^b parental bone marrow allografts in F1 mice (Raziuddin et al., 1996). This rejection of parental bone marrow cells can be abrogated by depleting the Ly49G2$^+$ NK cell subset in F1 mice (Raziuddin et al., 1996).

The role of Ly49 NK cell receptors in the regulation of virus infections is not known. Recently it has been demonstrated that MCMV, like HCMV, encodes a MHC class I homologue that may interfere with the ability of NK cells to regulate the virus (Farrell et al., 1997; Reyburn et al., 1997). It is possible that MCMV may exploit the inhibitory effects on NK cells caused by the binding of Ly49 NK cell receptors to class I molecules by providing pseudo class I molecules to the NK cells.

MHC CLASS I EXPRESSION AND NK CELLS

Before the discovery of NK cell receptors and their interaction with MHC class I molecules, the ability of *in vivo*-stimulated NK cells to lyse allogeneic targets and target cells that do not express MHC class I molecules was, in fact, initially used to differentiate virus-induced NK cell-mediated killing from CTL-mediated lysis (Welsh, 1978; Welsh et al., 1979; Kiessling and Welsh, 1980). The 'Missing Self Hypothesis' proposed that the susceptibility of target cells to NK cell-mediated lysis is inversely proportional to the amount of class I molecules on the target cell surface (Ljunggren and Karre, 1990; Sentman et al., 1995). Ljunggren et al showed that tumor cells that were class I negative were rejected by the NK cells in their syngeneic host and that the induction of MHC class I on the cell surfaces after transfection of β_2-microglobulin (β_2m) into β_2m negative [β_2m (-/-)] mutant tumor cells restored the tumorigenic potential (Glas et al., 1992b). They also showed that the IFN-mediated resistance of cells to NK cell-mediated lysis was in part due to the presence of class I molecules, as YAC-1 lymphoma β_2m (-/-) variants could only be protected by IFNs after transfection and expression of β_2m, which allowed for transport of class I molecules in stable form to the cell surface (Ljunggren et al., 1990). As the interactions between most of the known NK cell receptors and MHC class I molecules may have an inhibitory effect on the engaged NK cells, and because some virus infections markedly alter MHC class I expression, it has been speculated that virus-induced modifications of MHC class I expression on target cells may render the cells susceptible to NK cell killing (Storkus and Dawson, 1991; Brutkiewicz and Welsh, 1995).

1) Virus-Induced Downregulation of MHC Class I Molecules

Some viruses quantitatively alter the expression of MHC class I antigens by directly down-regulating cell surface expression and/or by interfering with the ability of IFNs to upregulate class I molecules (Brutkiewicz and Welsh, 1995). During virus infections *in vivo*, the induced IFN-α/β not only activates the NK cells but also transcriptionally induces the expression of MHC class I on many of the cells in the infected host (Bukowski and Welsh, 1985a; Bukowski and Welsh, 1986). This upregulation of class I expression renders the uninfected host cells resistant to NK cell-mediated lysis and more susceptible to allospecific CTL (Hansson et al., 1980; Bukowski and Welsh, 1986). It has been suggested that an impairment of IFN-induced protection in virus-infected cells may leave those cells susceptible to attack by the highly activated NK cells, while the uninfected cells in the host would be protected from such attack (Trinchieri and Santoli, 1978; Santoli and Koprowski, 1979; Bukowski and Welsh, 1986). A downregulation of class I molecules or an inhibition of IFN-induced upregulation of class I molecules on infected cells undoubtedly helps the virus to escape the immune surveillance by T cells, as T cells require the interactions between the viral peptide presented on MHC class I molecules with their cell surface T cell receptors to be activated. Whether these alterations directly influence their susceptibility to NK cells in the context of a viral infection *in vivo* is a question under investigation.

2) Mechanisms of Virus-Induced Downregulation of MHC Class I Expression

Different viruses have evolved various mechanisms to quantitatively reduce cell surface MHC class I expression, presumably in their attempt to escape CTL-mediated lysis. Blocks in class I heavy chain transcription, class I assembly or class I transport are some of the mechanisms described to date. HCMV infection affects the stability of class I heavy chains (Beersma et al., 1993; Warren et al., 1994; Yamashita et al., 1994; Jones and Sun, 1997). In the very early times post-infection, US3 glycoprotein, a 21 kDa immediate early gene product of HCMV, sequesters MHC class I heavy chains predominantly in the endoplasmic reticulum (ER). Subsequently, two early HCMV glycoproteins, US2 and US11 destablize the sequestered MHC class I molecules by misdirecting the class I molecules from the ER to the cytosol for degradation by proteosomes (Jones et al., 1995; Hengel et al., 1996; Wiertz et al., 1996a; Wiertz et al., 1996b; Jones and Sun, 1997). This retention and shunting of class I molecules out of the ER effectively prevents any form of class I from being expressed on the cell surface.

MCMV prevents antigen presentation by blocking the transport of peptide-loaded MHC class I molecules into the medial-golgi compartment (del Val et al., 1992). This block is carried out by MCMV's early gene products, and, in contrast to HCMV, these gene products also downregulate the synthesis of class I molecules but have no discernible effect on the rate of class I degradation (Campbell and Slater, 1994; Thale et al., 1995). Recently one of the early MCMV genes involved in the downregulation of MHC class I molecules was isolated. *m152*, a 40 kDa type I transmembrane MCMV glycoprotein has

been shown to arrest the transport of mouse MHC class I molecules in the ER-golgi intermediate compartment/cis-golgi compartment (Ziegler et al., 1997). This arrest of class I transport is specific as m152 glycoprotein does not prevent the expression of human MHC class I molecules (Ziegler et al., 1997). In addition to the its ability to prevent class I expression, MCMV also inhibits the ability of IFN to upregulate class I expression (Campbell and Slater, 1994).

In HSV-1-infected cells, class I molecules are not transported to the cell surface early in infection under conditions where class I synthesis remains normal (Hill et al., 1994). This class I deficiency is due to the HSV-1 immediate early gene product, ICP47, which blocks the presentation of viral and endogenous peptides to the CTL by physically associating with the transporter associated with antigen presentation (TAP), efficiently blocking the transport of peptides by TAP into the ER (Fruh et al., 1995; Hill et al., 1995). This block in peptide transport into the ER prevents normal MHC class I complexes to form and thus not be expressed on the cell surface.

Human immunodeficiency virus 1 (HIV-1) has been reported to downregulate class I antigens in a $CD4^+$ T cell line. The defect in the expression of class I is not due to a block in the transport or the assembly of class I molecules but is instead due to HIV-1 tat protein-induced reduction in class I heavy chain transcription (Scheppler et al., 1989). In a separate report, HIV-1 Nef protein is also shown to downregulate MHC class I expression in the same $CD4^+$ T cell line, as well as in monocytic and epithelial cells. MHC class I transcription is not reduced in Nef-expressing cells, but the expression of Nef stimulates

the rapid internalization and accumulation of cell surface class I molecules in the endosomal vesicles, which are subsequently degraded (Schwartz et al., 1996; Mangasarian et al., 1997). As tat may control Nef gene expression at the transcriptional level, these 2 viral gene products may work in concert to downregulate MHC class I expression (Cullen and Greene, 1989).

In Burkitt's lymphoma cells or Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines, there is a selective downregulation in the expression of one of several class I alleles (Masucci et al., 1987; Masucci et al., 1989; Imreh et al., 1995). Mutational analyses revealed that the internal glycine-alanine repeat of EBNA1, an EBV nuclear protein, has the ability to inhibit antigen processing and MHC class I presentation (Levitskyaya et al., 1995).

Adenovirus subgroups B through E prevent the expression of class I complexes by binding the E3 19 KDa glycoprotein to class I molecules and retaining them in the ER (Wold and Gooding, 1991; Hermiston et al., 1993). The E1A protein of adenovirus subgroup A, like HIV-1's tat protein, interferes primarily through the downregulation of class I heavy chain mRNA transcription (Friedman and Ricciardi, 1988; Shemesh et al. 1991;). A summary of MHC class I downregulation by viruses is shown in Table I-2.

3) Effects of Virus-Induced Downregulation of MHC Class I Molecules on NK Cell-Mediated Lysis

Although it is well-established that viral infections down-regulate class I expression, it has not yet been clearly shown that this affects the sensitivity of virus-

Table I-2. Virus-Induced Downregulation of MHC Class I Molecules

Virus	Gene/s Involved	Effects on MHC class I	References
HCMV	US3	Retains class I heavy chains in the ER	Ahn et al, 1993; Jones et al, 1993.
	US2, US11	Shunts class I heavy chains into the cytoplasm	Jones et al, 1995; Wiertz et al, 1996; Hengel et al, 1996; Wiertz et al, 1996.
MCMV	Early gene/s (*m152*)	Downregulation of class I synthesis and prevention of transport out of the ER-golgi intermediate compartment/cis-golgi compartment	del Val et al, 1992; Campbell and Slater, 1994; Thale et al, 1995; Ziegler et al, 1997
HSV	ICP47	Prevents peptide translocation into the ER	Fruh et al, 1995; Hill et al, 1993
HIV-1	tat	Dowregulation of class I heavy chain mRNA transcription	Scheppler et al, 1989
	Nef	Internalization of cell surface class I molecules in endosomal vesicles	Schwartz et al, 1996; Mangasarian et al, 1997
EBV	EBNA1	Inhibits antigen processing and expression of HLA-A11 allele	Masucci et al, 1987; Masuci et al, 1989; Imreh et al, 1995;

			Levitskaya et al, 1995
Adenovirus subgroups B through E	E3	Retention of class I heavy chain in the ER	Wold et al, 1991; Hermiston et al, 1993
Adenovirus subgroup A	E1A	Downregulation of class I heavy chain mRNA transcription	Friedman and Ricciardi, 1988; Shemesh et al, 1991.

infected cells to NK cells. A correlation between MHC class I downregulation by different strains of adenovirus and susceptibility of adenovirus-infected cells to NK cell-mediated lysis was made in one report, but no further work has supported this claim (Dawson et al., 1989). Cells infected by an adenovirus E3 mutant, which lacks the gene that retains class I molecules in the ER, express normal levels of class I molecules on the cell surface that can be further upregulated by treatment with IFN-γ, similar to normal uninfected cells (Routes, 1992). However, despite the high levels of class I molecules on the cell surfaces of IFN-γ-treated E3-mutant-infected cells, these cells remain relatively susceptible to NK cell-mediated lysis compared to untreated E3-mutant-infected cells and uninfected cells.

HIV-1-infected cells have enhanced sensitivity to lysis by NK cells, but the link has not yet been made between this enhanced sensitivity to lysis and the HIV-1 tat- or Nef-induced downregulation of class I molecules (Ruscetti et al., 1986; Bandyopadhyay et al., 1990). It is noteworthy that HIV-infected individuals have fewer NK cells than normal subjects and that these NK cells have defective NK cell cytolytic activity (Bonagura et al., 1992; Hu et al., 1995; Ullum et al., 1995). Therefore, even though HIV-1-infected targets may be susceptible to NK cell-mediated lysis, NK cells in these individuals would have poor ability to lyse the targets. It is possible that this HIV-1-induced defective natural immunity contributes to the host's susceptibility to opportunistic infections and the reactivation of herpes viruses thought to be controlled by NK cells (Biron et al., 1989; Ullum et al., 1995).

Substantial work has indicated that human fibroblasts infected with HCMV or HSV-1 are more susceptible to NK cell mediated lysis than uninfected cells (Ching and Lopez, 1979; Borysiewicz et al., 1985). Evidence that this is related to class I molecules was confined to one report showing that increased sensitivity of HSV-1-infected targets to NK cells was limited to cells expressing class I molecules and not to a class I negative cell line, which was quite sensitive to lysis even when uninfected (Kaufman et al., 1992). It has been speculated that the dowregulation of class I molecules by HSV-1 may have clinical significance in gestating mothers infected with the virus. HSV infections can sometimes be deleterious to the unborn child, as there is an association between the HSV-1 infection and spontaneous fetal loss (Zdravkovic et al., 1994). Human trophoblasts do not express the classical MHC class I molecules but instead express the non-classical class I molecule HLA-G, as well as a HLA-C-like molecule, HLA-C_{JED} (Schust et al., 1996). The interaction of HLA-G molecules and the p58 NK cell receptor proteins on cloned human NK cells have an inhibitory effect on NK cell-mediated lysis (Pazmany et al., 1996). Recently it has been shown that HSV-1 ICP47 blocks the intracellular transport of HLA-G, thereby preventing the expression of HLA-G on the cell surface of extravillious cytotrophoblast cell lines (Schust et al., 1996). It has been speculated that this prevention of HLA-G molecules from expressing on the cell surface may be linked to spontaneous fetal loss during HSV-1 infection, as the extravillous cytotrophoblasts would not be protected from maternal NK cell-mediated lysis. More information is required to evaluate this hypothesis.

4) Qualitative Alterations in MHC Class I Molecules Associated with Insertion of Viral Peptides

A second mechanism by which viruses might alter MHC class I expression is by the insertion of virus-encoded peptides into the class I peptide-binding groove. Studies using site-directed mutagenesis on human MHC class I molecules and with MHC-congenic strains of mice suggest that human and mouse NK cells interact with the class I molecules near the antigen peptide-binding groove and the surrounding α-helices, specifically the α1 and α2 domains, which cradle the peptide (Karlhofer et al., 1994; Kurago et al., 1995). Indeed, cloned NK cells have been shown to be inhibited by self peptides in the context of self MHC molecules (Correa and Raulet, 1995; Malnati et al., 1995). These results suggest that the self peptides presented by the class I molecules may be important in the inhibition of NK cell lysis and lead to the hypothesis that the insertion of foreign (i.e. viral) peptides into the peptide-binding groove may interfere with this interaction and render a virus-infected cell sensitive to NK cell killing. Studies on the sensitivity of target cells to lysis by NK cells after treatment of targets with immunodominant viral peptides for CTL have led to conflicting results. One study suggested that an influenza virus peptide enhanced the susceptibility of concanavalin A (Con A)-induced lymphoblasts to syngeneic NK cells (Chadwick et al., 1992). A second conflicting report showed that under conditions that would sensitize target cells to CTL

killing, immunodominant T-cell peptides from several viruses including influenza failed to sensitize these target cells to NK cell-mediated lysis (Brutkiewicz and Welsh, 1995). Studies using the Ly49A$^+$ NK cell subset and TAP-mutant RMA-S cells transfected with H-2D^d showed that any of a variety of peptides that enabled the H-2D^d molecule to form a stable complex on the cell surface delivered a negative signal to Ly49A$^+$ NK cells (Correa and Raulet, 1995). A recent report showed that empty MHC class I molecules which can be stably expressed on RMA-S cell surfaces at 26^{0}C are sufficient to confer protection to the target cells, implying that polymorphic structures on the class I molecules and not the peptides that are interacting with the NK cells (Mandelboim et al., 1996). It is thus likely that when peptides do alter the sensitivity of targets to NK cells, it is not because they are recognized by NK cells per se, but because they either change the conformation of the class I molecule or destabilize it on the cell surface.

Whether or not the insertion of immunodominant or even non-immunodominant peptides into MHC class I during a viral infection will alter the negative signal to NK cells and render a virus-infected target preferentially susceptible to NK cell-mediated lysis remains unclear. As VV-infected target cells become sensitive to lysis by VV-specific CTL, the infected targets have a period in time when they display enhanced sensitivity to NK cell-mediated lysis and markedly reduced sensitivity to killing by allospecific CTL, even though the quantitative expression of class I antigens remains high; it was suggested that the replacement of endogenous peptides presented by the MHC molecules by foreign VV-encoded peptides may have abrogated the abilities of class I molecules to be

recognized properly by either allospecific CTL or NK cells (Brutkiewicz et al., 1992). In a second similar study, HSV-1 infection was found to enhance the NK cell-sensitivity of class I-deficient C1R cells transfected with class I genes but had no effect on the NK cell-sensitivity of non-transfected cells (Kaufman et al., 1992). This enhanced sensitivity occurred under conditions where class I expression remained high on the cell surface, leading again to the speculation that viral peptides may have replaced endogenous peptides in the expressed class I molecules. While suggestive, neither of these studies formally proved the hypotheses.

B. NK CELLS AS EFFECTOR CELLS AGAINST PARASITIC INFECTIONS

In vivo and *in vitro* evidence has indicated that NK cells can contribute to the early resistance against *Toxoplasma gondii, Leishmania major and Schistosoma mansoni* infections by producing IFN-γ, which stimulates microbicidal activity in macrophages. To date there is little evidence of direct NK cell-mediated killing of parasites or parasite-infected cells. *T. gondii*-infected IFN-γ knockout mice or C57BL/6 mice treated with anti-IFN-γ Abs have good NK cell activity but cannot control the infection, whereas beige mice deficient in NK cell cytotoxic activity can control the parasitic infection (Pazmany et al., 1996). The first example of a protozoan stimulating the production of T cell-independent IFN-γ was shown using *T. gondii* (Sher et al., 1993). *In vitro* studies showed that SCID mouse splenocytes exposed to live tachyzoites of *T. gondii* or to just soluble

parasitic extracts could be stimulated to produce high levels of IFN-γ (Sher et al., 1993). Spleen cells from SCID mice when treated with anti-aGM$_1$ antisera, which deplete the NK cells, did not produce IFN-γ, suggesting that it was the NK cells that were the source of the IFN-γ. *In vivo*, the depletion of IFN-γ or NK cells in *T. gondii*-infected SCID mice abrogated the resistance to the parasite, adding further evidence that NK cell-produced IFN-γ is essential in the regulation of T. gondii (Gazzinelli et al., 1993). The protection afforded by NK cells in the early resistance to *T. gondii* was examined in β_2m (-/-) mice (Denkers et al., 1993). Depletion of NK1.1$^+$ cells in β_2m (-/-) mice enhanced the growth of the parasite, whereas the depletion of CD4$^+$ or CD8$^+$ T cells had no effect on this early resistance. As in the MCMV infection, NK cell-produced IFN-γ during *T. gondii* infection requires the help of macrophage-produced IL-12 and TNF-α (Gazzinelli et al., 1993).

IFN-γ produced by NK cells also contributes to the early innate resistance to *L. major* and *S. mansoni*. *In vivo* depletion of NK cells in C57BL/6 mice with anti-NK1.1 Abs reduced the amount of IFN-γ produced, resulting in enhanced growth of *L. major* (Laskay et al., 1993; Scharton-Kersten and Scott, 1995). In *S. mansoni*-infected C3H and C57BL/6 mice, depletion of NK cells or IFN-γ resulted in increased parasitic burden and granuloma formation (Wynn et al., 1994).

In addition to providing resistance to parasites during the early phase of infection, NK cells can also modulate the adaptive immune response. The depletion of NK cells, IFN-γ or IL-12 from *S. mansoni*-infected C3H or C57BL/6 mice resulted in the skewing of the initial Th1 response to a Th2 response (Oswald et al., 1994; Wynn et al., 1994)

The same principle was true for *L. major*-infected mice, as the removal of NK cells decreased IFN-γ levels and promoted IL-4 production, leading to higher parasitic burden and lesion development (Scharton and Scott, 1993). Depletion of aGM_1^+, $CD3^-$ cells or IL-12 in the genetically resistant C3H mice infected with *L. major* abrogated NK cell-produced IFN-γ, causing the mice to mount a Th2 response rather than the usual Th1 response (Scharton-Kersten et al., 1995; Scharton-Kersten and Scott, 1995). BALB/c mice cannot control *L. major* infection because they are genetically predisposed to mount a Th2 response to the parasite. Administration of IL-12 promoted $CD4^+$ Th1 development in *L. major*-infected BALB/c mice, and these Th1 cells failed to develop if the NK cells were depleted at the time of infection (Afonso et al., 1994).

NK cells are not always a determining factor in modulating Th1 versus Th2 responses, as removal of NK cells in *Candida albicans*-infected C57BL/6 mice or in *L. major*-infected C3H mice still allowed for the development of a Th1 response (Romani et al., 1993; Scharton-Kersten and Scott, 1995). However, there still was an increase in parasite burden in the L. major-infected C3H mice because the Th1 response was delayed (Scharton-Kersten and Scott, 1995). Both C57BL/6 and C3H mice are naturally high NK strains, and it is possible their high NK cell activity may predispose them to mount a Th1 instead of a Th2 response. The inability of BALB/c mice to mount a Th1 response may be because BALB/c mice have low NK cell activity and their NK cells are poor producers of IFN-γ (Glas et al., 1992b; Scharton and Scott, 1993). It would be interesting to see if

BALB/c mice made congenic with either the C3H or C57BL/6 NK gene complex would have a Th1 response instead of a Th2 response to *L. major* infection.

C. NK CELLS AS EFFECTORS AGAINST BACTERIAL INFECTIONS

NK cells have been reported to have the capacity to be directly bactericidal and to lyse bacterially-infected cells *in vitro*, but the more likely way NK cells control bacterial infections *in vivo* is by producing cytokines that activate the macrophages to degrade the bacteria. Purified human NK cells when mixed with *Salmonella typhimurium* were shown to inhibit the outgrowth of the bacterial colonies (Garcia-Penarrubia et al., 1989). Macrophages when exposed to bacteria or bacterial products are stimulated to produce NK cell-activating cytokines such as IFN-α/β, IL-12 and TNF-α, which in turn activate the NK cells to kill NK-sensitive targets (Wolf et al., 1976; Wold and Gooding, 1991; Guo et al., 1992). Direct incubation of NK cells with fixed bacteria can also activate the NK cells to kill NK-sensitive targets (Tarkkanen et al., 1986). NK cells have been shown to lyse *S. typhimurium*-, *Mycobacterium avium* complex-, and *Shigella flexneri*-infected cells *in vitro* (Klimpel et al., 1986; Katz et al., 1990; Griggs and Smith, 1994). However, experimental evidence *in vivo* has suggested that NK cell-produced cytokines are probably more important than the cytotoxic potential of NK cells in the control of bacterial infections. *Listeria monocytogenes*, *M. avium* and *S. typhimurium* infection in mice can stimulate the production of IFN-γ by NK cells, and this stimulation, as with viruses and

parasites, requires factors produced by macrophages (Ramarathinam et al., 1993; Tripp et al., 1993; Appelberg et al., 1994). *L. monocytogenes* infection in SCID mice induces the production of TNF-α and IL-12 from macrophages, and these cytokines subsequently activate the NK cells to produce IFN-γ (Tripp et al., 1993). Neutralization of IL-12, the cytokine that induces the production of IFN-γ, decreases the resistance to listeria in SCID mice, indicating the importance of NK cell-produced IFN-γ in the regulation of this intracellular bacterial infection (Tripp et al., 1994).

IFN-γ-mediated resistance to mycobacterial or listerial infections may involve the production of NO by macrophages and other cells (Glesch and Kaufmann, 1991; Beckerman et al., 1993). iNOS-deficient mice cannot control *L. monocytogenes* replication (MacMicking et al., 1995). The production of NO in *L. monocytogenes*-infected SCID mice is dependent on NK cell-produced IFN-γ, and inhibitors of NOS, N^G-monomethyl arginine or aminoguanidine inhibited the production of NO and increased the titers of listeria in these mice (Beckerman et al., 1993).

Even though there is strong evidence showing that the depletion of NK cell-produced IFN-γ increased the susceptibility of immunodeficient SCID mice to *L. major* infection, several studies using normal mice have ironically suggested that the depletion of NK cells *increased* host resistance to the bacteria. Depletion of NK cells in C57BL/6 mice by anti-NK1.1 mAb increased the resistance *to L. monocytogenes* (Takada et al., 1994). This result is surprising, as IFN-γ has been shown to be important for the control of the listerial infection, and anti-NK1.1 treatment decreases the number of IFN-γ producing

cells (Teixeira and Kaufmann, 1994). An explanation for this may reside in competition between host effector functions. One study suggests that NK cells may be inhibiting the proliferation of γδ T cells that may play a role in the enhanced clearance of *L. monocytogenes* in the early stage of infection (Takada et al., 1994) and a second study has shown that the presence of NK cells may inhibit the accumulation of neutrophils in bacterially-infected lesions (Newton et al., 1992).

OBJECTIVE OF THE THESIS

The major focus of this thesis project was to determine how NK cells regulate virus infections *in vivo*. The mechanisms used by NK cells in the early stages of virus infections were not well defined, and one of the major goals was to study the *in vivo* mechanisms by which NK cells regulate MCMV infections in adult mice as well as in suckling mice. The discovery of the innate MCMV resistance gene, *Cmv-1*, and the disparity in the spleen and liver regulation of MCMV in C57BL/6 and BALB/c mice stimulated us to also ask if NK cells used different mechanisms to control MCMV in different organs and if subsets of NK cells with the ability to use different mechanisms traffick to different organs to control the infection in that organ. As mouse genetic studies had indicated that the gene *Cmv-1* was found within the mouse NK gene locus, the second objective was to test the importance of mouse NK cell receptors and MHC class I molecules in the control of MCMV by NK cells. The specific aims discussed in this thesis are as follows:

1. What are the mechanisms utilized by NK cells to regulate MCMV infections in adult C57BL/6 mice?

2. What are the mechanisms utilized by NK cells to regulate MCMV infections in suckling mice?

3. What role does the innate MCMV resistance gene, *Cmv-1*, play in the regulation of MCMV by NK cells in the spleen and the liver?

4. Do the members of the Ly49 NK cell receptor family play any roles in the *in vivo* regulation of MCMV by NK cells?

5. Do the interactions between MHC class I molecules and NK cells influence the ability of the NK cells to control virus infections?

CHAPTER II

MATERIALS AND METHODS

A. CELLS

YAC-1 is a highly NK-sensitive Moloney leukemia virus-induced lymphoma cell line from A/Sn mice. D1R and R1E.D^b cells were derived from R1.1 (thymoma cells from C58/J mice) and R1E (β_2m-deficient derivative of R1.1) cells respectively, and transfected with the H-$2D^b$ gene (Allen et al., 1986; Waneck et al., 1987). YAC-1 cells were cultivated in suspension in RPMI 1640 medium (Sigma, St. Louis, Mo.) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Complete RPMI). D1R and R1E.D^b were grown in the above medium additionally supplemented with 500 mg/ml of G418 (GIBCO/BRL, Gaithersburg, MD). D1R and R1E.D^b cells were kindly provided by Dr. G. Waneck, Massachusetts General Hospital. Mouse embryonic fibroblasts (MEF) and vero cells (monkey kidney cells) were cultivated as monolayers in MEM (Gibco Laboratories, Grand Island, NY) with antibiotics and 20% or 10% FBS, respectively.

B. GENERATION OF MEF

MEF were generated from 12 - 14 day pregnant C57BL/6 mice. The embryos were aseptically removed, and only the torsos were used. The torsos were minced using a

pair of dissecting scissors and the resulting mixture was treated for 1 hour at 37^0C in 0.05% trypsin (GIBCO BRL, Grand Island, NY). MEM with 20% FBS and antibiotics were then added to the mixture and were used to seed T150 flasks (Falcon, Newark, NJ). After 4 - 5 days of culture, cells adhering to the bottom of the flasks were removed and expanded. Confluent monolayers of MEF formed after first passage were removed using 0.05% trypsin and were frozen in liquid nitrogen for future use.

C. MICE

C3H/HeSnJ (H-2^k), C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. These mice were conventionally housed and were used at 6 - 12 weeks of age. Breeder pairs of mice homozygous for a targeted mutation disrupting the gene for β_2m, originally derived by Drs. B. Koller and O. Smithies (Koller et al., 1990), University of North Carolina, Chapel Hill, NC, were kindly supplied by Dr. D. Roopenian (The Jackson Laboratory, Bar Harbor, ME). Breeder pairs of 129 wild type control and 129 mice homozygous for a targeted mutation disrupting the mouse interferon gamma receptor gene (IFN-$\gamma R^{0/0}$) were originally derived and kindly supplied by Dr. M. Aguet, University of Zurich, Switzerland (Huang et al., 1993). Breeder pairs of 129 x C57BL/6 mice heterozygous for a targeted mutation disrupting the gene for perforin were originally derived and kindly provided by Drs. C.M. Walsh and W.R. Clark, University of California, Los Angeles (Walsh et al., 1994). F1 offspring of the above

heterozygous breeding were typed using PCR. β_2m (-/-) mice, perforin deficient (perforin 0/0) and IFN-$\gamma R^{0/0}$ mice and their respective wild type controls were housed in a specific pathogen free (SPF) environment and were used at 6 - 12 weeks of age. Suckling mice of C57BL/6, 129 and 129/IFN-$\gamma R^{0/0}$ background were used at 4 - 6 days of age in the adoptive transfer experiments.

D. VIRUSES

To generate stocks of the Smith strain of MCMV, 6 week old BALB/c mice were injected i.p. with 5 x 10^3 PFU MCMV. The mice were sacrificed two weeks post-infection, and the salivary glands were removed. The salivary glands were ground using a mortar and pestle, and the resulting mixture was centrifuged at 2000 rpm at 4^0C for 20 min. The supernatant was removed, aliquoted and stored at -70^0C. MCMV, which is an NK cell-sensitive virus (Bukowski and Welsh, 1985b; Bukowski et al., 1985), was titrated by plaque assay on MEF. MCMV was given to adult and suckling mice i.p. at a dose of 1 - 3 x 10^4 PFU/adult mouse and 2 x 10^3 PFU/suckling mouse 3 days before use.

The Armstrong strain of LCMV was propagated in BHK21 cells. LCMV, which is an NK cell-resistant virus (Bukowski and Welsh, 1985b; Bukowski et al., 1985), was titrated by plaque assay on vero cells. LCMV was given i.p. at a dose of 5 x 10^4 PFU/mouse 3 days before assay.

E. PLAQUE ASSAYS

MCMV and LCMV were titrated by plaque assay on MEF and vero cells, respectively. Organs from MCMV- or LCMV-infected mice were removed and stored at -70^{0}C in complete RPMI. On the day of the plaque assay, the organs were ground using a mortar and pestle and the mixture was centrifuged at 2000 rpm at 4^{0}C for 20 min. The clarified supernatant was removed and was used to titrate the virus on the appropriate cells in 6 well plates (Costar, Cambridge, MA). Ten fold dilutions of 100 µl were added to the cells in 1 ml of media and incubated for 90 min at 37^{0}C with occasional agitation. A 1:1 mixture of 1% Seakemp agarose (FMC, Rockland, ME) and EMEM (BioWhittaker, Walkersville, MD) supplemented with antibiotics (overlay mixture) and 40% FBS (for the titration of MCMV on MEF) or 10% FBS (for the titration of LCMV on vero cells) was overlayed onto the cells. The plates were incubated at 37^{0}C for 5 days (MCMV) or 4 days (LCMV). To visualize MCMV plaques, the agarose overlays were removed, and the cells were stained with 0.1% crystal violet (Sigma, St. Louis, MO) in 1% formaldehyde (Banco, Fort Worth, TX)-PBS solution. To visualize LCMV plaques, 0.1% neutral red (Sigma, St. Louis, MO) was mixed with the overlay mixture and added onto the initial agarose overlay 3 days post-assay. LCMV plaques were read on day 4.

F. INFECTIVE CENTER PLAQUE ASSAYS

Infective center plaque assays were done by using a modification of the routine plaque assays. Spleens from virus-infected mice were aseptically removed and single cell suspensions were prepared using complete RPMI. One million spleen cells suspended in 1 ml of media were added to the appropriate cells (MEF for MCMV and vero cells for LCMV) in 6 well plates and incubated for 4 hours at 37^0C. Care was taken to make sure that the plates were not agitated during incubation. The plates were then washed 3 times to remove the spleen cells before the overlay mixture was added. The plates were incubated at 37^0C for 5 days (MCMV) or 4 days (LCMV). Visualization of the plaques were the same as in the routine plaque assays.

G. CYTOTOXICITY ASSAYS

Standard 4 - 6 h ^{51}Chromium-release microcytotoxicity assays (Bukowski et al., 1985) were used to determine NK cell activity on various targets. Target cells (1×10^6) were incubated with 20 μCi of $Na_2{}^{51}CrO_4$ (5 mCi/ml, Dupont NEN, Boston, MA) for 1 hour at 37^0C. The targets were then washed 3x in warm complete RPMI, and YAC-1 and other cultured cells were used at 10^4 targets/well and at a variety of E:T ratios. To avoid the high spontaneous release of ^{51}Cr, thymocyte targets were used at 4×10^4 cells per well. All assays were performed in U-bottom 96 well plates (Costar, Cambridge, MA).

H. IMMUNE REAGENTS

Rabbit antiserum to aGM_1 (Wako Labs, Dallas, TX) was used at a pretitrated dose which depleted LCMV-induced NK cell activity but not CTL activity in C57BL/6 mice. Anti-aGM_1 was inoculated into mice i.p. 12 h prior to or on the day of infection (Bukowski et al., 1983). Poly I:C was purchased from Sigma Chemical Co., St. Louis, MO, and was inoculated i.p. into mice at a dose of 100 μg/mouse 2 days before assay. Mouse IFN β was purchased from Lee Biomolecular, San Diego, CA. IFN β was used at 1000 U/ml to treat the appropriate cultured cells for 24 h at 37^0C prior to assay. The anti-NK1.1 mAb, PK136 (provided by Dr. G.C. Koo, Merck Sharpe and Dohme Research Laboratories, Rahway, NJ) (Koo and Peppard, 1984), was produced in ascites using BALB/c mice, and then NH_4SO_4-cut and affinity-purified before use. PK136 was inoculated into mice i.v. via the retro-orbital sinus at a dose of 200 μl of a 1:40 dilution/mouse 24 hours before the day of infection. The anti-IFN-γ mAb, R46A2, was obtained from American Type Culture Collection, Rockville, MD, and was inoculated i.p. in ascites form at 200 μl of a 1:5 dilution/mouse on the day of infection, and at day 1 and day 2 after infection. By ELISA, the amount of anti-IFN-γ ascites given in 3 days was enough to bind 0.9 μg/ml of recombinant mouse IFN-γ (Pharmingen, San Diego, CA). *In vivo* depletion of $CD4^+$ T cells was done by injecting i.p. 80 μl of a rat anti-mouse CD4 mAb in undiluted ascites (clone GK1.5). $CD8^+$ T cells were depleted by injecting i.p. 100 μl of a 1:40 dilution of an ascites preparation of rat anti-mouse CD8 mAb (clone 2.43). Clone 2.43 was provided by Dr. R. Fujinami, University of Utah, UT, and clone GK1.5

was purchased from American Type Culture Collection, Rockville, MD. The anti-CD4 and anti-CD8 mAbs were routinely used in our laboratory at these concentrations to deplete CD4 and CD8 cell numbers as well as virus-induced CTL activity *in vivo*. Anti-Ly49G2 ascites, 4D11 (Hazelton Technologies), was given iv at a dose of 200 μg/mouse 1 day before virus infection. Anti-Ly49D ascites, 12A8, was given iv at a dose of 300 μg/mouse at day -2 and day -1. 4D11 and 12A8 Abs were provided by Dr. J.R. Ortaldo, NCI, Frederick, MD. Anti-Ly49C ascites, 5E6, and anti-Ly49A ascites were given iv at a dose of 2 mg/mouse and 100 μl of a 1:2 dilution/mouse, respectively, 1 day before virus infection. Anti-Ly49A and anti-Ly49C Abs were provided by Dr. V. Kumar, University of Texas, Southwestern, Dallas, TX. The Ly49 antibodies were used at the doses recommended by our collaborators (Drs. V. Kumar and J.R. Ortaldo), who had titrated the antibodies *in vivo* to ensure the depletion of the respective NK cell subsets.

I. IMMUNOFLUORESCENCE

The rat anti-mouse H-2 class I mAb M1 (clone M1/42.3.9.8.HLK) is panreactive for all mouse H-2 class I molecules except H-2D^{d}. The 28-11-5S and the 28-14-8S hybridomas produce anti-H-2D^{b} alloantibodies that recognize conformation-dependent and conformation-independent determinants, respectively (Townsend et al., 1989). Clone M1, 28-11-5S, and 28-14-8S were obtained from the American Type Culture Collection, Rockville, MD. Fluorescein isothiocyanate (FITC)-labelled goat anti-rat IgG + IgM (H+L)

and FITC-labelled goat anti-mouse $F(ab)'_2$ Ig antiserum were purchased from Jackson Immunoresearch, West Grove, PA. Clone M1, 28-11-5S and 28-14-8S were used at 200 $\mu l/10^6$ cells. Cells were incubated with the above antibodies for 30 min. on ice, washed and FITC-labelled goat anti-rat IgG + IgM or FITC-labelled goat anti-mouse $F(ab)'_2$ Ig antiserum were added. The cells were then incubated for another 30 min. before they were washed again and fixed with 4% paraformaldehyde for 10 min in room temperature. After the final wash, the cells are kept at 4^0C until FACs analysis by the Univ. of Massachusetts Medical Center (UMMC) FACs facility.

FITC-labelled anti-CD8 and phycoerythrin (PE)-labelled anti-CD4 were purchased from GIBCO/BRL, Grand Island, NY. PE-labelled anti-Thy1.2 mAb, FITC-labelled B220 mAb, PE-labelled anti-NK1.1 mAb, FITC-labelled anti-Ly49A mAb and FITC-labelled Ly49C mAb were purchased from Pharmingen, San Diego, CA. The antibodies were used according to manufacturer's specifications. FITC-labelled anti-Ly49D (12A8) (50 µl of $1:80/10^6$ cells) and FITC-labelled anti-Ly49G2 (4D11) (50 µl of 1:40 dilution/10^6 cells) were provided by Dr. J.R. Ortaldo, NCI, Frederick, MD. Cells were incubated with the antibodies for 30 min on ice before they were washed and fixed with 4% paraformaldehyde for 10 min in room temperature. After the final wash, cells stained with these directly labeled antibodies were kept as above before FACs analysis.

To sort the lymphokine activated killer (LAK) cells into the different Ly49 NK cell subsets, LAK cells were stained with PE-labelled anti-NK1.1 mAb and one of the 4 FITC-labelled anti-Ly49 antibodies at a concentration stated above. The stained cells were

washed once, resuspended in RPMI-1640 supplemented with 4% FBS and antibiotics, and were immediately sent to the UMMC FACs facility for sorting. Typically, the sorted cells were 90% - 99% pure for the respective population.

Anti-Ly49D (12A8) Ab used in the *in vivo* depletion studies and the adoptive transfer studies was cross-reactive with Ly49A NK cell receptor. However a different FITC-labelled anti-Ly49D Ab which was not cross-reactive with Ly49A receptor was used in the study of NK cell distribution. This Ab was used at a concentration 50 µl of a 1:1000 dilution/10^6 cells.

To stain for MHC class II antigens on macrophages, 5 x 10^7 splenocytes were first treated with a rat monoclonal antibody, J11D (33 µl of 1:3 dilution) (Bruce et al., 1981; Natuk and Welsh, 1987a), + rabbit complement (Pel Freeze Chemical Inc., Brown Deer, WI) to remove the B cells and granulocytes. The residual cells were pre-treated with normal mouse serum for 10 min. and then stained with mouse anti-mouse I-A^b-FITC (Pharmingen, San Diego, CA).

J. GENERATION OF LAK CELLS

Ten ml syringes (Becton Dickinson & Company, Franklin Lakes, NJ) packed with 0.8 - 1.0 g nylon wool (Polysciences Inc, Warrington, PA)/syringe were used to generate LAK cells. The columns were first rinsed with 20 ml of prewarmed RPMI 1640 (Sigma, St. Louis, MO), supplemented with 10% FBS, L-glutamine, antibiotics, non-essential

amino acids, sodium pyruvate and 2-mercaptoethanol (MLC media). The columns were then filled with MLC media to cover the nylon wool and any air bubbles trapped within the columns were removed. The columns were incubated for at least 30 min or until use at 37^0C to allow the nylon wool to equilibrate with the media. In the above preparation steps, 18 gauge needles (Becton Dickinson & Company, Franklin Lakes, NJ) were used for rinsing the nylon wool. Six to eight week old C57BL/6 spleens were aseptically excised, and a single cell suspension was prepared using MLC media. Splenic leukocytes were washed, and 1 x 10^8 cells at a concentration of 5 x 10^7 cells/ml were incubated on a nylon wool column for 45 min at 37^0C. After incubation, nylon wool non-adherent cells were eluted at a rate of 1 drop/second with prewarmed media and then cultured in MLC media supplemented with 800 U/ml of recombinant human IL-2 (Cetus Corporation, Emeryville, CA) at a density of 2 x 10^6 cells/ml in 60 x 15 mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ). The elution step was performed using a 26 gauge needle (Becton Dickinson & Company, Franklin Lakes, NJ). After the initial 3 days of culture, only the adherent cells were expanded using the MLC media supplemented with 800 U/ml of recombinant IL-2 for another 4 - 6 days before use.

K. ADOPTIVE TRANSFERS

Adoptive transfer experiments were done by using a modification of a method previously described (Bukowski et al., 1985). Four to six day old suckling mice were pooled and randomly assigned to lactating females. Groups of 4 - 10 mice were given 5 x

10^5 LAK cells/mouse or 5 x 10^7 mouse spleen cells/mouse i.p. in 0.1 ml of complete RPMI using a 1-ml syringe and a 30 gauge needle (both purchased from Becton Dickinson & Company, Franklin Lakes, NJ). The following day, the suckling mice were challenged with 2000 PFU/mouse MCMV i.p. Three days post-infection, the mice were sacrificed and the spleens were removed for virus titration.

L. REAGENTS

N^{ω}-monomethyl-$_L$-arginine (L-NMA) and N^{ω}-monomethyl-$_D$-arginine (D-NMA) were obtained from Sigma Immunochemicals (St. Louis, MO). Both reagents except where indicated were given i.v. at a dose of 5 mg/mouse on the day of infection, and at day 1 and day 2 of the infection.

M. ISOLATION OF MOUSE TAIL DNA FOR PCR

Mouse tails approximately 1 cm in length were taken from mice 6 weeks old or less and cut into several pieces. The protein was digested in a 500 µl solution containing 0.1 M EDTA, 0.05 M Tris-Cl, pH 8.0, 0.5% SDS and proteinase K (final concentration 500 µg/ml) overnight at 55^0C. At the end of the digestion, NaCl was added at a final concentration of 150 mM. A phenol, chloroform and isoamyl alcohol mixture (25:24:1) was added and the resulting mixture was microfuged at 14000 rpm for 5 min in the cold (DNA extraction step). The aqueous phase was removed into a fresh tube, and the DNA

extraction step was repeated. After the second DNA extraction, NaCl with a final concentration of 0.5 M was added to the resulting aqueous phase before 100% ethanol at room temperature was added. The solution was mixed by inversion and spun in the microfuge at 14000 rpm for 15 min. The DNA pellet obtained after the first ethanol wash was washed the second time with 100% ethanol to remove any residual salt. DNA obtained after a second wash was dried in a SpeedVac (Savant SVC-100H, Farmingdale, NY) for 15 min. TE was then added to solublize the dried DNA in a 37^{0}C waterbath, and the DNA solution was stored at -20^{0}C.

N. TYPING OF PERFORIN-DEFICIENT MICE

The primer sequences of the perforin primers were as follows: CTCCGGCTCCTTCCCAGTGA and TTCTTCCAGTTCCTTTCTCC (Ransom Hill Bioscience Inc., Ramona,CA). Neomycin primer sequences 1 and 2 (ATGATTGAACAAGATGGATTGC and GACAAAAAGAACCGGGCG respectively) were provided by Dr. C.L. Sidman, University of Cincinnati Medical Center, Cincinnati, OH. The neomycin primers were made by Biosynthesis, Lewisville, TX, and were provided by Dr. A.M. Baird, Harvard University, Cambridge, MA. PCR were performed on the tail DNA obtained from the F1 offspring of the perforin heterozygous breeding. PCR were done in a 20 μl volume, consisting of 7.9 μl double distilled water, 4 μl of DNA, 0.4 μl each of the perforin primers (20 μM stock), 108 ng/reaction of neomycin

primer 1, 132 ng/reaction of neomycin primer 2, 2 µl of 10X PCR Buffer II, 2 µl 10 mM dNTP (2.5 mM dATP, dCTP, dGTP and dTTP each), 2.5 µl of 25 mM Mg^{2+} solution and 2 U of AmpliTaq DNA Polymerase (5 U/µl). All the PCR reagents were obtained from Perkin Elmer's GeneAmp PCR Core Reagents, manufactured by Roche Molecular Systems Inc., Branchburg, NJ. The 20 µl reaction mix was layered with 20 µl of mineral oil, placed in the Peltier Thermal Cycler (Model PTC-2000, MJ Research, Inc., Watertown, MA), and after 5 minutes at 94^0C, 35 cycles of the following were performed: 1 minute at 94^0C, 2 minutes at 56.5^0C and 2 minutes at 72^0C. This was followed by a 2 minutes at 33^0C resting stage and varying lengths of time at 4^0C. The reaction products were then loaded onto a gel (2% Metaphor Agarose, FMC, Rockland, ME, mixed with 1% Ultrapure Agarose, GIBCO BRL, Gaithersburg, MD), and after electrophoresis, the gel was stained with ethidium bromide and the DNA bands visualized by ultraviolet trans-illumination. The PCR would only amplify the 145 bp neomycin band from the DNA of perforin 0/0 and only the 414 bp perforin band from the perforin-intact mice. Tail DNA from perforin heterozygous mice was used as a positive control, and 2 bands corresponding to the 414 bp perforin band and the 145 bp neomycin band were obtained (Fig II-1).

O. GENERATION OF BONE MARROW CHIMERAS

Bone marrow cells were obtained from 6 - 12 week old C57BL/6 mice by flushing the femur with RPMI 1640 supplemented with 10% FBS and antibiotics. Prior to injection

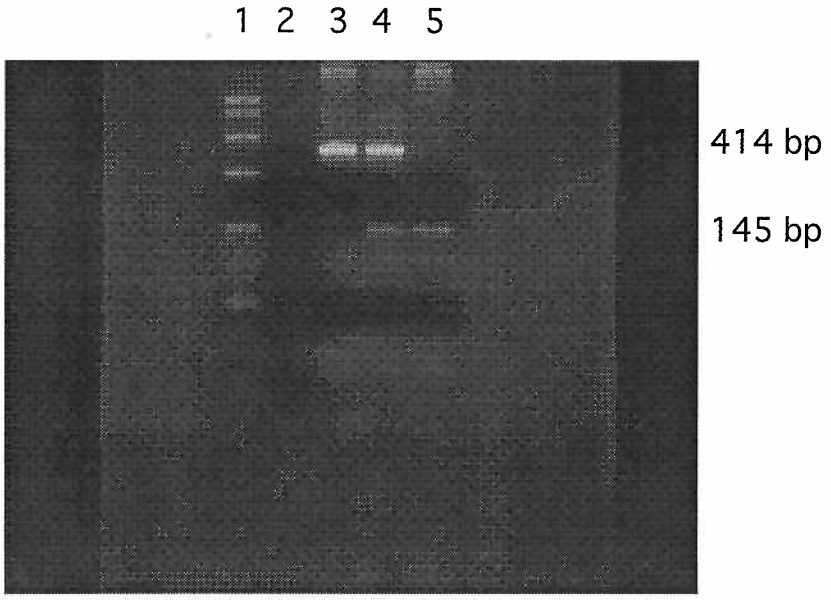

Figure II-1. Typing of perforin-deficient mice using PCR. Mice tail DNA was typed using perforin-specific and neomycin-specific primers in the same reaction vial. PCR will only amplify the 414-bp perforin band from perforin +/+ and only the 145-bp perforin band from perforin 0/0 mice. Lane 1: 100 bp PCR markers. Lane 2: No DNA control. Lane 3: Perforin +/+ DNA. Lane 4: Perforin +/0 DNA. Lane 5: Perforin 0/0 DNA.

with bone marrow cells, 6 - 12 week old 129 or IFN $\gamma R^{0/0}$ mice received 950 rads (1 rad = 0.01 gray) of γ-irradiation. The irradiated mice were allowed to rest for 1 hour before they were injected i.v. with 5 x 10^7 bone marrow cells via the retro-orbital sinus. These bone marrow-reconstituted mice were housed in microisolator cages and were given sterile food and tetracycline-treated water and were used 6 weeks after reconstitution. The ability of anti-NK1.1 mAb to deplete the NK cell activity in the reconstituted mice was used as an indication that the bone marrow reconstitution with $NK1.1^+$ C57BL/6 cells was complete, as the 129 strain does not express the NK1.1 antigen.

P. STATISTICAL ANALYSES

All the data shown except in Table III-2 were analyzed by Student's *t*-test for each individual experiment. For Table III-2, the calculation of the average $\log_{10}$ PFU increase or decrease in MCMV titers compared to the appropriate controls, taking into consideration all of the relevant experiments was determined. Data obtained from individual mice were used, and a statistical test analyzing the variance (ANOVA) for mixed models using restricted estimation by maximum likelihood (REML) was applied. All computations were performed using the "Proc Mixed" procedure in the SAS statistical software package (SAS, Cary, NC). The results are shown as average $\log_{10}$ PFU ± SE in the text and in Table III-2.

CHAPTER III

MECHANISMS OF MCMV REGULATION BY NK CELLS IN ADULT C57BL/6 MICE

The mechanisms utilized by NK cells to regulate MCMV infections *in vivo* have until recently been poorly understood. Recent studies have indicated that NK cells produce anti-viral cytokines like IFN-γ to control the virus in the liver (Orange et al., 1995; Orange and Biron, 1996a) but there are no reports on how the virus is regulated in the spleen, another major organ of early MCMV infection. Findings by Scalzo et al have shown that the *Cmv-1* gene product, a non-MHC-linked resistance gene to MCMV which maps within the NK gene complex (Scalzo et al., 1990; Scalzo et al., 1992; Scalzo et al., 1995b), confers resistance to MCMV in the spleen but not in the liver. As the effects of *Cmv-1* are mediated by $NK1.1^+$ cells, it is possible that NK cells may utilize a *Cmv-1*-dependent mechanism to control MCMV in the spleen but another mechanism to control the virus in the liver. To address this possibility, I examined MCMV infections in perforin 0/0 mice and IFN-$\gamma R^{0/0}$ mice, as well as in normal mice treated with anti-IFN-γ Abs in an effort to delineate the mechanisms utilized by NK cells in the regulation of MCMV in different organs. I will show in this chapter that in *Cmv-1*^{r}, C57BL/6 mice, there is a dichotomy in the mechanisms utilized by NK cells in the regulation of MCMV in different organs.

A. REPLICATION OF MCMV IN PERFORIN +/+ AND 0/0 MICE

Perforin is a key effector molecule in cell-mediated cytolysis mediated by $CD8^+$ T cells and NK cells, and the molecule has been shown to be important in the *in vivo* T cell-dependent regulation of virus infections and in the clearance of tumors (Walsh et al., 1994; Kagi et al., 1994a). To study the role of perforin in the early regulation of MCMV by NK cells, perforin +/+ and 0/0 mice were infected with MCMV for 3 days, and the NK cell activity and MCMV titers in the spleens and livers were measured. Spleen and liver viral titers were chosen, because these are the primary organs of infection early in the infection. Figure III-1 shows the NK cell activity elicited in these mice 3 days post infection. Perforin +/+ mice had good NK cell activity, whereas the perforin 0/0 mice exhibited no NK cell activity, confirming both the genotype of these mice and the importance of perforin in cell-mediated cytolysis. Replication of MCMV in the spleens of perforin 0/0 mice was in several experiments markedly elevated compared to perforin +/+ mice (Table III-1). Interestingly, in both strains of mice there was no appreciable difference in the liver MCMV titers (Table III-1). In 4 experiments, perforin 0/0 mice had an average of 1.8 ± 0.4 $\log_{10}$ PFU more MCMV in the spleen than in the perforin +/+ mice ($p = 0.0002$; Table III-2). In the liver, there was a statistically insignificant 0.1 ± 0.5 $\log_{10}$ PFU MCMV increase in perforin 0/0 mice when compared to perforin +/+ mice (Table III-2). These results suggest that perforin plays an important role in the regulation of MCMV in the spleen but not in

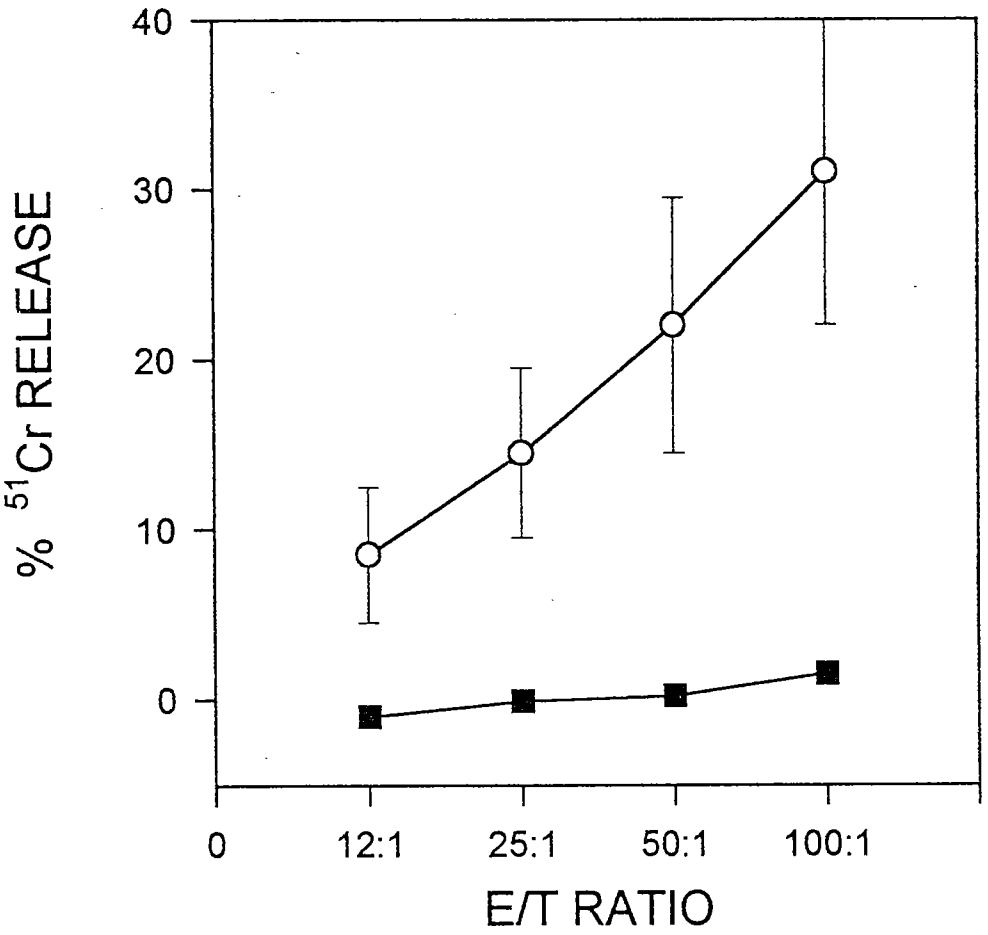

Figure III-1. NK cell activity from perforin +/+ and perforin 0/0 mice. Three days post-infection, spleen cells from MCMV-infected perforin +/+ (O) and perforin 0/0 (■) mice were used as effectors against YAC-1 targets in a standard 5-h ^{51}Cr release assay.

Table III-1. Regulation of MCMV Infection in Perforin +/+ and 0/0 Mice[a]

	Log_{10} PFU/organ ± SD[b]							
	Exp 1[c]		Exp 2[d]		Exp 3		Exp 4	
Group	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver
Perforin +/+	1.5±0.3	3.6±0.2	2.6±0.3	5.3±0.04	3.4±0.0	4.5±0.3	1.5±0.2	3.4±0.1
Perforin 0/0	3.9±0.1[#]	4.0±0.1	4.3±0.3[#]	5.4±0.3	4.2±0.04[#]	3.9±0.3	3.6±0.1[#]	3.7±0.1

[a] Age matched perforin +/+ and 0/0 mice were given i.p. 5×10^3 MCMV PFU/mouse.
[b] Spleen and liver MCMV were titrated on C57BL/6 MEF 3 days after infection.
[c] Splenic NK cells were used as effectors against YAC-1 targets. The results are depicted in Figure III-1.
[d] Anti-CD4 and anti-CD8 Abs were given i.p. 1 day prior to MCMV infection.
[#] Virus titers were significantly higher compared to perforin +/+ mice, $p < 0.005$.

Table III-2. Summary of Experiments in Chapter III[a]

Group/Treatment[b,c] (N)[d]	Control[b,c] (N)[d]	Increase in Log_{10} PFU MCMV/organ ± SE over controls: Spleen	p[e]	Liver	p[e]
Perforin 0/0 (11)	Perforin +/+ (11)	1.8±0.4	0.0002	0.1±0.5	0.6 (NS)[f]
Perforin 0/0 + anti-NK1.1 (9)	Perforin 0/0 (9)	0.1±0.3	0.4 (NS)	0.6±0.2	0.0002
B6 + anti-IFN-γ (15)	B6 (15)	0.1±0.2	1.0 (NS)	0.7±0.2	0.0003
B6 + anti-NK1.1 (14)	B6 (15)	1.6±0.2	0.0003	0.7±0.2	0.0003
B6 + anti-IFN-γ + anti-NK1.1 (14)	B6 (15)	1.6±0.2	0.0003	0.7±0.2	0.0003
B6→129 + anti-NK1.1 (9)	B6→129 (10)	1.2±0.2	0.0002	0.6±0.3	0.0002
B6→IFN-γR$^{0/0}$ + anti-NK1.1 (15)	B6→IFN-γR$^{0/0}$ (16)	1.5±0.4	0.0002	0.03±0.3	1.0 (NS)
B6 + L-NMA (9)	B6 (8)	-0.2±0.4	0.3 (NS)	0.6±0.4	0.003
B6 + L-NMA (9)	B6 + D-NMA (6)	-0.1±0.4	1.0 (NS)	0.8±0.4	0.0004

[a] Table III-2 provides an analysis of data encompassing the individual experiments presented in Tables III-1, III-3, III-4, III-5 and III-6. Shown is the increase in least square mean titers (Log_{10} PFU/organ ± SE) between the treatment group and the control group.

[b] Age-matched mice were infected i.p. with 10^4 PFU/mouse of MCMV.

[c] Mice were either left untreated, or treated with anti-NK1.1 Abs i.v. 1 day prior to infection. Anti-IFN-γ Abs were given i.p. at day 0, 1 and 2 of the infection. L-NMA or D-NMA were given i.v. at day 0, 1 and 2 of the infection.

[d] Total number of mice used in each group.

[e] p values were obtained by applying the statistical test analysing the variance for mixed models using restricted estimation by maximum likelihood using the "Proc Mixed" procedure in the SAS statistical software package.

[f] Not Significant

the liver. Although the control of MCMV infection at 3 days has been shown to be mediated by NK cells and not T cells (Bukowski et al., 1985; Bukowski et al., 1984; Welsh et al., 1994), in one experiment mice were depleted of T cells with mAbs to CD4 and CD8. Perforin 0/0 mice treated with the anti-CD4 and anti-CD8 mAbs also exhibited similar splenic titer increases with no change in the liver MCMV titers when compared to T-cell depleted perforin +/+ mice (Table III-1, Exp. 2).

B. EFFECTS OF ANTI-NK1.1 MAB TREATMENT ON THE REPLICATION OF MCMV BY PERFORIN 0/0 MICE

The results from the experiments performed above suggest that NK cells utilize perforin to regulate MCMV in the spleen but not in the liver. However, these mice were of a mixed genetic background (129 x C57BL/6), and 129 mice and C57BL/6 mice have different susceptibilities to MCMV infection (Scalzo et al., 1995b). To prove that any effects seen were indeed due to NK cells, the mice were first shown to express the NK1.1 antigen by immunofluoresence, and then they were depleted of NK cells to test for the enhancement in virus titers. Results depicted in Table III-3 show that MCMV-infected, perforin 0/0 mice depleted of $NK1.1^+$ NK cells synthesized more virus in the liver compared to MCMV-infected, NK cell-intact perforin 0/0 mice. However, splenic MCMV titers remained the same in both sets of mice. In 3 experiments, the average $\log_{10}$ PFU increase in virus titers in the liver was 0.6 ± 0.2 $\log_{10}$ PFU ($p = 0.0002$; Table III-2), while

Table III-3. Effect of Anti-NK1.1 mAb Treatment on the Regulation of MCMV by Perforin +/+ and 0/0 Mice.[a]

Exp	Group	Log_{10} PFU/organ ± SD[b] Spleen	Liver
1	Perforin 0/0	4.4±0.1	4.5±0.1
	Perforin 0/0 + anti-NK1.1	4.5±0.1	5.0±0.1[#]
2	Perforin 0/0	3.9±0.1	4.0±0.1
	Perforin 0/0 + anti-NK1.1	3.8±0.2	4.5±0.1[#]
	Perforin +/+	1.5±0.3	3.6±0.2
	Perforin +/+ + anti-NK1.1	2.6±0.2[#]	4.3±0.04[*]
3	Perforin 0/0	4.6±0.1	4.1±0.1
	Perforin 0/0 + anti-NK1.1	4.6±0.1	4.5±0.2[$]

[a] Age-matched perforin +/+ and 0/0 mice were given i.p. 5 x 10^3 MCMV PFU/mouse. Anti-NK1.1 was given i.v. 1 day prior to infection.

[b] Spleen and liver MCMV titers were measured using C57BL/6 MEF 3 days after infection.

[*] Virus titers increased significantly after NK cell depletion when compared to infected controls, $p < 0.01$.

[#] Virus titers increased significantly after NK cell depletion when compared to infected controls, $p < 0.005$.

[$] Virus titers increased significantly after NK cell depletion when compared to infected controls, $p = 0.08$.

there was only a slight increase in MCMV titers in the spleens (0.1 ± 0.3 $\log_{10}$ PFU; Table III-2) of anti-NK1.1-treated perforin 0/0 mice. These data suggest that at 3 days post-MCMV infection, the virus in the spleen is controlled by perforin, while in the liver, MCMV is controlled by NK cells in a perforin-independent manner.

C. SPLENIC CELL TYPES INFECTED WITH MCMV

To determine the phenotype of spleen cells are infected by MCMV *in vivo*, splenocytes from 3 day MCMV-infected C57BL/6 mice were sorted into Thy1.2$^+$, B220$^+$ and Thy1.2$^-$-B220$^-$ populations. These cells were then placed into an infectious center assay, and the number of productively infected cells were measured. All 3 cell types were productively infected. In 2 experiments, more B220$^+$ cells were infected by MCMV than were Thy1.2$^+$ cells (Exp 1: 61 PFU/10^6 B220$^+$ cells vs 11 PFU/10^6 Thy1.2$^+$ cells; Exp 2: 13.5 PFU/10^6 B220$^+$ cells vs 4.5 PFU/10^6 Thy1.2$^+$ cells). In the only experiment performed, Thy1.2$^-$-B220$^-$ cells were also infected by MCMV (Exp 2: 160 PFU/10^6 Thy1.2$^-$-B220$^-$ cells). These results suggest that B and T cells can be infected by MCMV.

D. EFFECTS OF ANTI-IFN-γ TREATMENT ON MCMV REPLICATION IN C57BL/6 MICE

One possible mechanism that NK cells can use to control MCMV in the liver is via the production of anti-viral cytokines like IFN-γ. IFN-γ produced by NK cells protects the

liver against MCMV infection, and this protection can be abrogated by treating mice with anti-IFN-γ Abs (Orange et al., 1995). C57BL/6 mice were infected with MCMV and simultaneously treated with anti-IFN-γ Abs to determine whether the depletion of IFN-γ would raise virus titers in both the liver and the spleen. Treatment of MCMV-infected C57BL/6 mice with anti-NK1.1 mAb or anti-aGM_1 antiserum depleted the NK cell activity below the levels of the uninfected controls, while the NK cell activity of mice depleted of IFN-γ was similar to that of the infected controls (Figure III-2). This shows that the anti-IFN-γ treatment had no effect on the virus-induced augmentation of NK cell activity, results similar to that recently reported by Orange et al (Orange et al., 1995). However, the effector function of NK cells *in vivo* was compromised, as MCMV-infected C57BL/6 mice depleted of IFN-γ had more virus growth in the liver (Table III-4). In contrast, the splenic titers remained the same as the infected untreated controls (Table III-4). There was an average of 0.7±0.2 $\log_{10}$ PFU more MCMV in the livers ($p = 0.0003$) but only 0.1±0.2 $\log_{10}$ PFU more virus in the spleens of anti-IFN-γ treated mice when compared to the infected untreated controls (Table III-2). The amounts of MCMV present in the livers of anti-IFN-γ-treated mice were similar to those in MCMV-infected mice that were either given anti-NK cell Abs only (anti-NK1.1 or anti-aGM_1) or a combination of anti-NK cell Abs and anti-IFN-γ Abs (Table III-4). Although anti-IFN-γ treatment had no effect on splenic viral titers, mice that were given anti-NK1.1 Abs exhibited 1.6±0.2 $\log_{10}$ PFU more virus in the spleen compared to infected controls ($p = 0.0003$; Table III-2).

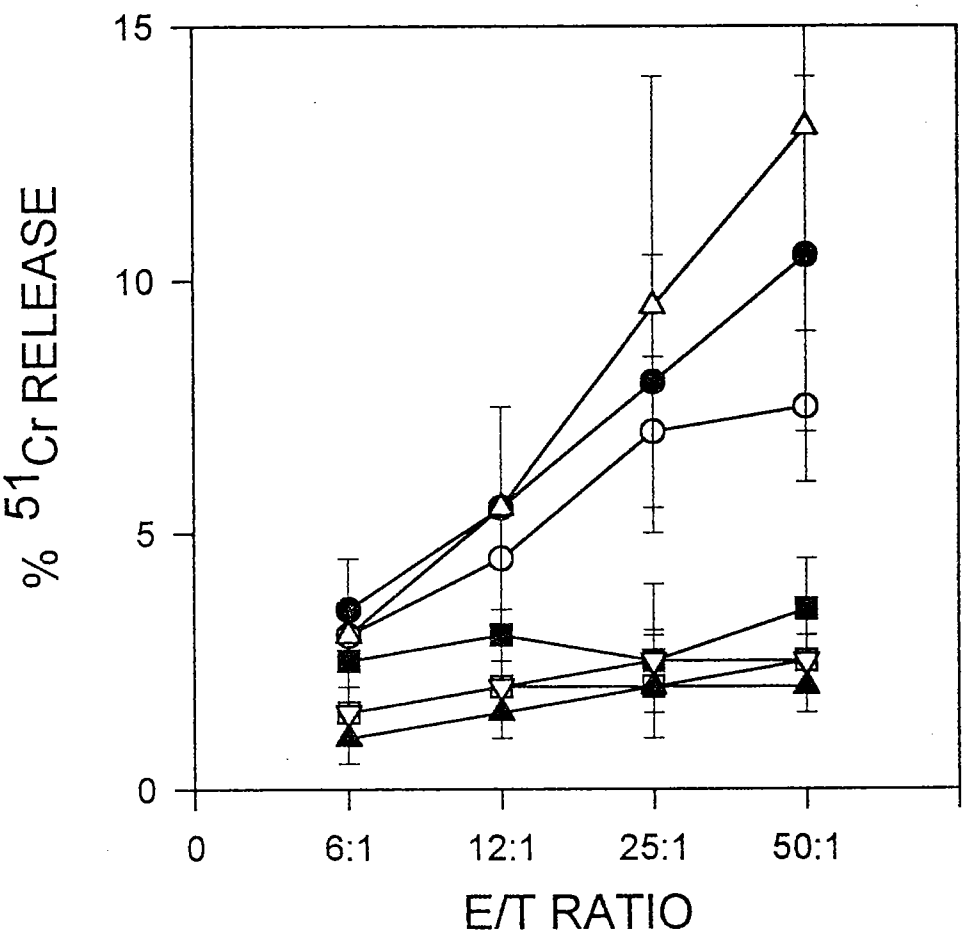

Figure III-2. NK cell activity from anti-IFN-γ-treated C57BL/6 mice. Three days post-infection, spleen cells from uninfected (○), MCMV-infected (●), MCMV-infected, anti-aGM_1-treated (□), MCMV-infected, anti-NK1.1-treated (■), MCMV-infected, anti-IFN-γ-treated (△), MCMV-infected, anti-aGM_1-treated, anti-IFN-γ-treated (▲) and MCMV-infected, anti-NK1.1-treated, anti-IFN-γ-treated (▽) C57BL/6 mice were used as effectors against YAC-1 targets in a standard 5-h ^{51}Cr release assay.

Table III-4. Effects of Anti-NK Cell and/or Anti-IFN-γ mAb Treatment in the Regulation of MCMV in C57BL/6 Mice[a]

	Log_{10} PFU/organ ± SD[b]					
	Exp 1[c]		Exp 2		Exp 3	
Group	Spleen	Liver	Spleen	Liver	Spleen	Liver
Untreated	<2.3±0.0	4.4±0.4	3.2±0.2	4.0±0.4	2.7±0.4	4.3±0.1
Treated with						
+ anti-IFN-γ	<2.3±0.0	5.0±0.2*	3.2±0.6	4.7±0.04*	3.0±0.3	4.9±0.3*
+ anti-NK1.1	4.5±0.1#	5.1±0.2*	4.4±0.2#	4.6±0.1	4.3±0.5#	4.9±0.2*
+ anti-NK1.1 + anti-IFN-γ	4.4±0.1#	5.0±0.02*	4.5±0.2#	4.8±0.3*	4.1±0.2#	4.9±0.3*
+ anti-aGM_1	4.6±0.1#	5.3±0.3*	N.D.[d]	N.D.	N.D.	N.D.
+ anti-aGM_1 + anti-IFN-γ	4.1±0.2#	5.2±0.2*	N.D.	N.D.	N.D.	N.D

[a] Age matched C57BL/6 mice were infected i.p. with 10^4 PFU/mouse of MCMV. Anti-NK1.1 or anti-aGM_1 was given i.v or i.p., respectively, 1 day prior to infection. Anti-IFN-γ was given i.p. at day 0, 1 and 2 of the infection.
[b] Spleen and liver MCMV PFU were titrated on C57BL/6 MEF 3 days after infection.
[c] Splenic NK cells were used as targets against YAC-1 targets. The results are shown in Figure III-2.
[d] N.D. Not Done
* NK cell depletion or anti-IFN-γ treatment significantly increased the virus titers compared to infected controls, $p < 0.05$.
# NK cell depletion or anti-IFN-γ treatment significantly increased the virus titers compared to infected controls, $p < 0.005$.

To make sure that the anti-IFN-γ treatment was altering IFN-γ-induced functions in the spleen, splenocytes enriched for macrophages from uninfected, MCMV-infected and MCMV-infected, anti-IFN-γ-treated C57BL/6 mice were stained for MHC class II expression, which MCMV-induced IFN-γ has been shown to upregulate (Heise and Virgin IV, 1995). MCMV infection increased MHC class II expression on spleen leukocytes, but in MCMV-infected mice treated with the same dose of anti-IFN-γ Abs, MHC class II levels on the spleen leukocytes were at the same levels as the uninfected control (Fig. III-3). This indicated that the anti-IFN-γ treatment blocked IFN-γ-mediated functions in the spleen, yet it did not affect MCMV replication in that organ.

E. REPLICATION OF MCMV IN IFN-γR$^{0/0}$ MICE RECONSTITUTED WITH C57BL/6 BONE MARROW CELLS

To further clarify the role of IFN-γ in the *in vivo* regulation of MCMV, we made use of the IFN-γR$^{0/0}$ mice. These mice lack the receptor for IFN-γ, and, even though they retain the ability to produce IFN-γ, the cells in these animals cannot respond to that cytokine (Huang et al., 1993). The 129 background of these knockout mice did not allow us to perform satisfactorily direct analyses on the NK cell control of MCMV infection, because the 129 strain has a low NK cell activity and is NK1.1$^-$ and *Cmv-1*^{s} (Scalzo et al., 1995b). Depletion of NK cell activity in these mice with anti-aGM$_1$ antiserum led to only marginal increases in the spleen and liver MCMV titers. In 5 experiments each with 4 - 5 mice/group, anti-aGM$_1$-treated, MCMV-infected 129 mice had an average of 0.5±0.4

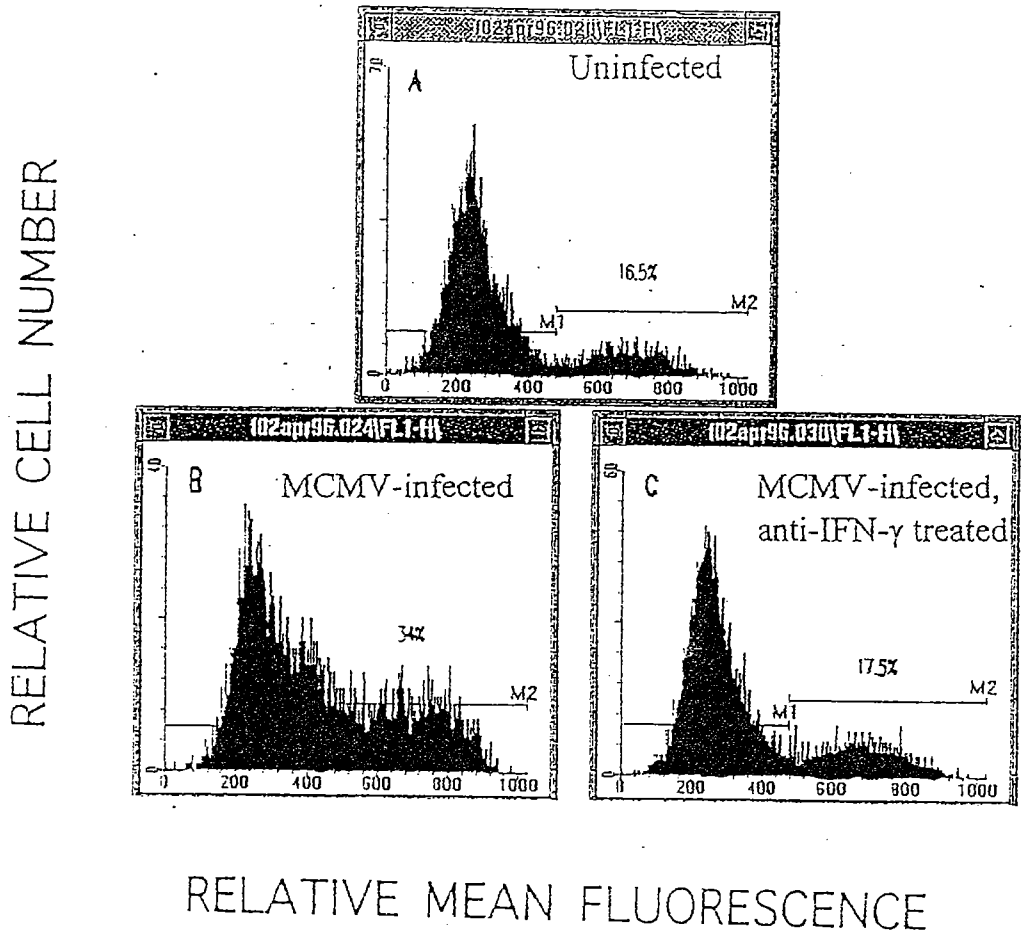

Figure III-3. MHC class I expression of splenic macrophages. Spleen cells from uninfected (A), MCMV-infected (B), and MCMV-infected, anti-IFN-γ-treated (C) mice were partially enriched for macrophages by treatment with J11D plus complement and then stained with a mouse anti-mouse I-A^b-FITC Ab. The number above the positive peak reflects the percentage of cells that stained positively for MHC class II expression.

$\log_{10}$ PFU increase in splenic titers and only 0.1±0.2 $\log_{10}$ PFU increase in liver titers when compared to infected controls. In 7 experiments, anti-aGM_1-treated, MCMV-infected 129/IFN-$\gamma R^{0/0}$ mice had only a 0.2±0.2 $\log_{10}$ PFU increase in splenic titers and a 0.1±0.1 $\log_{10}$ PFU MCMV decrease in liver titers when compared to infected controls. Scalzo et al had shown that, in BALB/c mice congenic for C57BL/6 *Cmv-1* and the NK gene complex, susceptibility of these mice to MCMV changed from a *Cmv-1*^{s} to a *Cmv-1*^{r} phenotype (Scalzo et al., 1995a). Therefore, to circumvent the 129/*Cmv-1*^{s} background problem, we reconstituted the IFN-$\gamma R^{0/0}$ mice with C57BL/6 bone marrow cells, thereby creating a strain of mouse (B6→IFN-$\gamma R^{0/0}$) that has C57BL/6 NK cells, is *Cmv-1*^{r}, and retains its IFN-γR deficient phenotype, at least among cells not of the bone marrow origin.

Figure III-4 shows the NK cell activity from B6→129 and B6→IFN-$\gamma R^{0/0}$ chimeras 3 days after MCMV infection, with or without anti-NK1.1 mAb treatment. Both strains of mice exhibited robust NK cell activity, which was depleted with the anti-NK1.1 mAb. B6→129 mice depleted of $NK1.1^+$ cells had higher virus titers in both their spleens and livers when compared to infected controls, further showing the efficacy of the bone marrow reconstitution (Table III-5). In 2 experiments, NK cell-depleted B6→129 mice had 1.2±0.2 $\log_{10}$ PFU more virus in the spleen (p = 0.0002) and 0.6±0.3 $\log_{10}$ PFU more virus in the liver (p = 0.0002) compared to NK cell-intact, MCMV-infected controls (Table III-2). However, NK cell-depleted B6→IFN-$\gamma R^{0/0}$ mice showed only an increase in splenic titers with no appreciable increase of MCMV in the liver (Table III-5). In 4 experiments performed, there was an average 1.5±0.4 $\log_{10}$ PFU increase in splenic titers

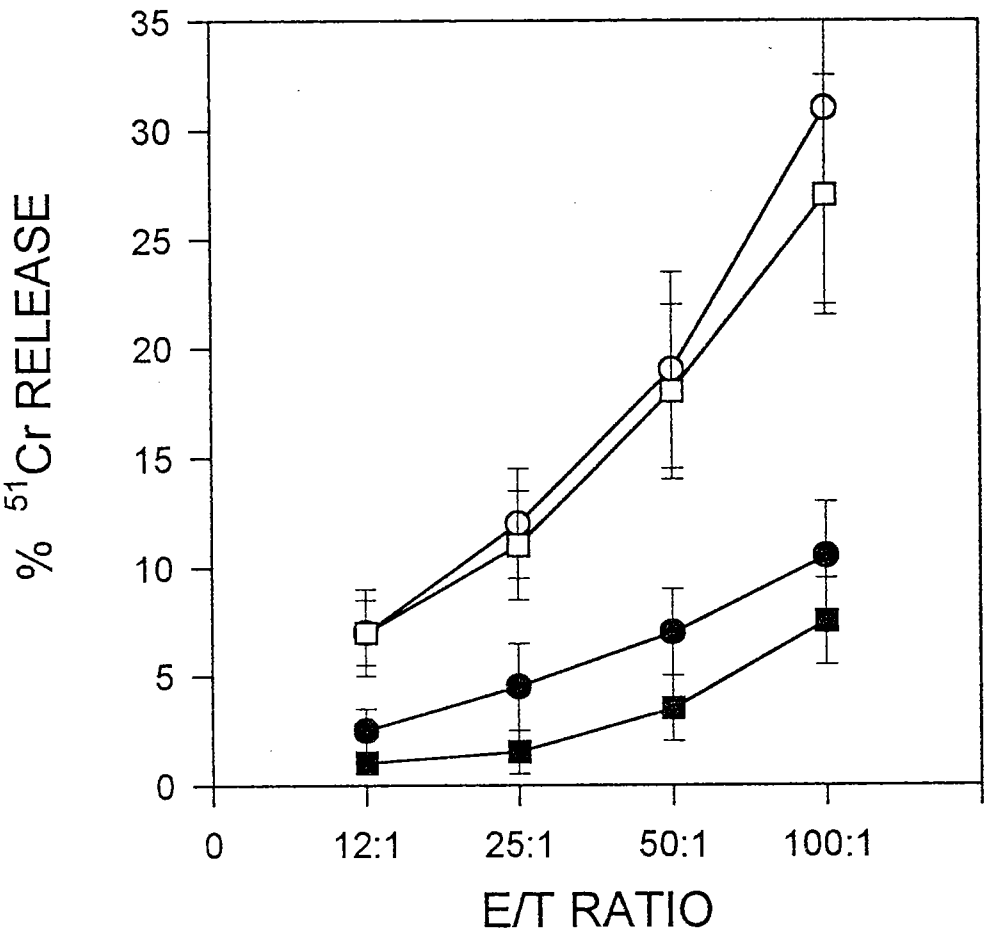

Figure III-4. NK cell activity from B6→IFN-γR$^{0/0}$ and B6→129 mice. Three days post-infected, spleen cells from MCMV-infected B6→IFN-γR$^{0/0}$ control (○), MCMV-infected, anti-NK1.1-treated B6→IFN-γR$^{0/0}$ (●), MCMV-infected B6→129 control (□), and MCMV-infected, anti-NK1.1-treated B6→129 (■) mice were used as effectors against YAC-1 targets in a standard 5-h ^{51}Cr release assay.

Table III-5. Effects of Anti-NK1.1 mAb Treatment on the Regulation of MCMV in B6→IFN-γR$^{0/0}$ and B6→129 Mice[a]

Exp	Group	Log_{10} PFU/organ ± SD[b] Spleen	Liver
1	B6→IFN-γR$^{0/0}$	<1.4±0.2	4.8±0.2
	B6→IFN-γR$^{0/0}$ + anti-NK1.1	3.8±0.3[#]	4.6±0.5
2	B6→IFN-γR$^{0/0}$	<1.1±0.2	4.0±0.2
	B6→IFN-γR$^{0/0}$ + anti-NK1.1	2.6±0.5[#]	4.3±0.4
3	B6→IFN-γR$^{0/0}$	<2.5±0.3	4.4±0.4
	B6→IFN-γR$^{0/0}$ + anti-NK1.1	3.6±0.5[#]	4.8±0.1
4[c]	B6→IFN-γR$^{0/0}$	<1.4±0.2	3.8±0.6
	B6→IFN-γR$^{0/0}$ + anti-NK1.1	2.6±0.3[#]	3.9±0.2
	B6→129	1.3±0.0	3.4±0.3
	B6→129 + anti-NK1.1	2.8±0.4[#]	4.1±0.1[*]
5	B6→129	<1.9±0.5	3.9±0.1
	B6→129 + anti-NK1.1	2.9±0.3[#]	4.4±0.2[#]

[a] Age matched B6→IFN-γR$^{0/0}$ mice or B6→129 mice were infected i.p. with 10^4 MCMV PFU/mouse. Anti-NK1.1 was given i.v. 1 day prior to infection.

[b] Spleen and liver MCMV PFU were titrated on C57BL/6 MEF 3 days post infection.

[c] Splenic NK cells were used as effectors against YAC-1 targets. The results are shown in Figure III-4.

[*] NK cell depletion caused a significant increase in virus titers compared to the appropriate controls, $p < 0.01$.

[#] NK cell depletion caused a significant increase in virus titers compared to the appropriate controls, $p < 0.005$.

(p = 0.0002) but only a 0.03±0.3 $\log_{10}$ PFU increase in the livers of NK cell-depleted B6→IFN-γR$^{0/0}$ over the infected untreated controls (Table III-2). These results are compatible with the concept that IFN-γ is the NK cell-dependent mechanism that regulates MCMV synthesis in the liver but not in the spleen. To be certain that the effect seen in the spleens of NK1.1-depleted B6→IFN-γR$^{0/0}$ mice was not due to the donor cells' ability to respond to IFN-γ, perforin 0/0 bone marrow cells were used to reconstitute IFN-γR$^{0/0}$ mice, and the effects of depleting NK1.1^{+} cells in MCMV-infected, perforin 0/0→IFN-γR$^{0/0}$ chimeric mice were measured. Three days post-MCMV infection, NK cell-depleted perforin 0/0→IFN-γR$^{0/0}$ mice (spleen: 3.2±0.2 $\log_{10}$ PFU; liver: 4.8±0.2 $\log_{10}$ PFU) had about the same amount of virus in the spleen and liver when compared to the NK cell-intact, untreated control (spleen: 3.4±0.1 $\log_{10}$ PFU; liver: 4.9±0.2 $\log_{10}$ PFU). This result strongly suggests that the early regulation of MCMV in the spleen is controlled by perforin.

F. EFFECTS OF AN *INOS* INHIBITOR, L-NMA, ON THE REPLICATION OF MCMV IN C57BL/6 MICE

One of the ways IFN-γ can inhibit virus replication is by inducing the expression of the gene *iNOS*, which encodes nitric oxide synthase (NOS), which in turn catalyzes the guanidino nitrogen of L-arginine into a free radical gas, nitric oxide (NO) (Nathan, 1992). NO production by NOS has been shown both *in vivo* and *in vitro* to inhibit ectromelia

virus, VV and HSV-1 replication (Karupiah et al., 1993b; Harris et al., 1995). To see if MCMV is regulated by IFN-γ via the production of NO and to determine if this regulation is organ-dependent, we treated MCMV-infected mice with a competitive inhibitor of NOS, L-NMA, and measured the NK cell activity as well as the viral titers in both spleens and livers of these mice. Three days post-MCMV infection, L-NMA or its stereoisomer D-NMA had no effect on the NK cell-mediated lysis (Figure III-5). L-NMA-treated mice had more virus in the liver compared to the infected controls, but the splenic titers remained the same in these mice (Table III-6). The enantiomeric analogue of L-NMA, D-NMA, had no effect on the virus titers in either the spleen or the liver (Table III-6). In 3 experiments, L-NMA treatment induced an average of 0.6±0.4 $\log_{10}$ PFU increase in liver virus titers ($p = 0.003$) but caused a 0.2±0.4 $\log_{10}$ PFU decrease in the spleen compared to untreated controls (Table III-2). Compared to the D-NMA control, L-NMA-treated mice had 0.8±0.4 $\log_{10}$ PFU more virus in the liver ($p = 0.0004$), but there was no appreciable difference in splenic MCMV titers (Table III-2). This suggests that MCMV synthesis in the liver but not in the spleen is regulated by IFN-γ-induced nitric oxide and adds further evidence to the concept that the control of MCMV infection is mediated by different mechanisms in the spleen and liver.

In this chapter, I provided evidence that in the early phase of MCMV infection, NK cells in *Cmv-1*^{r}, C57BL/6 mice have the ability to use different mechanisms to control MCMV in different organs. In the spleen, NK cells may control MCMV via a perforin-

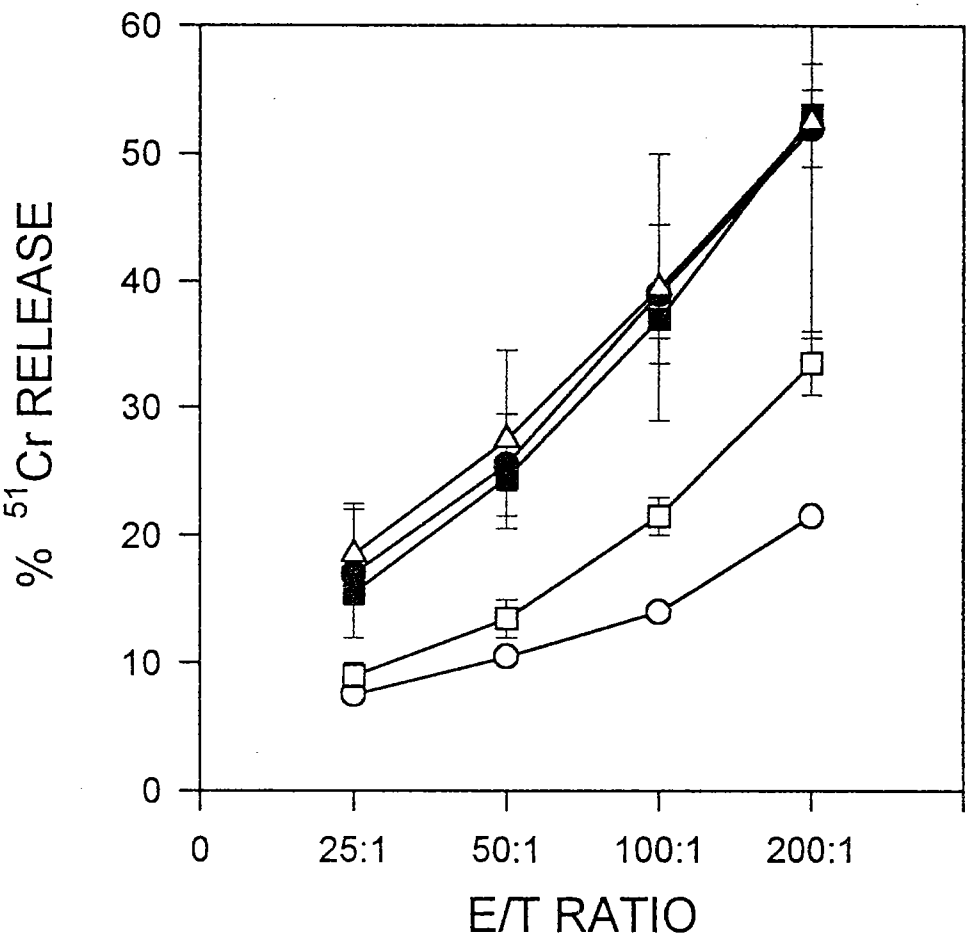

Figure III-5. NK cell activity from L-NMA- and D-NMA-treated C57BL/6 mice. Three days post-infection, spleen cells from uninfected (○), MCMV-infected (●), MCMV-infected, anti-NK1.1-treated (□), MCMV-infected, L-NMA-treated (■), and MCMV-infected, D-NMA-treated (△) C57BL/6 mice were used as effectors against YAC-1 targets in a standard 5-h ^{51}Cr release assay.

Table III-6. Effects of L-NMA and D-NMA Treatment on the Regulation of MCMV in C57BL/6 Mice[a]

	Log_{10} PFU/organ ± SD[b]					
	Exp 1[c]		Exp 2[c, d]		Exp 3[e]	
Group	Spleen	Liver	Spleen	Liver	Spleen	Liver
Untreated	2.5±0.2	4.2±0.04	1.8±0.2	3.0±0.4	1.9±0.2	3.6±0.5
Treated with:						
+ anti-NK1.1	3.5±0.2*	4.9±0.2*	2.6±0.2#	4.1±0.2*	2.9±0.1#	4.5±0.2*
+ L-NMA	2.5±0.2	4.7±0.1*	1.4±0.2	3.7±0.03*	1.8±0.0	4.2±0.2$
+ D-NMA	N.D.	N.D.	1.4±0.2	2.6±0.6	2.0±0.2	3.7±0.3

[a] Age matched C57BL/6 mice were infected i.p. with 10^4 MCMV PFU/mouse of MCMV. Anti-NK1.1 was given i.v. 1 day prior to infection. L-NMA and D-NMA were given i.v. at day 0, day 1 and day 2 of the infection.
[b] Spleen and liver MCMV titers were titrated on C57BL/6 MEF 3 days post infection.
[c] For experiments 1 and 2, L-NMA and D-NMA were used at a dose of 5 mg/mouse/day.
[d] Splenic NK cells were used as effectors against YAC-1 targets. The results are shown in Figure III-5.
[e] L-NMA was used at a dose of 10 mg/mouse/day while D-NMA was used at a dose of 5 mg/mouse/day.
* NK cell depletion or treatment with L-NMA resulted in increases of virus titers compared to infected controls, $p < 0.05$.
# NK cell depletion or treatment with L-NMA resulted in increases of virus titers compared to infected controls, $p < 0.005$.
$ Treatment with L-NMA resulted in increases of virus titers compared to the infected controls, $p=0.1$

dependent cytotoxic mechanism, while in the liver IFN-γ produced by NK cells is a major mediator in regulating the virus in that organ.

CHAPTER IV

MECHANISMS OF MCMV REGULATION IN SUCKLING MICE BY ADOPTIVELY TRANSFERRED NK CELLS

Three week old or younger suckling mice are very susceptible to MCMV infections because NK cells take about 3 weeks from birth to reach maturity (Boos and Wheelock, 1971; Kiessling et al., 1975a). Bukowski et al have shown that the adoptive transfer of syngeneic adult spleen cells into suckling mice prior to MCMV infection can protect the suckling mice from a lethal MCMV infection (Bukowski et al., 1985). Analyses of splenic MCMV titers indicated that suckling mice adoptively reconstituted with adult spleen cells have lower MCMV titers compared to control suckling mice. This protection is mediated by NK cells as the depletion of NK cells from the adoptively transferred splenic population abrogated this protection. Splenocytes depleted of T cells do not confer protection in suckling mice. However, a T cell clone with NK cell-like activity also protected suckling mice from MCMV (Bukowski et al., 1985). Taken together, these results show that suckling mice are good models to study the mechanisms used by NK cells in the regulation of MCMV synthesis.

In Chapter III, I have presented evidence that NK cells use a perforin-dependent mechanism to control splenic MCMV synthesis in adult C57BL/6 mice. The mechanisms of protection afforded by the adoptively transferred NK cells in the suckling mouse system

are not known. To examine this, adoptive transfers of C57BL/6, 129 or perforin 0/0 adult spleen cells and LAK cells into MCMV-infected C57BL/6, 129 or IFN-$\gamma R^{0/0}$ suckling mice were performed. In this chapter, I will present evidence that unlike the adult mouse system, adoptively transferred NK cells protect the spleens of suckling mice from MCMV via an IFN-γ-dependent mechanism.

A. EFFECTS OF ADOPTIVELY TRANSFERRED C57BL/6 OR 129 ADULT SPLEEN CELLS AND LAK CELLS ON THE REGULATION OF MCMV IN C57BL/6 SUCKLING MICE

Four to six day old C57BL/6 suckling mice were adoptively reconstituted with C57BL/6 or 129 spleen cells and LAK cells prior to infection, and splenic MCMV titers were measured 3 days post-infection. As expected, C57BL/6 spleen cells or C57BL/6 LAK cells when transferred into the suckling mice significantly lowered MCMV titers in the spleen compared to the media controls (Table IV-1). 129 spleen cells when adoptively transferred into C57BL/6 suckling mice had a protective effect in the spleen compared to the media controls (Table IV-1, Exp 1). Adoptive transfer of 129 LAK cells into C57BL/6 suckling mice also lowered splenic MCMV titers to the levels similar to that mediated by C57BL/6 LAK cell-reconstituted suckling mice (Table IV-1, Exp 2). These results are surprising in view of the fact that the 129 strain is *Cmv-1*^{s}, and the regulation of splenic MCMV in adult C57BL/6 mice is *Cmv-1*-dependent (Chapter III, Tay and Welsh, 1997).

Table IV-1. Mechanisms of MCMV Regulation in Adoptively Reconstituted C57BL/6 Suckling Mice[a]

Exp	Group[b]	# of cells Transferred	Log_{10} PFU MCMV per Spleen ± SD[c]	*p* value[d]
1	Media Control		4.1±0.1	
	+ 129 spleen cells	5×10^7	3.4±0.3	$p < 0.01$
	+ C57BL/6 spleen cells	5×10^7	3.3±0.1	$p < 0.001$
2	Media Control		3.0±0.1	
	+ 129 LAK cells	5×10^5	2.3±0.2	$p < 0.001$
	+ C57BL/6 LAK cells	5×10^5	2.2±0.1	$p < 0.001$
3	Media Control		3.1±0.1	
	+ Perforin 0/0 spleen cells	5×10^7	2.2±0.3	$p < 0.001$
	+ C57BL/6 spleen cells	5×10^7	2.0±0.3	$p < 0.001$
4	Media Control		3.4±0.2	
	+ C57BL/6 spleen cells	5×10^5	3.3±0.2	$p = 0.5$
	+ Perforin 0/0 LAK cells	5×10^5	2.6±0.4	$p < 0.005$
	+ C57BL/6 LAK cells	5×10^5	2.5±0.3	$p < 0.001$
5	Media Control		2.6±0.2	
	+ Perforin 0/0 LAK cells	5×10^5	2.1±0.4	$p = 0.1$
	+ C57BL/6 LAK cells	5×10^5	2.2±0.2	$p < 0.05$

[a] Four to six day old C57BL/6 suckling mice were given i.p. 2000 PFU MCMV per mouse.

[b] Age-matched C57BL/6 mice were given 0.1 ml complete RPMI/mouse (Media control) or were adoptively reconstituted i.p. with spleen cells or LAK cells in 0.1 ml complete RPMI at the cell concentration shown in the table. All these transfers were performed one day prior to infection.

[c] Splenic MCMV PFU were titrated on C57BL/6 MEF 3 days after infection.

[d] *p* values were generated by comparing the virus titers from adoptively reconstituted groups to the media control.

These data therefore suggest that at 3 days post-MCMV infection, the protection afforded by adoptively transferred NK cells in the spleens of C57BL/6 suckling mice is not *Cmv-1*-dependent.

B. EFFECTS OF ADOPTIVELY TRANSFERRED C57BL/6 OR PERFORIN 0/0 MICE ADULT SPLEEN CELLS AND LAK CELLS ON THE REGULATION OF MCMV IN C57BL/6 SUCKLING MICE

In adult mice, *Cmv-1*-dependent, NK cell regulation of MCMV in the spleen is mediated by a perforin-dependent mechanism (Chapter III, Tay and Welsh, 1997). The results presented above suggested that the regulation of MCMV in the spleen of the suckling mouse model was not *Cmv-1*-dependent, but the role of perforin was not evaluated. To test the role of perforin in the regulation of MCMV in suckling mice, perforin 0/0 spleen cells and perforin 0/0 LAK cells were adoptively transferred, and their abilities to control MCMV synthesis in the spleens of C57BL/6 suckling mice were tested.

Perforin 0/0 spleen cells or LAK cells when adoptively transferred into 4 - 6 day old C57BL/6 suckling mice significantly lowered MCMV titers in the spleens compared to control spleens (Table IV-1, Exp 3 - 5). The levels of protection afforded by perforin 0/0 spleen cells and LAK cells were similar to that of C57BL/6 spleen cells and C57BL/6 LAK cells respectively. Results presented in Table IV therefore suggest that adoptively transferred spleen cells or LAK cells protect C57BL/6 suckling mice from MCMV via a *Cmv-1*-independent, perforin-independent mechanism.

C. EFFECTS OF ADOPTIVELY TRANSFERRED C57BL/6 SPLEEN CELLS ON THE REGULATION OF MCMV IN 129 AND IFN-$\gamma R^{0/0}$ MICE

In the above experiments, the results show that adoptively transferred NK cells do not protect the spleens of MCMV-infected suckling mice via perforin. As NK cells can regulate MCMV via the production of anti-viral cytokines, one possibility is that the transferred NK cells control splenic MCMV titers in the suckling mice via the production of IFN-γ. To test this, adult C57BL/6 spleen cells were adoptively transferred into 4 - 6 day old 129 or IFN-$\gamma R^{0/0}$ suckling mice, and their abilities to reduce MCMV synthesis in the spleens were measured.

Adult C57BL/6 spleen cells when adoptively transferred into MCMV-infected 129 suckling mice significantly reduced splenic MCMV titers compared to infected control spleens (Table IV-2, Exp 1 and 2). However, the same C57BL/6 spleen cells had no effect on MCMV splenic titers in MCMV-infected IFN-$\gamma R^{0/0}$ suckling mice. These data suggest that the control of MCMV synthesis in suckling mice by adoptively transferred cells is IFN-γ-dependent.

Taken together, the results presented in this chapter strongly suggest, that unlike the adult mouse/MCMV system, the regulation of MCMV in the spleens of suckling mice by adoptively transferred NK cells is *Cmv-1*-independent and perforin-independent but is instead IFN-γ-dependent.

Table IV-2. Mechanisms of MCMV Regulation in Adoptively Reconstituted 129 and IFN-γR$^{0/0}$ Mice[a]

Exp	Group[b]	Log_{10} PFU MCMV/Spleen[c]	*p* value[d]
1	MCMV-infected 129		
	+ Media	3.0±0.1	
	+ C57BL/6 spleen cells	2.5±0.1	$p < 0.001$
	MCMV-infected IFN-γR$^{0/0}$		
	+ Media	3.2±0.2	
	+ C57BL/6 spleen cells	3.3±0.1	$p = 0.05$
2	MCMV-infected 129		
	+ Media	3.0±0.1	
	+ C57BL/6 spleen cells	1.7±0.4	$p < 0.001$
3	MCMV-infected IFN-γR$^{0/0}$		
	+ Media	3.9±0.1	
	+ C57BL/6 spleen cells	3.9±0.1	$p = 0.2$

[a] Four to six day old 129 and IFN-γR$^{0/0}$ suckling mice were given i.p. 2000 MCMV PFU per mouse.

[b] One day prior to infection, suckling mice were either given i.p. 0.1 ml complete RPMI per mouse or 5 x 10^7 C57BL/6 spleen cells per mouse.

[c] Splenic MCMV PFU were titrated on C57BL/6 MEF 3 days post infection.

[d] *p* value is calculated by comparing the MCMV titers between the adoptively reconstituted suckling mice to the media control within the same experiment.

CHAPTER V

THE ROLE OF LY49 NK CELL RECEPTORS IN THE REGULATION OF MCMV

In Chapter III, I have provided evidence that in adult C57BL/6 mice, NK cells used different mechanisms to regulate MCMV synthesis in different organs. The presence of the innate resistance gene *Cmv-1* dictates that in C57BL/6 mice, the regulation of MCMV in the spleen is perforin-dependent while the control of MCMV in the liver is IFN-γ-dependent. Investigators working on the identification of the *Cmv-1* gene product have indicated that *Cmv-1* is closely linked to the Ly49 receptor family, suggesting that *Cmv-1* may be an existing or undefined member of the Ly49 multigene family. Since *Cmv-1* is closely linked to the Ly49 family and may also be a possible member, it is possible that its gene product, like the rest of the Ly49 family members, be differentially expressed in the various subsets of NK cells. These different subsets of NK cells may then use different mechanisms to control MCMV in different organs. Subsets of NK cells that traffic to the liver may use an IFN-γ-dependent mechanism to control MCMV therein, while the subsets of NK cells that remain or traffic to the spleen may predominantly use a perforin-dependent mechanism.

In an effort to understand the roles played by Ly49 NK cell receptors in the regulation of MCMV, I will present experiments in this chapter looking at a) the

distribution of Ly49 NK cell receptors in the spleens and peritoneal cavity of uninfected and 3 day MCMV-infected C57BL/6 mice, b) the regulation of MCMV infections in adult C57BL/6 mice treated with mAbs to the different members of the Ly49 family, and c) the control of MCMV in C57BL/6 suckling mice adoptively reconstituted with various $Ly49^+$ LAK cells.

A. DISTRIBUTION OF $NK1.1^+$-$LY49^+$ NK CELL SUBSETS IN THE SPLEENS OF UNINFECTED AND 3 DAY MCMV-INFECTED C57BL/6 MICE

To examine if there were any differences in the distribution of the NK cell subsets before and after MCMV infection, spleen cells from uninfected and 3 day MCMV-infected C57BL/6 mice were stained for NK1.1, Ly49A, C, D and G2 NK cell receptors. Three days post-MCMV infection, the number of spleen leukocytes in MCMV-infected mice was similar to that in uninfected mice (data not shown). However, FACs analyses based on 10^4 gated lymphocytes showed that there were fewer $NK1.1^+$ cells (2 -3 fold decrease) in the spleens of MCMV-infected mice compared to that in uninfected mice (Figure V-1, Table V-1). Analyses of the Ly49 NK cell subset distribution within the $NK1.1^+$ population before and after MCMV infection revealed that there were increases in the percentages of $NK1.1^+$-$Ly49A^+$ and $NK1.1^+$-$Ly49G2^+$ NK cell subsets and decreases in the percentages of $NK1.1^+$-$Ly49C^+$ and $NK1.1^+$-$Ly49D^+$ NK cell subsets (Table V-2). These results suggest that, compared to uninfected mice, 3 day MCMV infection of C57BL/6 mice

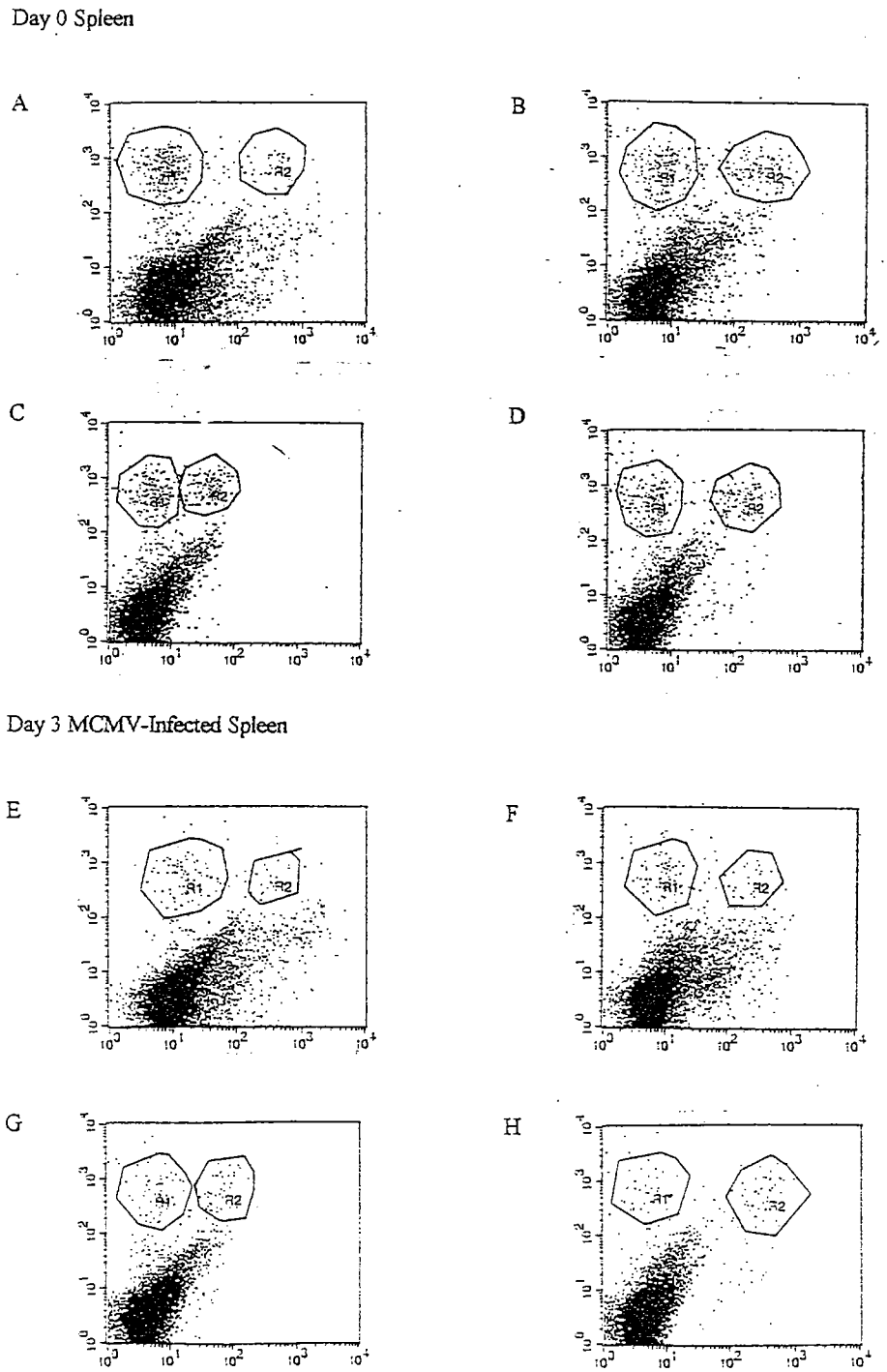

Figure V-1. Distribution of NK1.1$^+$-Ly49$^+$ NK cells in uninfected and 3 day MCMV-infected C57BL/6 spleen cells. Spleen cells were stained with anti-NK1.1 mAb (y-axis) and anti-Ly49A (A, E), anti-Ly49C (B, F), anti-Ly49D (C, G) and anti-Ly49G2 (D, H) mAbs (x-axis). Data from these FACs profiles are reported in Table V-2, Exp 1.

Table V-1. Frequency of NK1.1$^+$ Cells in the Spleens and the Peritoneal Cavities of Uninfected, MCMV-Infected and LCMV-Infected C57BL/6 Mice[a]

	Spleen[b]		Peritoneal Cavity[b]	
	NK1.1$^+$ cells per 10^4 lymphocytes[c]	p value[d]	NK1.1$^+$ cells per 10^4 lymphocytes[c]	p value[d]
Exp 1				
Uninfected	285±23		307±33	
MCMV-infected	101±8	$p < 0.001$	1281±68	$p < 0.001$
LCMV-infected	294±20	$p = 0.6$	828±56	$p < 0.001$
Exp 2				
Uninfected	349±38		559±134	
MCMV-infected	108±6	$p < 0.001$	947±86	$p < 0.001$
LCMV-infected	283±9	$p < 0.05$	383±30	$p < 0.05$
Exp 3				
Uninfected	341±51		246±46	
MCMV-infected	149±29	$p < 0.001$	598±36	$p < 0.001$
Exp 4				
Uninfected	188±22		245±47	
LCMV-infected	155±29	$p < 0.5$	267±74	$p = 0.6$

[a] Age-matched C57BL/6 mice were either uninfected or given i.p. 1 x 10^4 PFU/MCMV per mouse or 5 x 10^4 PFU/LCMV per mouse.

[b] Spleen cells and peritoneal cavity cells were removed from uninfected, 3 day MCMV-infected or 3 day LCMV-infected C57BL/6 mice and stained with anti-NK1.1-PE mAb. A lymph gate was used to analyze the cells.

[c] The number of NK1.1$^+$ cells was based on 10^4 lymphocytes.

[d] *p* value is calculated by comparing the number of NK1.1$^+$ cells from infected mice to the number of NK1.1$^+$ cells in the uninfected controls within the same experiment.

Table V-2. Expression of NK1.1 and Ly49 Receptors in Uninfected, MCMV-Infected and LCMV-Infected splenocytes of C57BL/6 Mice[a]

Group[b]	NK1.1$^+$-Ly49$^-$ (R1)[c] per 10^4 lymphocytes	NK1.1$^+$-Ly49$^+$ (R2)[c] per 10^4 lymphocytes	[R2/(R1+R2)] x 100[d]	Relative change compared to uninfected control
Exp 1:				
Day 0				
NK1.1-Ly49A	222	38	15%	
NK1.1-Ly49C	186	130	41%	
NK1.1-Ly49D	158	128	45%	
NK1.1-Ly49G2	172	111	39%	
Day 3 MCMV				
NK1.1-Ly49A	84	17	17%	Increase
NK1.1-Ly49C	79	24	23%	Decrease
NK1.1-Ly49D	66	44	40%	Decrease
NK1.1-Ly49G2	41	49	54%	Increase
Day 3 LCMV				
NK1.1-Ly49A	208	66	24%	Increase
NK1.1-Ly49C	184	107	37%	Decrease
NK1.1-Ly49D	168	154	48%	Decrease
NK1.1-Ly49G2	109	180	62%	Increase
Exp 2:				
Day 0				

NK1.1-Ly49A	261	61	19%	
NK1.1-Ly49C	242	161	40%	
NK1.1-Ly49D	203	150	42%	
NK1.1-Ly49G2	183	137	43%	
Day 3 MCMV				
NK1.1-Ly49A	82	28	25%	Increase
NK1.1-Ly49C	83	16	16%	Decrease
NK1.1-Ly49D	79	33	29%	Decrease
NK1.1-Ly49G2	50	60	55%	Increase
Day 3 LCMV				
NK1.1-Ly49A	231	62	21%	Increase
NK1.1-Ly49C	165	109	40%	No change
NK1.1-Ly49D	141	136	49%	Increase
NK1.1-Ly49G2	121	167	58%	Increase

Exp 3:				
Day 0				
NK1.1-Ly49A	259	54	17%	
NK1.1-Ly49C	152	142	48%	
NK1.1-Ly49D	160	251	61%	
NK1.1-Ly49G2	148	200	57%	
Day 3 MCMV				
NK1.1-Ly49A	83	85	51%	Increase
NK1.1-Ly49C	83	36	30%	Decrease
NK1.1-Ly49D	72	58	45%	Decrease
NK1.1-Ly49G2	54	125	70%	Increase

Day 3 MCMV*				
NK1.1-Ly49A	58	88	60%	Increase
NK1.1-Ly49C	56	42	43%	Decrease
NK1.1-Ly49D	31	30	49%	Decrease
NK1.1-Ly49G2	35	140	80%	Increase
Exp 4:				
Day 0				
NK1.1-Ly49A	150	32	18%	
NK1.1-Ly49C	118	90	43%	
NK1.1-Ly49D	115	87	43%	
NK1.1-Ly49G2	101	58	36%	
Day 3 MCMV*				
NK1.1-Ly49A	22	6	21%	Increase
NK1.1-Ly49C	37	3	8%	Decrease
NK1.1-Ly49D	36	11	23%	Decrease
NK1.1-Ly49G2	27	15	36%	No change
Day 3 LCMV				
NK1.1-Ly49A	164	29	15%	Decrease
NK1.1-Ly49C	87	37	30%	Decrease
NK1.1-Ly49D	85	60	41%	Decrease
NK1.1-Ly49G2	67	93	58%	Increase

[a] Age-matched C57BL/6 mice were given i.p. 1 x 10^4 PFU MCMV per mouse, 2 x 10^4 PFU MCMV per mouse (*), or 5 x 10^4 PFU LCMV per mouse.

[b] Spleen cells were removed from uninfected, 3 day MCMV-infected or 3 day LCMV-infected C57BL/6 mice and stained with anti-NK1.1-PE mAb and anti-Ly49A-FITC, -Ly49C-FITC, -Ly49D-FITC or -Ly49G2-FITC mAbs. A lymph gate was used to analyze the cells.

[c] R1 and R2 gates were drawn around $NK1.1^{+}$-$Ly49^{-}$ and $NK1.1^{+}$-$Ly49^{+}$ cells respectively, in the FACs profiles. An example of this can be seen in Figure V-2. The numbers reflected in each column are the number of positive events per 10^{4} lymphocytes.

[d] This percentage reflects the number of $NK1.1^{+}$ cells that stained positive for respective Ly49 NK cell receptors.

resulted in a decrease in the number of NK1.1$^+$ cells per 10^4 lymphocytes and changes in the percentages of NK1.1$^+$-Ly49$^+$ NK cell subsets within the spleen.

B. DISTRIBUTION OF NK1.1$^+$-LY49$^+$ NK CELL SUBSETS IN THE PERITONEAL CAVITY OF UNINFECTED AND 3 DAY MCMV-INFECTED C57BL/6 MICE

Analyses of the NK1.1$^+$-Ly49$^+$ NK cell subsets in the spleen indicated that even though the absolute number of splenocytes was similar between uninfected and infected mice, FACS analyses based on 10^4 lymphocytes showed that there were decreases in the number of NK1.1$^+$ cells, and changes in the proportion of the NK1.1$^+$-Ly49$^+$ subsets after MCMV infection. To examine if there were any changes in the NK cell population in the peritoneal cavity, peritoneal exudate cells (PEC) from uninfected and 3 day MCMV-infected C57BL/6 mice were stained with antibodies to NK1.1, Ly49A, C, D and G2 NK cell receptors. Compared to the uninfected controls, there was usually about a 2 fold increase in the absolute number of PEC in MCMV-infected mice (data not shown). FACs analyses showed that there were significantly more NK1.1$^+$ cells per 10^4 lymphocytes (2 - 4 fold increase) in the peritoneal cavity of 3 day MCMV-infected mice (Table V-1, Figure V-2). These data suggest that either there is an influx of NK1.1$^+$ cells into the peritoneal cavity during MCMV infection or else the resident NK cells are proliferating. Three days post-MCMV infection, FACs analyses of the Ly49 NK cell subsets within the NK1.1$^+$ population showed that there was a decrease in the percentage of NK1.1$^+$-Ly49A$^+$ cells

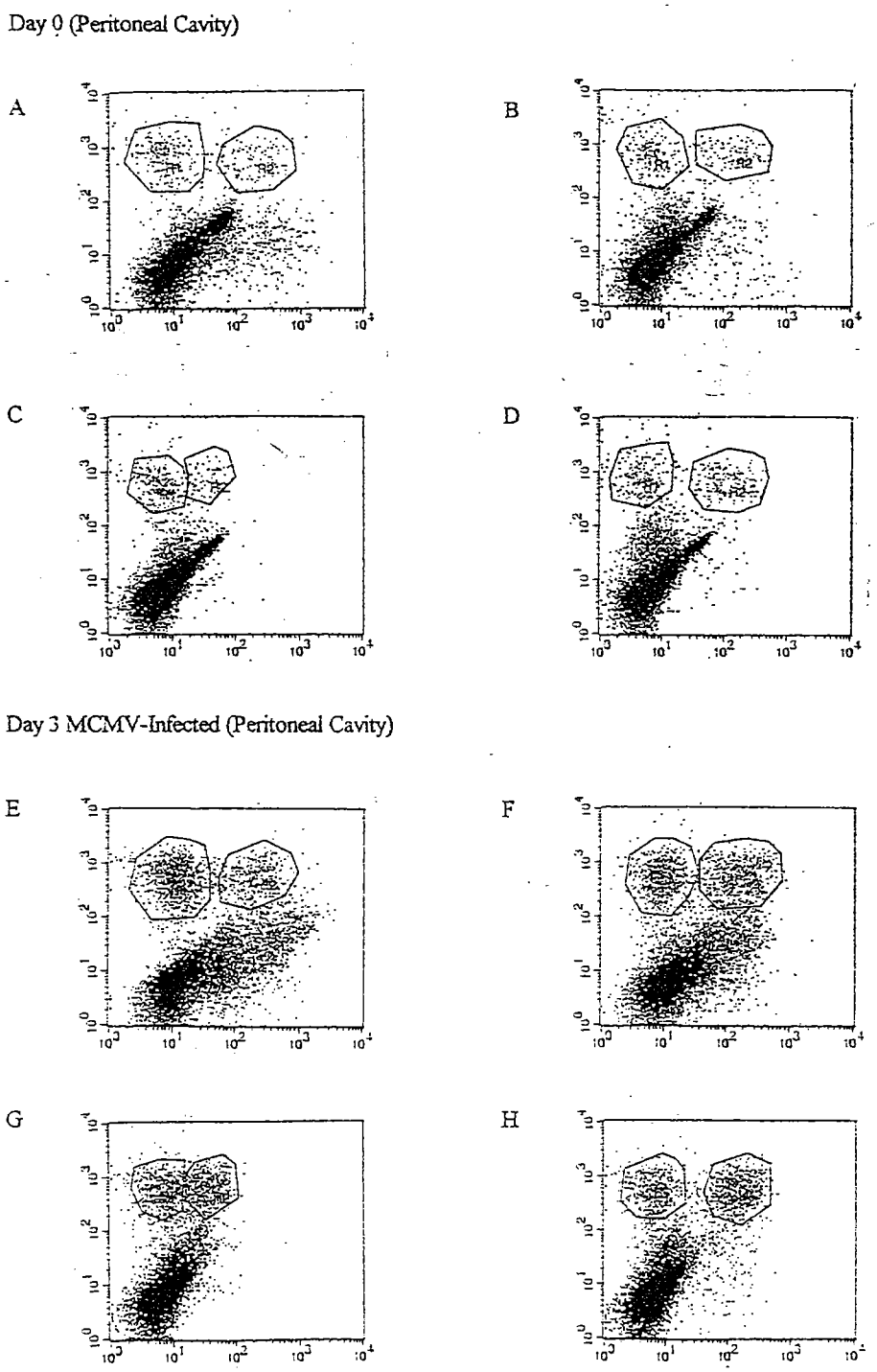

Figure V-2. Distribution of NK1.1$^+$-Ly49$^+$ NK cells in uninfected and 3 day MCMV-infected C57BL/6 peritoneal exudate cells (PECs). PECs were stained with anti-NK1.1 mAb (y-axis) and anti-Ly49A (A, E), anti-Ly49C (B, F), anti-Ly49C (C, G), or anti-Ly49G2 (D, H) mAbs (x-axis). Data from these FACs profiles are reported in Table V-3, Exp 1.

while there were increases in the percentages of NK1.1$^+$-Ly49C$^+$, NK1.1$^+$-Ly49D$^+$ and NK1.1$^+$-Ly49G2$^+$ cells (Table V-3).

Results from Tables V-2 and V-3 indicated that 3 days post-MCMV infection there was within the NK1.1$^+$ population an increase in the percentage of NK1.1$^+$-Ly49A$^+$ cells while there were decreases in the percentages of NK1.1$^+$-Ly49C$^+$ and NK1.1$^+$-Ly49D$^+$ cells in the spleen. This was accompanied by the concomitant decrease in the percentage of NK1.1$^+$-Ly49A$^+$ PECs and increase in the percentage of NK1.1$^+$-Ly49C$^+$ and NK1.1$^+$-Ly49D$^+$ PEC. In both the spleen and the peritoneal cavity, the percentage of NK1.1$^+$-Ly49G2$^+$ cells increased after MCMV infection. These results strongly suggest that 3 days post-MCMV infection, there was movement of NK1.1$^+$-Ly49$^+$ NK cell subsets in the spleen and the peritoneal cavity. A summary of the results is shown in Figure V-3.

C. DISTRIBUTION OF NK1.1$^+$-LY49$^+$ NK CELLS IN THE SPLEEN AND PERITONEAL CAVITY IN UNINFECTED AND 3 DAY LCMV-INFECTED C57BL/6 MICE

To determine if the above mentioned pattern of NK cell trafficking only occurred during MCMV infection, C57BL/6 mice were infected with a different virus, LCMV, and spleen cells and PEC were then stained with the various NK cell receptor antibodies. There were few differences in the absolute number of splenic leukocytes and PEC from 3 day LCMV-infected mice compared to uninfected mice (data not shown). FACs analyses also showed that there were few differences in the number of NK1.1$^+$ cells in the spleen

Table V-3. Expression of NK1.1 and Ly49 Receptors on Peritoneal Exudate Cells in Uninfected, MCMV-Infected and LCMV-Infected C57BL/6 Mice[a]

Group[b]	$NK1.1^+$-$Ly49^-$ (R1)[c] per 10^4 lymphocytes	$NK1.1^+$-$Ly49^+$ (R2)[c] per 10^4 lymphocytes	[R2/(R1+R2)] x 100[d]	Relative change compared to uninfected control
Exp 1:				
Day 0				
NK1.1-Ly49A	195	102	34%	
NK1.1-Ly49C	197	100	34%	
NK1.1-Ly49D	212	67	24%	
NK1.1-Ly49G2	192	163	46%	
Day 3 MCMV				
NK1.1-Ly49A	1012	354	26%	Decrease
NK1.1-Ly49C	710	596	46%	Increase
NK1.1-Ly49D	613	611	50%	Increase
NK1.1-Ly49G2	484	744	61%	Increase
Day 3 LCMV				
NK1.1-Ly49A	626	174	21%	Decrease
NK1.1-Ly49C	499	411	45%	Increase
NK1.1-Ly49D	435	380	47%	Increase
NK1.1-Ly49G2	315	472	60%	Increase
Exp 2:				
Day 0				

NK1.1-Ly49A	388	205	36%	
NK1.1-Ly49C	345	266	44%	
NK1.1-Ly49D	229	135	37%	
NK1.1-Ly49G2	357	311	47%	
Day 3 MCMV				
NK1.1-Ly49A	602	285	32%	Decrease
NK1.1-Ly49C	515	548	52%	Increase
NK1.1-Ly49D	466	495	52%	Increase
NK1.1-Ly49G2	380	495	57%	Increase
Day 3 LCMV				
NK1.1-Ly49A	289	56	16%	Decrease
NK1.1-Ly49C	243	174	42%	Decrease
NK1.1-Ly49D	202	180	47%	Increase
NK1.1-Ly49G2	203	184	48%	Increase +/-

Exp 3:

Day 0				
NK1.1-Ly49A	116	95	45%	
NK1.1-Ly49C	213	93	30%	
NK1.1-Ly49D	190	67	26%	
NK1.1-Ly49G2	112	97	46%	
Day 3 MCMV				
NK1.1-Ly49A	514	135	21%	Decrease
NK1.1-Ly49C	302	262	46%	Increase
NK1.1-Ly49D	274	312	53%	Increase
NK1.1-Ly49G2	236	357	60%	Increase

Day 3 MCMV*				
NK1.1-Ly49A	688	231	25%	Decrease
NK1.1-Ly49C	410	338	45%	Increase
NK1.1-Ly49D	465	406	47%	Increase
NK1.1-Ly49G2	250	571	70%	Increase
Exp 4:				
Day 0				
NK1.1-Ly49A	159	33	17%	
NK1.1-Ly49C	186	58	24%	
NK1.1-Ly49D	242	65	21%	
NK1.1-Ly49G2	143	115	45%	
Day 3 MCMV*				
NK1.1-Ly49A	160	44	22%	Increase
NK1.1-Ly49C	136	54	28%	Increase
NK1.1-Ly49D	143	112	44%	Increase
NK1.1-Ly49G2	114	133	54%	Increase
Day 3 LCMV				
NK1.1-Ly49A	244	83	25%	Increase
NK1.1-Ly49C	182	110	38%	Increase
NK1.1-Ly49D	150	139	48%	Increase
NK1.1-Ly49G2	112	147	58%	Increase

[a] Age-matched C57BL/6 mice were given i.p. 1 x 10^4 PFU MCMV per mouse, 2 x 10^4 PFU MCMV per mouse (*), or 5 x 10^4 PFU LCMV per mouse.

[b] Peritoneal exudate cells were removed from uninfected, 3 day MCMV-infected or 3 day LCMV-infected C57BL/6 mice and stained with anti-NK1.1-PE mAb and anti-Ly49A-FITC, -Ly49C-FITC, -Ly49D-FITC or -Ly49G2-FITC mAbs. A lymph gate was used to analyze the cells.

[c] R1 and R2 gates were drawn around $NK1.1^+$-$Ly49^-$ and $NK1.1^+$-$Ly49^+$ cells respectively, in the FACs profiles. An example of this can be seen in Figure V-2. The numbers reflected in each column are the number of positive events per 10^4 lymphocytes.

[d] This percentage reflects the number of $NK1.1^+$ cells that stained positive for respective Ly49 NK cell receptors.

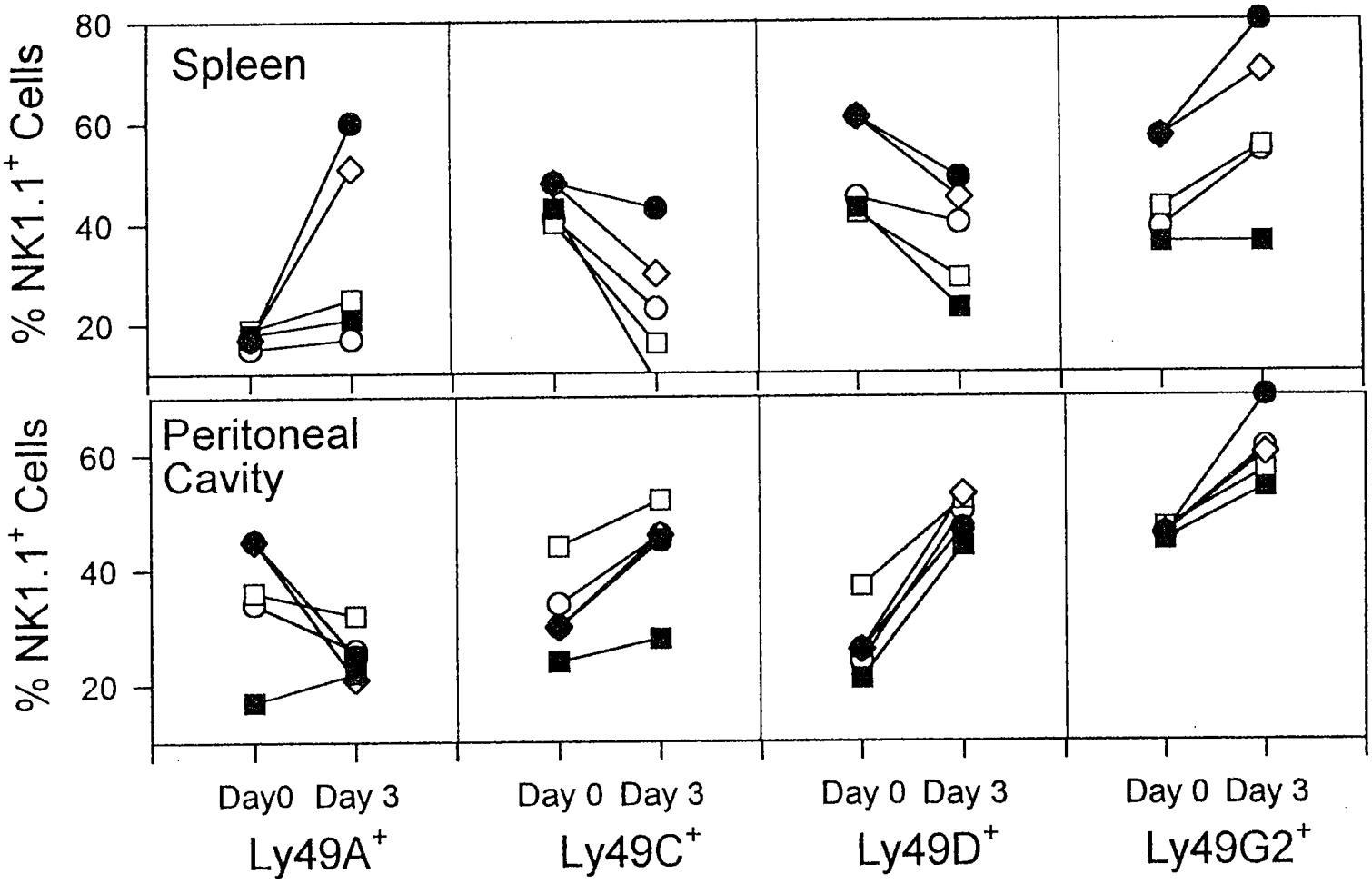

Figure V-3. Summary figure showing the changes in the % of NK1.1$^+$-Ly49$^+$ cells in the spleens and the peritoneal cavities of uninfected and 3 day MCMV-infected C57BL/6 mice. The percentages reflected in this figure are taken from Tables V-2 and V-3, Exp 1 (○), Exp 2 (□), Exp 3 (1 x 10^4 PFU MCMV/mouse, ◇), Exp 3 (2 x 10^4 PFU MCMV/mouse, ●) and Exp 4 (■).

(Table V-1), and in only 1 out of 3 experiments, there was an increase in NK1.1$^+$ cells per 10^4 lymphocytes in the peritoneal cavity (Table V-1). The distribution of the various Ly49 NK cell receptors in the spleen and the peritoneal cavity did not follow a reproducible pattern after 3 days of LCMV infection (Table V-2, Table V-3, Figure V-4). There were little to no changes in the percentages of NK1.1$^+$-Ly49A$^+$, NK1.1$^+$-Ly49C$^+$ and NK1.1$^+$-Ly49D$^+$ cells in the spleen while the changes in the percentage of these 3 (A, C, and D) Ly49 NK cell subsets were at best erratic in the peritoneal cavity. There was, however, an increase in the percentage of Ly49G2$^+$ NK cell subset in both the spleen and the peritoneal cavity (Table V-2, Table V-3, Figure V-4). Thus, the distribution of Ly49 NK cell subsets after MCMV infection is distinct for that particular virus infection, thereby suggesting that the Ly49 NK cell subsets may play a role in the regulation of MCMV in C57BL/6 mice.

D. EFFECTS OF *IN VIVO* ANTI-LY49 MAB TREATMENT ON THE REGULATION OF MCMV IN C57BL/6 MICE

Three days post-MCMV infection, there was a shift in the percentages of Ly49$^+$ NK cell subsets in the spleen and peritoneal cavity, suggesting that there was trafficking of the different Ly49 subsets to different locations after MCMV infection. To examine the importance of the different Ly49 subsets in the early regulation of MCMV, one of the known Ly49 subsets or a combination of Ly49 subsets were depleted with mAbs, and NK cell activity and MCMV titers in the spleen and the liver were then measured. The anti-Ly49C and anti-Ly49G2 Abs were previously titrated *in vivo* by our collaborators (Drs.

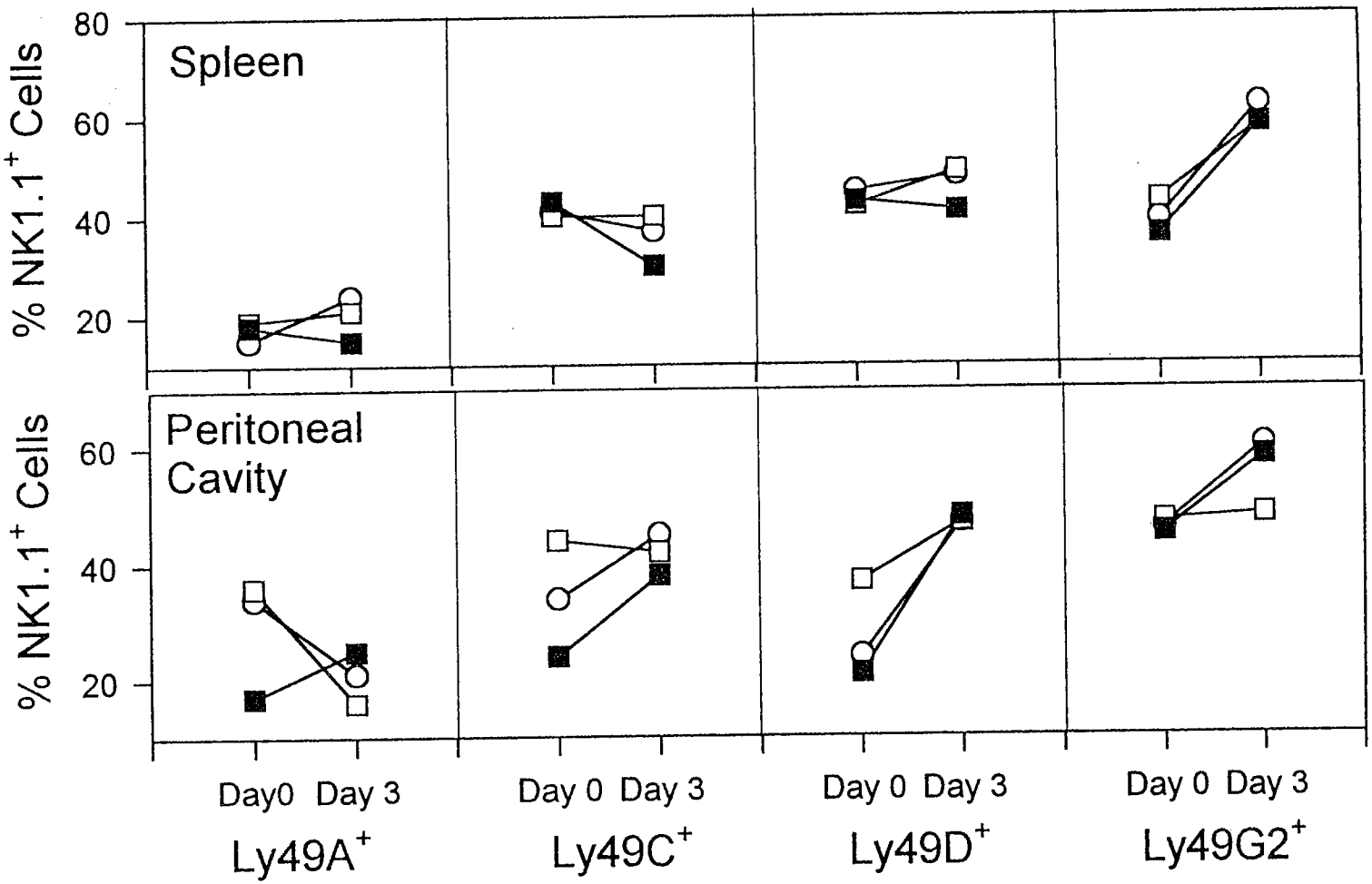

Figure V-4. Summary figure showing the changes in the % of NK1.1$^+$-Ly49$^+$ cells in the spleens and the peritoneal cavities of uninfected and 3 day LCMV-infected C57BL/6 mice. The percentages reflected in this figure are taken from Tables V-2 and V-3, Exp 1 (○), Exp 2 (□), Exp 4 (■).

V. Kumar and J.R. Ortaldo) by measuring the rejection of parental bone marrow cells by Ly49C$^+$ and Ly49G2$^+$ cells in F1 (b x d) mice. Anti-Ly49A and anti-Ly49D Abs were also previously titrated *in vivo* by our collaborators to ensure that the correct doses were used to *in vivo* deplete the respective Ly49 NK cell subsets. Depletion of a single Ly49 NK cell subset (Ly49A, Ly49C, Ly49D or Ly49G2 alone) had very little effect on NK cell cytotoxicity compared to MCMV-infected mice treated with anti-NK1.1 mAb (Figure V-5 A, B and C). When compared to the infected controls, 3 day MCMV-infected C57BL/6 mice treated with either anti-Ly49A, C, D or G2 mAbs alone exhibited no increase in virus titers in the spleen and the liver (Table V-4, Exps 1 - 5). Depletion of 2 subsets of Ly49$^+$ NK cells (Ly49C and Ly49D, Ly49C and Ly49G2, Ly49D and Ly49G2) had some effect on NK cell cytotoxicity (Figure V-5 D and E), but these depletions had no effect on the regulation of MCMV in the spleen and the liver (Table V-4, Exps 6 and 7). In 2 separate experiments, depletion of 3 Ly49$^+$ NK cell subsets (Ly49C, Ly49D and Ly49G2) resulted in diminished NK cell activity (Figure V-5) but in only 1 experiment did such a depletion resulted in the increase of MCMV titers in the spleen and the liver (Table V-4, Exps 7 and 8). As a positive control in all the experiments, C57BL/6 mice treated with anti-NK1.1 mAb to deplete the NK cells exhibited the expected increase in MCMV titers in the spleen and the liver. These results suggest that in adult C57BL/6 mice the absence of any one particular Ly49 NK cell subset does not affect the ability of the residual NK cells to control MCMV.

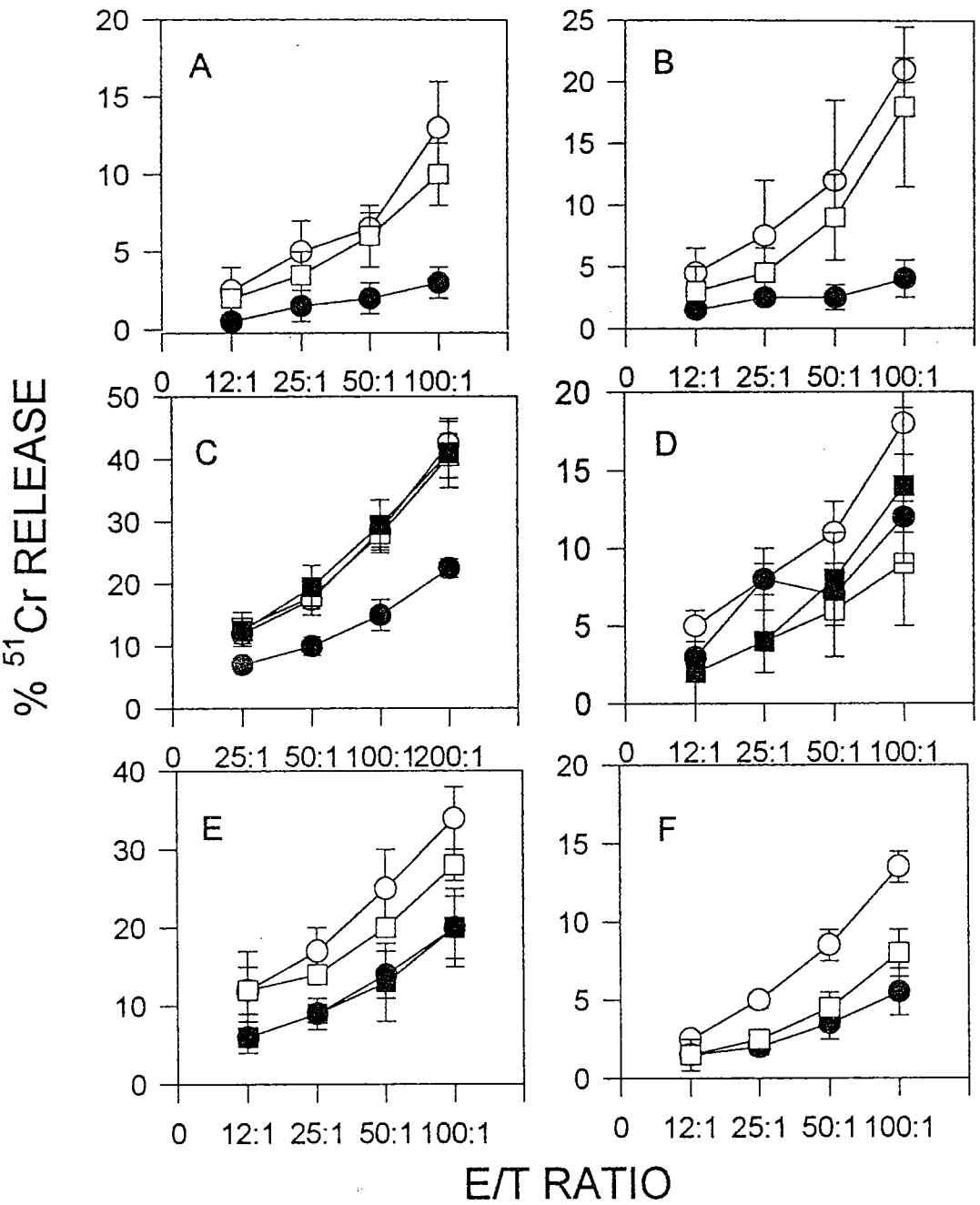

Figure V-5. NK cell activity from C57BL/6 mice treated with anti-Ly49 Abs. Three days post-infection, spleen cells from MCMV-infected (A - F, O), MCMV-infected, anti-NK1.1-treated (A - F, ●), anti-Ly49A-treated (A, □), anti-Ly49C-treated (B, □), anti-Ly49D-treated (C,□), anti-Ly49G2-treated (C, ■), anti-Ly49C-, -Ly49D-treated (D, ■), anti-Ly49C-, -Ly49G2-treated (D, □), anti-Ly49D-, -Ly49G2-treated (E, □), anti-Ly49C-, -Ly49D-, -Ly49G2-treated (E, ■), and anti-Ly49C-, -Ly49D-, -Ly49G2-treated (F, □) C57BL/6 mice were used as effectors against YAC-1 targets in a standard 5-h ^{51}Cr release assay.

Table V-4. Effects of *in vivo* Anti-Ly49 mAb Treatment on the Replication of MCMV in C57BL/6 Mice[a]

Exp	Group	Log_{10} PFU/Organ ± SD[b] Spleen	Liver
1	MCMV-infected Control	<2.4±0.1	4.4±0.2
	MCMV-infected + anti-NK1.1	3.9±0.3[c]	4.8±0.1[d]
	MCMV-infected + anti-Ly49A	<2.4±0.1[e]	4.3±0.1[f]
2	MCMV-infected Control	<2.3±0.0	<3.3±0.8
	MCMV-infected + anti-NK1.1	3.4±0.1[c]	4.3±0.1[d]
	MCMV-infected + anti-Ly49C	<2.4±0.1[f]	3.4±0.8[f]
3	MCMV-infected Control	2.7±0.1	4.2±0.2
	MCMV-infected + anti-NK1.1	4.2±0.4[c]	4.5±0.1[d]
	MCMV-infected + anti-Ly49C	2.8±0.3[e]	4.0±0.2[f]
4	MCMV-infected Control	2.4±0.2	5.2±0.1
	MCMV-infected + anti-NK1.1	4.0±0.1[c]	5.8±0.1[c]
	MCMV-infected + anti-Ly49C	2.5±0.3[f]	5.3±0.1[f]
5	MCMV-infected Control	<1.3±0.0	3.4±0.4
	MCMV-infected + anti-NK1.1	2.6±0.7[d]	4.1±0.7[f]
	MCMV-infected + anti-Ly49D	1.5±0.2[f]	3.6±0.3[f]
	MCMV-infected + anti-Ly49G2	1.6±0.7[f]	3.8±0.1[f]
6	MCMV-infected Control	2.5±0.1	4.1±0.1
	MCMV-infected + anti-NK1.1	3.1±0.1[c]	4.8±0.2[c]
	MCMV-infected + anti-Ly49C + anti-Ly49D	2.9±0.2[f]	4.3±0.2[f]
	MCMV-infected + anti-Ly49C + anti-Ly49G2	2.6±0.2[d]	4.2±0.2[f]
7	MCMV-infected Control	1.6±0.2	3.4±0.3
	MCMV-infected + anti-NK1.1	2.5±0.1[c]	4.6±0.1[c]
	MCMV-infected + anti-Ly49D + anti-Ly49G2	1.6±0.3[e]	3.4±0.6[e]
	MCMV-infected + anti-Ly49C + anti-Ly49D + anti-Ly49G2	1.5±0.3[e]	3.9±0.01[f]

8	MCMV-infected Control	1.5±0.3	4.3±0.3
	MCMV-infected + anti-NK1.1	3.7±0.1	4.9±0.1
	MCMV-infected + anti-Ly49C + anti-Ly49D + anti-Ly49G2	2.5±0.3	4.3±0.3

[a] Age-matched C57BL/6 mice were infected i.p. with 10^4 MCMV PFU per mouse. Anti-NK1.1, anti-Ly49A, anti-Ly49C and anti-Ly49G2 were given i.v. 1 day prior to infection. Anti-Ly49D was given i.v. at day -2 and day -1 prior to infection.

[b] Splenic and Liver MCMV PFU were titrated on C57BL/6 MEF 3 days post-infection.

[c] Compared to the infected control, anti-NK cell treatment resulted in a $p < 0.005$.

[d] Compared to the infected control, anti-NK cell treatment resulted in a $p< 0.05$.

[e] Compared to the infected control, anti-NK cell treatment resulted in a $p> 0.5$.

[f] Compared to the infected control, anti-NK cell treatment resulted in a $p\leq 0.5$.

E. EFFECTS OF ADOPTIVELY TRANSFERRED LY49$^+$ LAK CELLS ON THE REGULATION OF MCMV IN SUCKLING MICE

To further clarify the role of Ly49 NK cell receptors in the regulation of MCMV by NK cells, we tested the ability of NK1.1$^+$, Ly49$^+$ LAK cells to protect suckling mice from a lethal MCMV infection. LAK cells were generated using 6 - 12 week old C57BL/6 spleen cells. Figure V-6 shows the typical distribution of the different Ly49 subsets within the NK1.1$^+$ LAK cell population. The percentages of the different Ly49 NK cell subsets in the LAK cell cultures are similar to the percentages seen in spleen NK cells taken from naive C57BL/6 mice (Table V-2). Prior to adoptive transfers, the LAK cells were sorted into NK1.1$^+$-Ly49$^-$ or NK1.1$^+$-Ly49$^+$ for all the 4 Ly49 subsets (A, C, D, G2). These sorted LAK cells were adoptively transferred into 4 - 6 day old C57BL/6 suckling mice which were subsequently infected with MCMV, and splenic MCMV titers were measured 3 days later. Suckling mice adoptively reconstituted with any of the different combinations of NK1.1$^+$ LAK cells had lower splenic MCMV titers compared to the media control (Table V-5). For example, both NK1.1$^+$-Ly49A$^-$ LAK cells and NK1.1$^+$-Ly49A$^+$ LAK cells significantly decrease the amount of MCMV in the spleen of the suckling mice compared to the media control (Table V-5, Exp 2). These results suggest that the presence or absence of any one of the Ly49 NK cell receptors does not have an effect on NK cell's ability to protect suckling mice from MCMV.

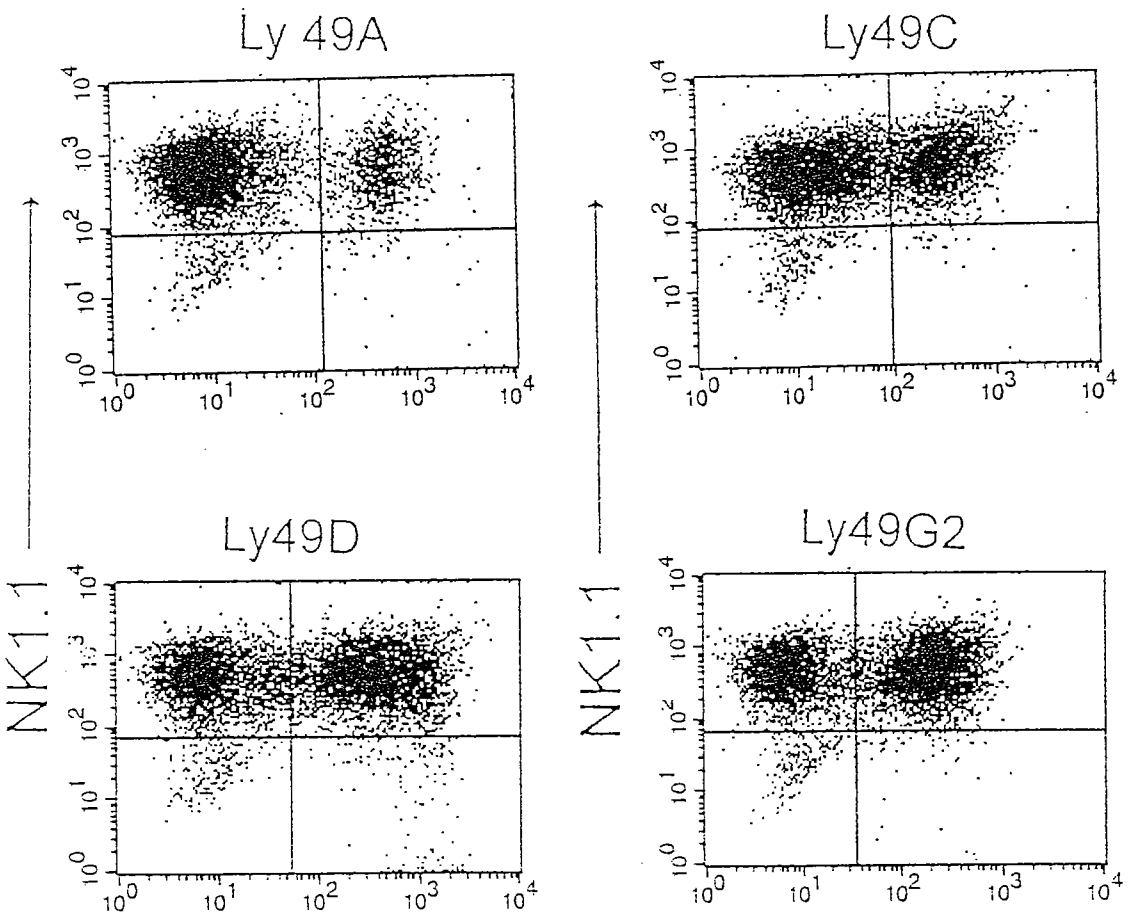

Figure V-6. FACs profiles of LAK cells. LAK cells stained with anti-NK1.1-PE mAbs (y-axis) and anti-Ly49A-FITC, -Ly49C-FITC, -Ly49D-FITC, or -Ly49G2-FITC (x-axis). LAK cells were generated from nylon-wool-passaged naive C57BL/6 spleen cells treated with 800 U/ml IL-2 for 7 - 9 days. LAK cultures are 96% - 99% $NK1.1^+$. $Ly49A^+$ cells make up 15%; $Ly49C^+$, 30%; $Ly49D^+$, 54%; and $Ly49G2^+$, 57% of all $NK1.1^+$ cells.

Table V-5. Control of MCMV by NK1.1$^+$ LAK Cells in MCMV-Infected C57BL/6 Suckling Mice[a]

Exp	Group[b]	Log_{10} PFU MCMV/Spleen[c]	p value[d]
1	Media Control	3.3 ± 0.2	
	Unsorted LAKs	2.5 ± 0.3	$p < 0.001$
2	Media Control	4.0 ± 0.2	
	NK1.1$^+$, Ly49A$^-$ LAKs	3.2 ± 0.3	$p = 0.001$
	NK1.1$^+$, Ly49A$^+$ LAKs	3.4 ± 0.3	$p = 0.005$
3	Media Control	4.1 ± 0.1	
	NK1.1$^+$, Ly49C$^-$ LAKs	3.1 ±0.6	$p = 0.01$
	NK1.1$^+$, Ly49C$^+$ LAKs	3.5 ± 0.5	$p < 0.05$
4	Media Control	3.1 ± 0.1	
	NK1.1$^+$, Ly49D$^-$ LAKs	2.6 ± 0.1	$p = 0.001$
	NK1.1$^+$, Ly49D$^+$ LAKs	2.7 ± 0.04	$p = 0.01$
5	Media Control	3.1 ± 0.04	
	NK1.1$^+$, Ly49D$^-$ LAKs	2.1 ± 0.1	$p < 0.001$
	NK1.1$^+$, Ly49D$^+$ LAKs	2.4 ± 0.1	$p < 0.001$
6	Media Control	2.9 ± 0.02	
	NK1.1$^+$, Ly49G2$^-$ LAKs	2.1 ± 0.2	$p = 0.01$
	NK1.1$^+$, Ly49G2$^+$ LAKs	2.2 ± 0.2	$p = 0.01$

7	Media Control	3.1 ± 0.04	
	$NK1.1^+$, $Ly49G2^-$ LAKs	2.3 ± 0.3	$p < 0.001$
	$NK1.1^+$, $Ly49G2^+$ LAKs	2.2 ± 0.5	$p = 0.001$

[a] Four to six day old C57BL/6 suckling mice were given i.p. 2000 PFU MCMV per mouse.
[b] One day prior to infection, the suckling mice were given i.p., 0.1 ml complete RPMI per mouse, 5×10^5 unsorted $NK1.1^+$ C57BL/6 LAK cells or sorted $NK1.1^+$ C57BL/6 LAK cells.
[c] Splenic MCMV titers were measured using C57BL/6 MEF 3 days post-infection.
[d] p value is calculated by comparing the MCMV titers of the adoptively reconstituted group to the media control group within the same experiment.

Taken together, these results strongly suggest that a single Ly49 NK cell receptor, Ly49A, C, D or G2 does not play an essential role in the control of MCMV synthesis by NK cells in both adult and suckling mice.

CHAPTER VI

NK CELL RESPONSE TO VIRAL INFECTIONS IN β_2-MICROGLOBULIN-DEFICIENT MICE*

Sensitivity of target cells to lysis by NK cells is inversely proportional to the amount of MHC class I expressed on the cell surfaces of the target cells (Missing Self Hypothesis), and *in vitro* studies have indicated that the interaction between most murine NK cell receptors and MHC class I molecules are inhibitory on the NK cells. As some viruses e.g. MCMV, have the ability to downregulate class I molecules, this virus-induced downregulation of MHC class I on infected cells could sensitize the infected cells to NK cell cytotoxicity by removing the inhibitory signal/s sent to the NK cells. We have shown that during a virus infection *in vivo*, the IFN response (and possibly other cytokines) causes a major up-regulation of MHC class I molecules throughout the body, and the uninfected cells in the host become more resistant to NK cells as they become substantially more susceptible to lysis by allospecific CTL (Hansson et al., 1980; Bukowski and Welsh, 1986). It is possible that NK cells may fail to regulate the LCMV infection because IFN, via the up-regulation of class I antigens, protects the LCMV-infected targets from NK cells; in contrast, NK cells may regulate MCMV infection because IFN cannot protect the MCMV-infected targets, as the virus downregulates MHC class I expression and IFN

* Work performed in this chapter was done in collaboration with Dr. R. R. Brutkiewicz (Tay et al., 1995).

cannot induce the levels of MHC class I on infected cells (Bukowski and Welsh, 1985b). In both cases, uninfected target cells would be spared the lytic effects of NK cells, because these normal cells would also be protected by IFN from NK cells. To understand the importance of class I antigens in the regulation of viral infections by NK cells, I examined viral infections in mice whose β_2m gene has been deleted by homologous recombination (Koller et al., 1990). Cells from these β_2m (-/-) mice do not express class I α-chain detectable by conformation-dependent Abs on their plasma membranes, although some reports suggest that there are some conformationally normal class I molecules present in low amounts (Bix and Raulet, 1992; Glas et al., 1992a). These mice have low levels of endogenous NK cell activity, but it can be augmented by treatment with the IFN inducer poly I:C (Hoglund et al., 1991b; Liao et al., 1991). Spleen lymphocytes of β_2m (-/-) mice, when stimulated by Con A, are highly sensitive to lysis by normal β_2m intact mouse NK cells, which preferentially lyse targets low in class I MHC expression. These targets are much less sensitive to β_2m (-/-) NK cells.

In this chapter, I will present evidence showing that, despite their inability to express MHC class I molecules in the correct conformation, and despite the resistance of these cells to IFN-mediated protection against NK cells, β_2m (-/-) mice still retain the ability to control MCMV.

A. ACTIVATION OF NK CELLS IN VIRUS-INFECTED MICE

C57BL/6 and β_2m (-/-) mice, either PBS-treated or depleted of NK cells with antiserum to aGM_1, were infected i.p. with MCMV (Figure VI-1 A, B) or LCMV (Figure VI-1 C, D) and tested 3 days later for spleen NK cell activity. Figure VI-1 (A, B) shows representative experiments indicating that the infection of either C57BL/6 or β_2m (-/-) mice with MCMV elicited an augmentation of NK cell activity compared to the uninfected controls. The levels of NK cell activity in the uninfected β_2m (-/-) mice were quite low, confirming the work of others (Hoglund et al., 1991; Liao et al., 1991). Administration of antiserum to aGM_1 significantly reduced the NK cell activity in the MCMV-infected C57BL/6 or β_2m (-/-) mice. Figure VI-1 (C, D) also shows that LCMV induced a more profound NK cell activation in both C57BL/6 and β_2m (-/-) mice, and, again, the NK cell response was completely ablated by antiserum to aGM_1.

B. REPLICATION OF MCMV IN NK CELL-DEPLETED β_2M (-/-) MICE

Replication of MCMV was, in several experiments, markedly elevated in the NK cell-depleted C57BL/6 mice, confirming earlier reports (Bukowski et al., 1983; Bukowski and Welsh, 1985b; Bukowski et al., 1985; Table VI-1). The synthesis of MCMV was comparable in the β_2m (-/-) and the C57BL/6 mice, and depletion of NK cells with antiserum to aGM_1, as with the C57BL/6 mice, resulted in substantially higher levels of MCMV synthesis in the β_2m (-

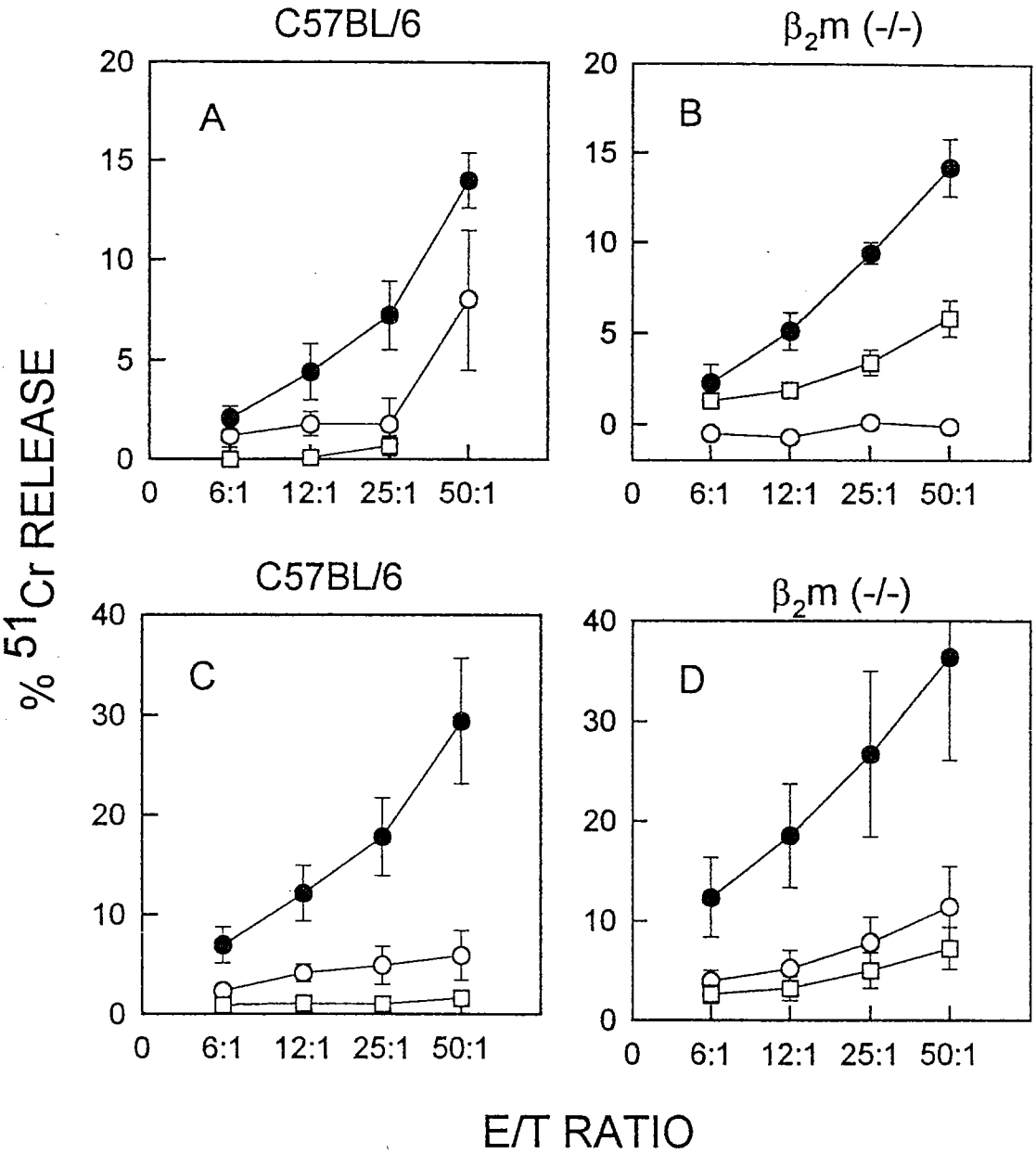

Figure VI-1. NK cell activity from C57BL/6 (+/+) and β_2m (-/-) mice. NK cells from uninfected (O), virus-infected (●) and virus-infected and anti-asialo GM_1-treated (□) mice were used as effectors against YAC-1 targets in a standard 4 h ^{51}Cr release assay. Three days before the assay, mice were infected with either MCMV (A and B) or LCMV (C and D).

TABLE VI-1. Splenic MCMV Titers in Intact and Anti-aGM$_1$-Treated β_2m (-/-) and C57BL/6 (+/+) Mice[a]

		Log_{10} PFU/spleen ± SD[b]	
Exp[c]	anti-aGM$_1$[d]	β_2m (-/-)	C57BL/6
1	-	3.0±0.3	3.3±0.1
	+	4.6±0.5	4.5±0.2
2	-	3.3±0.3	N.D.[e]
	+	4.1±0.2	N.D.
3[f]	-	4.0±0.8	3.4±0.2
	+	5.2±0.3	4.6±0.2

[a] Age-matched β_2m (-/-) and C57BL/6 (+/+) mice were infected with 1 x 10^5 PFU of MCMV i.p.

[b] Splenic MCMV was titrated on C57BL/6 mouse embryo fibroblasts, 3 days post infection.

[c] p values of the different experiments are as follows: Exp 1: β_2m (-/-), $p < 0.01$, C57BL/6, $p < 0.005$. Exp 2: β_2m (-/-), $p < 0.07$. Exp 3: β_2m (-/-), $p < 0.02$; C57BL/6, $p < 0.00003$.

[d] Mice received anti-aGM$_1$ antiserum, i.p., or no treatment at the time of infection.

[e] Not determined.

[f] Splenic NK cells from uninfected, MCMV-infected and MCMV-infected, anti-aGM$_1$-treated mice were used as effectors against YAC-1 targets. The results are shown in Figures VI-1A and VI-1B.

/-) mice (Table VI-1). These experiments therefore indicate that β_2m expression on either effector NK cells or their targets is not required for the anti-viral properties of NK cells *in vivo*.

C. REGULATION OF MCMV BY C57BL/6 AND β_2M (-/-) SPLEEN CELLS IN MCMV-INFECTED C57BL/6 SUCKLING MICE

To further show that the regulation of MCMV by NK cells is not dependent on MHC class I expression, the ability of β_2m (-/-) spleen cells to control MCMV synthesis 3 days post-infection in C57BL/6 suckling mice was measured. Adoptively transferred β_2m (-/-) adult spleen cells significantly lowered MCMV titers in the suckling mice when compared to the media control (Table VI-2). The level of protection afforded by the β_2m (-/-) spleen cells was similar to that of C57BL/6 adult spleen cells (Table VI-2). These experiments indicate that despite the differences in the expression of MHC class I molecules between the effector cell and the target cell, β_2m (-/-) spleen cells can still protect C57BL/6 suckling mice from MCMV.

D. REPLICATION OF LCMV IN NK CELL-DEPLETED β_2M (-/-) MICE

By a number of criteria, LCMV is considered an NK cell-resistant virus (Bukowski et al., 1983; Bukowski and Welsh, 1985b; Bukowski et al., 1985), and it has been speculated that LCMV may be resistant to NK cells because IFN can upregulate class I MHC on LCMV-infected target cells and protect these infected targets from NK cell-mediated lysis (Brutkiewicz

Table VI-2. Control of MCMV by C57BL/6 and β_2m (-/-) Adult Spleen cells in MCMV-Infected C57BL/6 Suckling Mice[a]

Exp	Group[b]	Log_{10} PFU MCMV/spleen[c]	*p* value[d]
1	Media Control	3.0±0.1	
	β_2m (-/-) spleen cells	2.2±0.4	$p < 0.05$
	C57BL/6 spleen cells	2.2±0.6	$p < 0.05$
2	Media Control	2.7±0.1	
	β_2m (-/-) spleen cells	1.7±0.1	$p < 0.005$
	C57BL/6 spleen cells	2.0±0.2	$p < 0.005$

[a] Four to six day old C57BL/6 suckling mice were given i.p. 5000 PFU MCMV per mouse.

[b] One day prior to infection, the suckling mice were given i.p., 0.1 ml complete RPMI, 5 x 10^7 C57BL/6 spleen cells or 5 x 10^7 β_2m (-/-) spleen cells.

[c] Splenic MCMV titers were measured using C57BL/6 MEF 3 days post-infection.

[d] *p* value is calculated by comparing the MCMV titers of the adoptively reconstituted group to the media control group within the same experiment.

et al., 1992; Bukowski and Welsh, 1985b). If, indeed, this were the mechanism of the resistance of LCMV to NK cells, one might expect LCMV to be an NK-sensitive virus in the β_2m (-/-) mice. In agreement with previous reports (Bukowski et al., 1983; Bukowski and Welsh, 1985b; Bukowski et al., 1985), C57BL/6 mice synthesized similar levels of LCMV, whether or not the mice had been NK cell-depleted (Table VI-3, Exp 1). Synthesis of LCMV in the β_2m (-/-) mice was virtually identical to the levels in the C57BL/6 mice, and depletion of NK cells had no effect on the LCMV titers in the β_2m (-/-) mice (Table VI-3). These experiments therefore argue against the hypothesis that the upregulation of class I MHC antigens by virus-induced IFN protects LCMV-infected targets from the anti-viral effects of NK cells.

E. EXPRESSION OF CLASS I MHC ANTIGENS IN VIRUS-INFECTED MICE

The possibility existed that viral infections induced cell surface expression of class I MHC antigens in the β_2m (-/-) mice. To test for this, thymocytes from uninfected and virus-infected mice were examined for surface class I expression using both MHC class I conformation-dependent and conformation-independent allo-antibodies. Figures VI-2 and VI-3 show that MCMV and LCMV infections up-regulated the expression of class I MHC antigens on thymocytes from virus-infected C57BL/6 mice. In contrast, no significant expression of MHC class I on thymocytes from the virus-infected β_2m (-/-) mice was detected with either the conformation-dependent or conformation-independent allo-antibodies. Similar

TABLE VI-3. Splenic LCMV Titers in Intact and Anti-aGM$_1$-Treated β_2m (-/-) and C57BL/6 (+/+) Mice[a]

		$\log_{10}$ PFU/spleen ± S.D.[b]	
Exp[c]	anti-aGM$_1$[d]	β_2m (-/-)	C57BL/6
1[e]	-	5.5±0.2	5.5±0.2
	+	5.6±0.2	5.5±0.1
2	-	5.4±0.5	N.D.[f]
	+	5.9±0.2	N.D.
3	-	5.3±0.3	N.D.
	+	5.1±0.8	N.D.
4	-	5.1±0.1	N.D.
	+	5.1±0.2	N.D.

[a] Age-matched β_2m (-/-) and C57BL/6 (+/+) mice were infected with 5 x 10^4 PFU of LCMV i.p.

[b] Splenic LCMV was titrated on Vero cells, 3 days post-infection.

[c] p values of the different experiments are as follows: Exp 1: β_2m (-/-), $p < 0.3$; C57BL/6, $P < 0.7$. Exp 2: $p < 0.2$. Exp 3: $p < 0.6$. Exp 4: $p < 0.7$.

[d] Mice received anti-aGM$_1$ antiserum, i.p. or no treatment at the time of infection.

[e] Splenic NK cells from uninfected, LCMV-infected and LCMV-infected, anti-aGM$_1$-treated mice were used as effectors on YAC-1 targets. The results are shown in Figures VI-1C and VI-1D.

[f] Not determined.

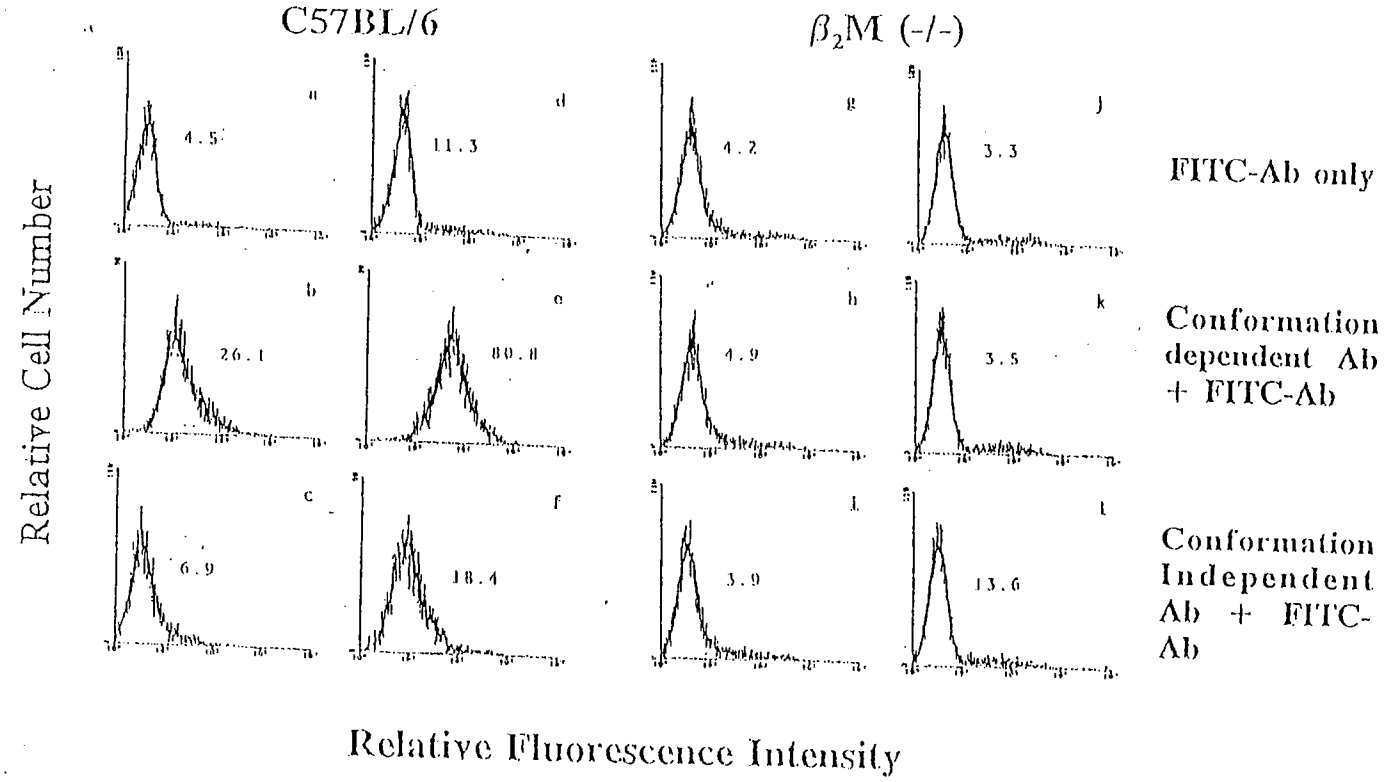

Figure VI-2. Expression of H-2D^b on thymocytes from uninfected or MCMV-infected C57BL/6 or β_2m (-/-) mice. Single cell suspensions of thymocytes were prepared from uninfected or MCMV-infected mice and were stained with the conformation-dependent 28-11-5S (b, e, h, and k) or conformation-independent 28-11-8S (c, f, i, and l) anti-H-2D^b on thymocytes from uninfected (a - c) or MCMV-infected (d - f) C57BL/6 mice. g - l, staining of H-2D^b on thymocytes from uninfected (g - i) or MCMV-infected (j - l) β_2m (-/-) mice. a, d, g, and j: Staining with second Ab only. The mean channel fluorescence is indicated next to the peak. This collaborative experiment was also shown in Brutkiewicz R.R., 1993.

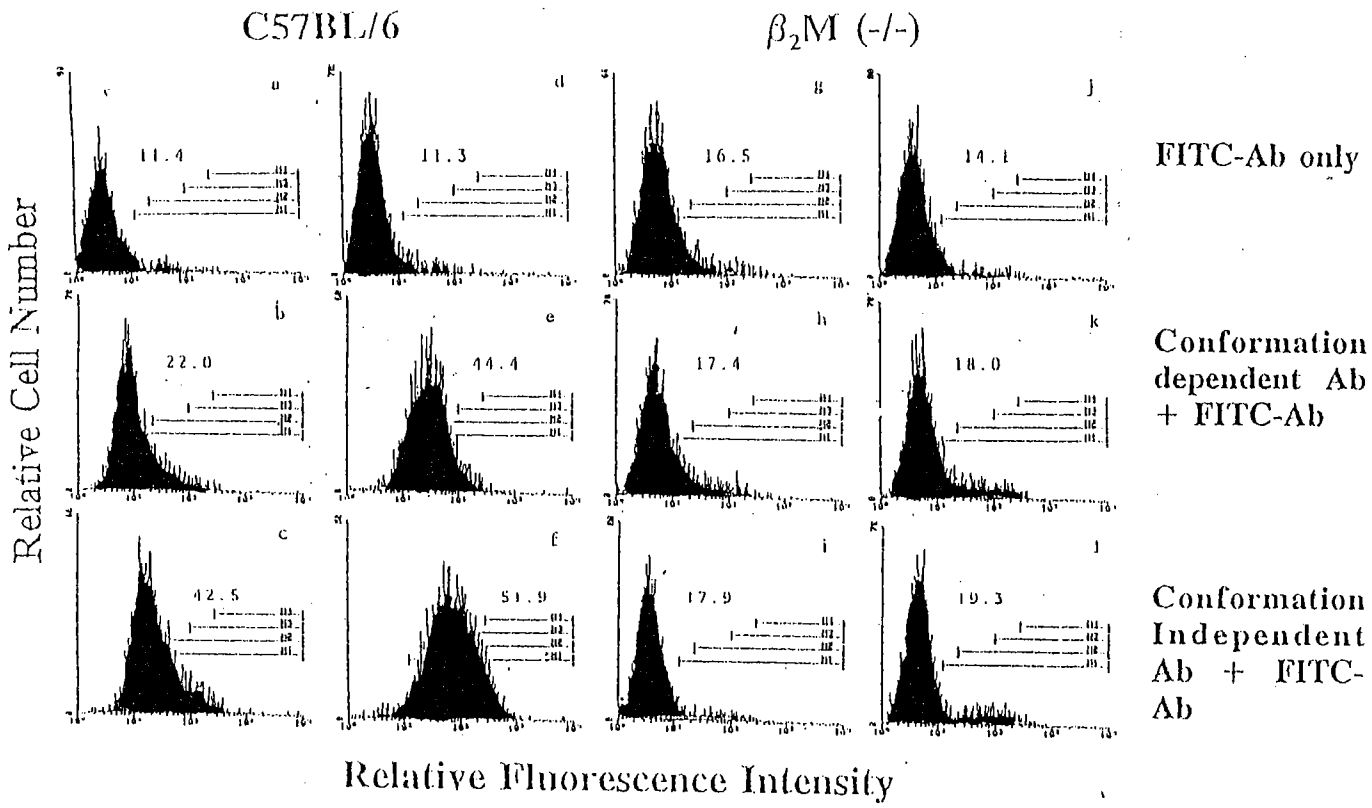

Figure VI-3. Expression of H-2D^b on thymocytes from uninfected or LCMV-infected C57BL/6 and β_2m (-/-) mice. Single cell suspensions of thymocytes were prepared from uninfected LCMV-infected mice and were stained with conformation-dependent 28-11-5S (b, e, h, and k) or conformation-independent 28-14-8S (c, f, i, and l) anti-H2D^b mAbs, followed by FITC-conjugated goat anti-mouse Ig antiserum. Analysis was performed by FACStar. Panels a - f: staining of H-2D^b on thymocytes from uninfected (a - c) or LCMV-infected (d - f) C57BL/6 mice. g - l: Staining of H-2D^b on thymocytes from uninfected (g - i) or LCMV-infected (j - l) β_2m (-/-) mice. Panels a, d, g, and j: Staining with second Ab only. The mean channel fluoresence is indicated next to the peak.

results not depicted were shown using the pan-H-2 class I-specific mAb, M1. This demonstrates that the β_2m (-/-) mice failed to express surface class I antigens detectable by antibody even during a virus infection.

F. CYTOTOXIC CAPACITY OF NK CELLS FROM VIRUS-INFECTED β_2M-DEFICIENT MICE

Splenocytes from β_2m (-/-) mice were tested for their cytolytic capacity against a variety of targets to determine the specificity and magnitude of their lytic function. Large, cortical, cortisone-sensitive thymocytes appear to be natural targets for NK cells and can be shown *in vitro* to be highly sensitive to NK cell-mediated lysis (Hansson et al., 1980). Therefore, thymocytes, in addition to continuous cell lines, were tested as targets. Table VI-4 shows that β_2m (-/-) splenocytes from LCMV-infected mice displayed only moderate levels of cytotoxicity against the prototypic YAC-1 NK cell target and very little cytotoxicity against a variety of other targets, including β_2m (-/-) and β_2m (+/+) cell lines and β_2m (-/-) and C57BL/6 thymocytes. These targets were much more sensitive to lysis by splenocytes from the LCMV-infected genetically high NK cell strain, C3H. Thus, even though NK cells from β_2m (-/-) mice could mediate a normal anti-viral effect, their ability to lyse their own naturally NK cell-sensitive targets was very poor. It should be noted that when β_2m (-/-) thymocytes were used as targets for C3H mouse splenocytes, they were more sensitive than C57BL/6 thymocytes to

Table VI-4. Lysis of Targets by NK Cells From LCMV-Infected β_2m (+/+) and β_2m (-/-) Mice

	Exp 1[a]			Exp 2[a]		
Target/Effector[b]	C3H/HeSnJ	C57BL/6	β_2m (-/-)	C3H/HeSnJ	C57BL/6	β_2m (-/-)
YAC-1	61	25	24	75	44	41
C3H thymocytes	15	<1	<1	20	N.D.[c]	4
C57BL/6 thymocytes	22	1	2	42	N.D.	6
β_2M (-/-) thymocytes	26	5	<1	39	N.D.	2
MC57G	21	8	3	20	N.D.	3
D1R (+/+)	17	5	<1	23	5	<1
R1E.D[b] (-/-)	23	5	7	23	6	4

[a] % ^{51}Cr release is shown at E/T ratio of 50:1.
[b] Effector cells were splenic cells from day 3 LCMV-infected C3H/HeSnJ (+/+) , C57BL/6 (+/+) and β_2m (-/-) mice.
[c] Not determined.

NK cell mediated lysis. This confirms work of others showing that cells lacking class I MHC expression have enhanced sensitivity to NK cell-mediated lysis (Ljunggren and Karre, 1990).

G. INTERFERON-MEDIATED PROTECTION OF β_2M-DEFICIENT TARGET CELLS FROM NK CELL-MEDIATED LYSIS

Some cell lines lacking class I MHC expression have been shown to be sensitive to NK cells and are resistant to the protective effects of IFN (Ljunggren and Karre, 1990; Sandberg et al., 1994). We sought to confirm these reports and used a previously untested β_2m-deficient cell line as a target for IFN-protection studies (Allen et al., 1986; Waneck et al., 1987). Table VI-5 showed that the β_2m (+/+) cell line, D1R, an H-2D^b-transfectant, expressed moderate to high levels of class I MHC and were moderately sensitive to activated NK cells taken from the spleens of LCMV-infected C3H mice. After treatment with IFN-β, D1R expressed higher levels of class I MHC and became resistant to lysis by NK cells. In contrast, the R1E.D^b β_2m (-/-) cell line was highly sensitive to NK cells and expressed little detectable class I antigen. After IFN-β treatment, the β_2m (-/-) cells remained sensitive to NK cells and failed to upregulate the surface expression of conformationally normal class I, as detectable by antibody. This and previous work (Sandberg et al., 1994; Bukowski and Welsh, 1986; Hansson et al., 1980), suggest that the β_2m (-/-) mice should be resistant to the IFN-mediated protection of targets that normally occurs *in vivo* during virus infections.

TABLE VI-5. Sensitivity of IFNβ-Treated Normal and β_2m-Deficient Cell Lines to NK Cell-Mediated Lysis

Exp	Target Cells	Treatment[b]	% Lysis[c]	MHC Class I Expression Mean Channel Fluorescence[a] Control	28-11-5S
1	D1R (D^b)	Mock	37	9	266
		IFNβ	20	8	447
	R1E.D^b (β_2m-def)	Mock	36	20	29
		IFNβ	36	12	17
2	D1R	Mock	28	ND[d]	ND
		IFNβ	12	ND	ND
	R1E.D^b	Mock	35	ND	ND
		IFNβ	37	ND	ND
3	D1R	Mock	22	ND	ND
		IFNβ	9	ND	ND
	R1E.D^b	Mock	25	ND	ND
		IFNβ	30	ND	ND

[a] Cells were stained with 28-11-5S (anti-D^b, conformation- dependent, mouse anti-mouse) antibodY, followed by a FITC conjugated anti-mouse Ig antiserum. Analysis was by FACs.

[b] Cells were either mock-treated or exposed to 1000 U/ml of murine IFNβ for 24 h at 37°C.

[c] Percent lysis by LCMV-activated splenic C3H/HeSnJ NK cells in a 4-h ^{51}Cr release assay. E:T ratio = 100:1.

[d] Not determined.

H. PROTECTION OF β_2M (-/-) THYMOCYTES FROM NK CELLS BY *IN VIVO* POLY I:C TREATMENT

To determine whether an IFN inducer would be able to protect natural thymocyte targets of β_2m (-/-) mice from NK cells, we treated mice with poly I:C, a potent *in vivo* IFN-inducer previously shown to upregulate class I MHC expression on thymocytes and to render thymocytes resistant to NK cell-mediated lysis (Hansson et al., 1980; Bukowski and Welsh, 1986). Figure VI-4A shows a representative experiment indicating that poly I:C treatment rendered thymocytes from C57BL/6 mice relatively resistant to lysis by activated NK cell populations derived from the spleens of LCMV-infected C3H mice. Similar protection was seen when C57BL/6 splenocytes were used as effector cells, but the data from the C3H mice are presented because the NK cell response in these mice is much higher than that in C57BL/6 mice. In general, β_2m (-/-) effector cell populations lysed all thymocyte target types very poorly (Table VI-4).

Thymocytes from untreated β_2m (-/-) mice had higher sensitivity to NK cell-mediated lysis relative to C57BL/6 controls, and poly I:C-treatment only marginally reduced their sensitivity to lysis. In a total of 3 experiments, poly I:C-treatment resulted in a 30% $\pm$ 6 (standard deviation) reduction in the proportion of C57BL/6 thymocytes lysed, but only a 10% $\pm$ 7 reduction (5 experiments) in the killing of β_2m (-/-) thymocytes. Using the mAb M1, there was no increase in the MHC class I expression in the β_2m (-/-) thymocytes after poly I:C

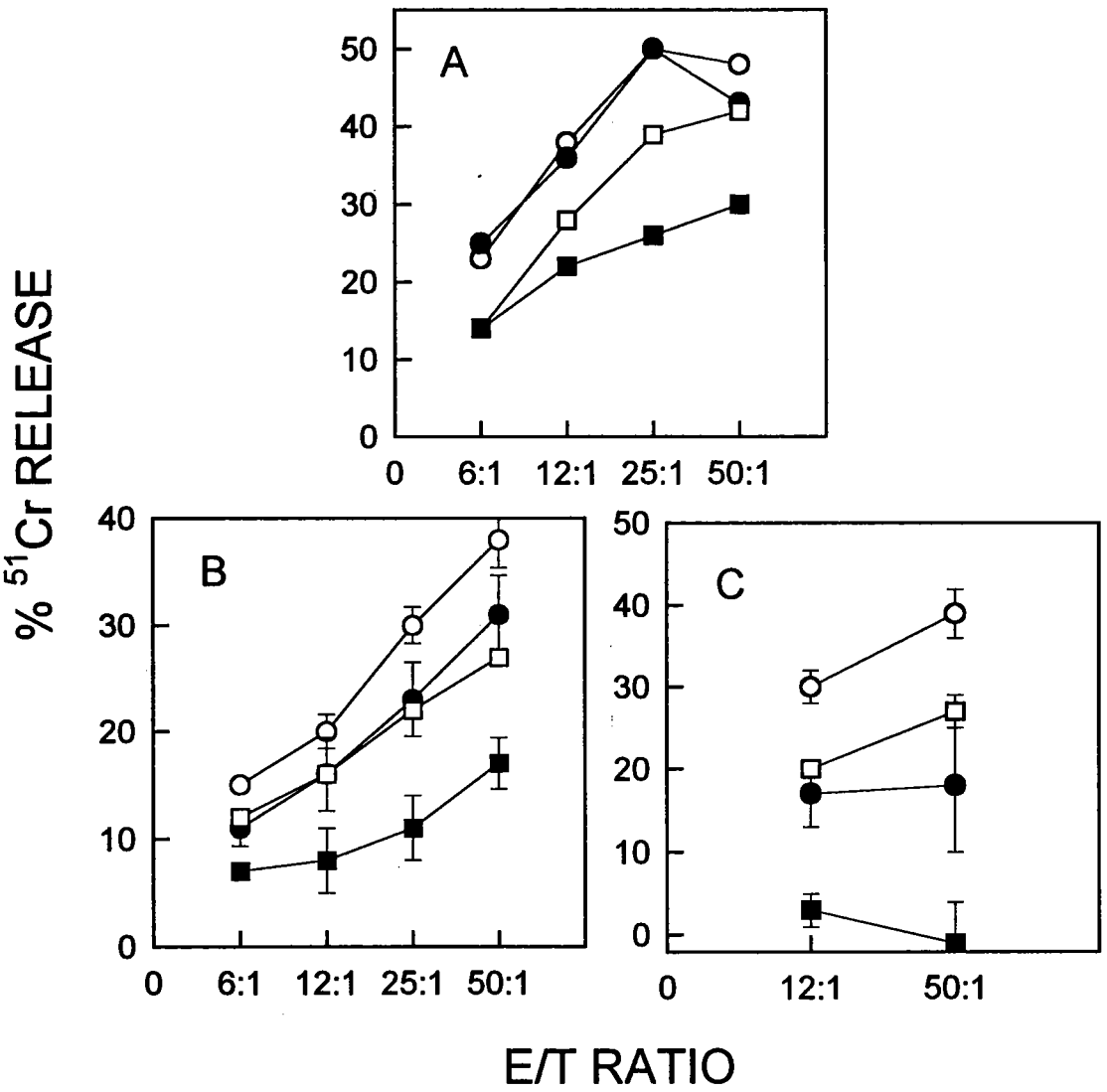

Figure VI-4. Sensitivity of thymocytes to NK cell killing. Thymocytes from uninfected or mock-treated C57BL/6 (+/+) (□), β_2m (-/-) (○) mice and from 3 day virus-infected (LCMV, figure V1-4B; MCMV, figure V1-4C) or 2 day poly I:C-treated (figure V1-4A) C57BL/6 (+/+) (■), β_2m (-/-) (●) mice were harvested and used as targets in a 4-h ^{51}Cr release assay. Effectors were splenocytes taken from day 3 post LCMV-infected C3H/HeSnJ mice.

treatment whereas thymocytes taken from C57BL/6 mice treated with poly I:C displayed a substantial increase in class I expression, as previously shown (Bukowski and Welsh, 1986). These experiments suggest that, similar to the results with β_2m (-/-) cells in Table VI-5, natural β_2m (-/-) thymocyte targets for NK cells are resistant to the protective effects of IFN (and possibly other cytokines) induced by poly I:C.

I. SUSCEPTIBILITY OF THYMOCYTES FROM VIRUS-INFECTED MICE TO LYSIS BY ACTIVATED NK CELLS

Thymocytes from uninfected or virus-infected C57BL/6 and β_2m (-/-) mice were tested for their sensitivity to activated NK cells. All thymocyte targets were relatively resistant to LCMV-induced β_2m (-/-) NK cells, so the data are again presented using spleen leukocytes from LCMV-infected C3H mice as sources of NK cells. Figures VI-4B and C show representative data indicating that infections with LCMV (Figure VI-4B) and MCMV (Figure VI-4C) each caused thymocyte populations from C57BL/6 mice to become considerably more resistant than untreated control thymocytes to lysis by NK cells. In 4 experiments, the LCMV infection caused a 38% $\pm$ 19 reduction in the proportion of C57BL/6 thymocytes lysed, whereas in each of 3 experiments, the MCMV infection resulted in the complete resistance of C57BL/6 thymocytes from NK cell-mediated lysis. Thymocytes from LCMV-infected β_2m (-/-) mice were slightly more resistant than control β_2m (-/-) thymocytes to NK cells, as in 7 experiments there was a reduction in lysis of 21% $\pm$ 13, but within an experiment, this

resistance was significantly less than that of thymocytes from LCMV-infected C57BL/6 mice (Figure VI-4B). Thymocytes from MCMV-infected β_2m (-/-) mice were much more resistant than control β_2m (-/-) thymocytes to NK cells, as, in 3 experiments, there was a reduction in lysis of 48% $\pm$ 14, but they were still significantly less resistant than thymocytes from MCMV-infected C57BL/6 mice, which completely resisted lysis (Figure VI-4C).

Hansson et al have shown that in thymocytes, the cortisone-sensitive $CD4^+CD8^+$ thymocyte subpopulation is the most sensitive to NK cell lysis (Hansson et al., 1979). To determine if the moderate reduction in sensitivity to NK cell killing after MCMV infection was due to a change in the thymocyte distribution, thymocytes from 3 day MCMV- and LCMV-infected β_2m (-/-) mice were stained with anti-CD4 and anti-CD8 mAbs. Thymocyte profiles from MCMV-infected β_2m (-/-) mice revealed that there was a loss of $CD4^+CD8^+$ cells (Figure VI-5 B). This loss of $CD4^+CD8^+$ cells was even more severe in MCMV-infected, anti-aGM_1-treated β_2m (-/-) mice (Figure VI-5 C). However, this change in phenotype and distribution of the thymocyte subpopulations was not apparant after LCMV infection or in LCMV-infected, anti-aGM_1-treated β_2m (-/-) mice (Figure VI-5 E, F). These data suggest that in MCMV-infected β_2m (-/-) mice, the loss of the $CD4^+CD8^+$ thymocyte population may be the cause of the increased resistance to NK cells when compared to uninfected controls.

Data presented here showed that the sensitivity to NK cells by MCMV and the resistance to NK cells by LCMV are unchanged in the β_2m (-/-) mice, although detectable cell surface class I MHC expression is not induced, and the IFN protection mechanism is

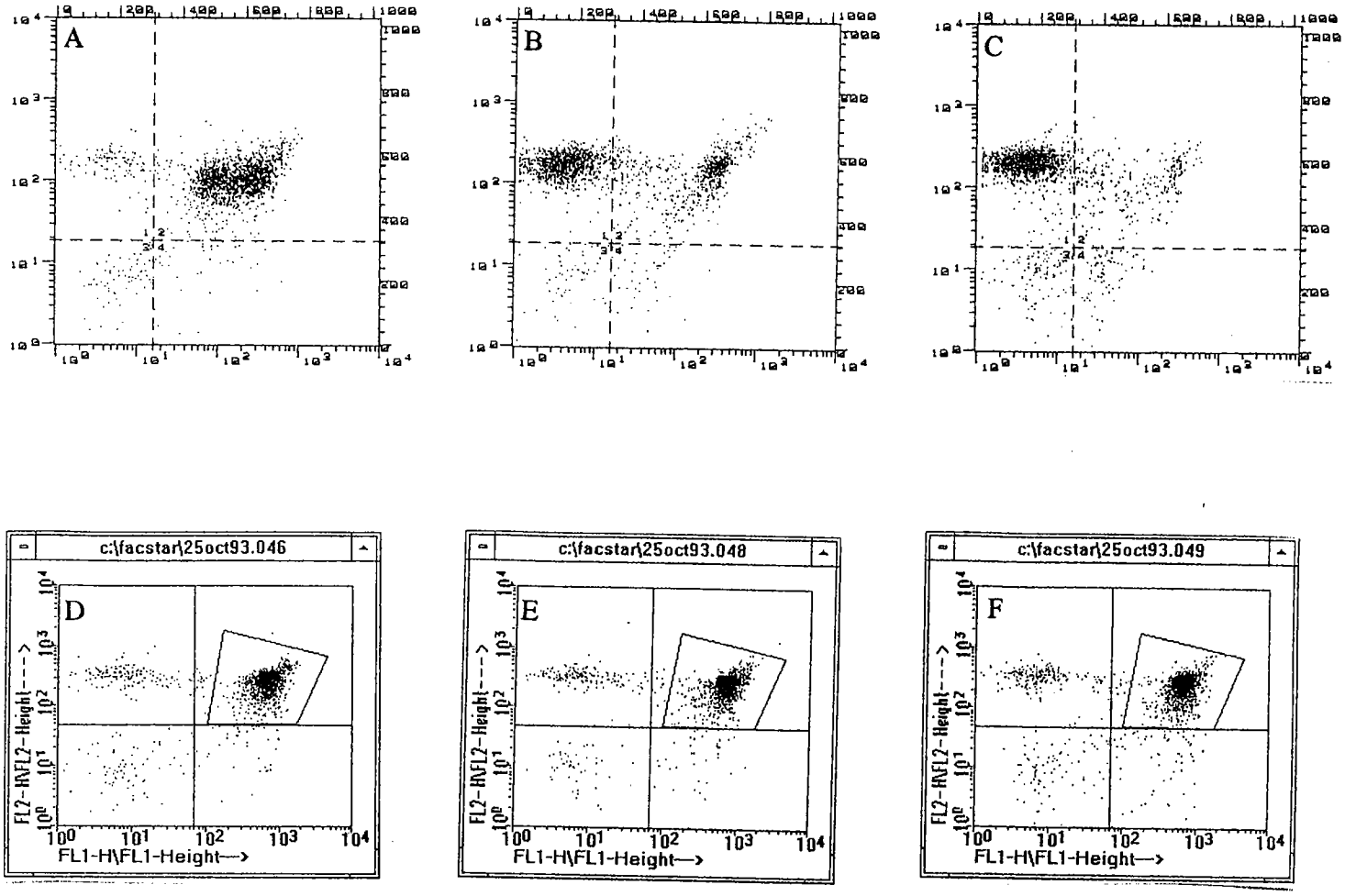

Figure VI-5. Thymocyte FACs profiles from MCMV-infected and LCMV-infected β_2m (-/-) mice. Thymocytes from uninfected (A, D), 3 day MCMV-infected (B), MCMV-infected, anti-aGM$_1$-treated (C), 3 day LCMV-infected (E) or LCMV-infected, anti-aGM$_1$-treated C57BL/6 mice were stained with anti-CD4-PE (y-axis) mAb and anti-CD8-FITC (x-axis).

depressed. Using the adoptive transfer protocol, I provided evidence that adult β_2m (-/-) spleen cells can control MCMV synthesis in C57BL/6 suckling mice, despite the fact that the effector cells and target cells have different levels of MHC class I expression. Taken together, these results suggest that the regulation of MCMV by NK cells is independent on the expression of MHC class I molecules either on the target cells or on the effector cells.

CHAPTER VII

DISCUSSION

My observation that MCMV is controlled in the spleen by a perforin-dependent mechanism and in the liver by an IFN-γ-dependent mechanism is the first demonstration of NK cells using different mechanisms to regulate viral infections in different organs. Some recent work in viral and bacterial systems has suggested that the phenomenon of the same effector cells using different mechanisms to control infections may also be occurring with other immune cells (Lucin et al., 1992; Kagi et al., 1994b; von Herrath et al., 1997). Kagi et al (1994b) have shown that the amount of bacteria controlled by T cells in the spleens of 5 or 8 day *L. monocytogenes*-infected perforin 0/0 mice was significantly higher than in perforin +/+ mice, but the bacterial load in the livers of both strains of mice were similar. Their results indicated that the control of *L. monocytogenes* by T cells in the spleen was perforin-dependent but the authors did not investigate the anti-bacterial mechanisms used by these T cells in the liver. von Herrath et al (1997) reported that within the $CD8^+$ T cells, low affinity LCMV-specific CTLs, and not the high affinity LCMV-specific CTLs required IFN-γ to clear an acute LCMV infection (it is not clear if the low affinity CTLs produce the IFN-γ). The need for IFN-γ by low affinity CTLs to clear an acute LCMV infection may be due to the fact that the high affinity CTLs were highly cytotoxic while the low affinity CTLs were not (measured by cytotoxicity assays). In another system, the

control of MCMV in the different organs of BALB/c mice was dependent on different mechanisms provided by different cell types (Lucin et al., 1992). Here, $CD8^+$ T cells utilized an IFN-γ-independent mechanism to control MCMV in the peripheral organs (spleen and lung), but the control of MCMV in the salivary glands was not dependent on $CD8^+$ T cells. It was instead dependent on IFN-γ produced by $CD4^+$ T cells (Lucin et al, 1992). Kagi et al and von Herrath et al only provided partial evidence that the same effector cells may use different mechanisms to control a pathogen, and Lucin et al showed that there may be a preferential use of one mechanism over another in the control of a pathogen in a particular organ. The work presented in this thesis is the first definitive study showing that at a given time point, the same effector cells can utilize different mechanisms in different organs to effectively control an infection.

In addition to showing that NK cells may use different mechanisms to control MCMV in the spleen and the liver, this report is also the first demonstration of NK cells using perforin to control virus infections. Perforin 0/0 mice had more virus in the spleen compared to perforin +/+ mice 3 days post-MCMV infection, while the liver titers in both groups of mice remained similar. This suggests that the perforin-dependent mechanism is of greater importance in the spleen than in the liver. Because perforin is related to the cytotoxic function of NK cells, this may indicate that the cytotoxic function is important for the control of MCMV in the spleen but not in the liver. Depletion of NK cells in perforin 0/0 mice with anti-NK1.1 mAb resulted only in the increase of liver MCMV titers (Table III-3). Such a result when taken in context with the above mentioned experiments

is consistent with the regulation of MCMV in the spleen being mediated via a perforin-dependent mechanism, and the removal of NK cells in the perforin 0/0 mice should not have affected the splenic titers. However, MCMV in the livers of perforin 0/0 mice was controlled by NK cells via a perforin-independent mechanism, as the removal of NK cells in the mice abrogated the resistance in that organ.

A perforin-independent mechanism that NK cells use to control MCMV infection in the liver is the production of the anti-viral cytokine IFN-γ. During the course of my studies, Orange et al showed that NK cells in C57BL/6 mice synthesize IFN-γ and regulate MCMV in the liver, but they did not report data with the spleen (Orange et al., 1995). My results are in agreement with their work with the liver, but further showed that this cytokine has a negligible anti-MCMV role in the spleen. Depletion of IFN-γ in MCMV-infected C57BL/6 mice with mAbs only resulted in increases in MCMV titers in the liver but not in the spleen. These results together with the data obtained from the perforin 0/0 mice strongly suggest that 3 days post-MCMV infection, NK cells utilized a perforin-dependent mechanism in the spleen and an IFN-γ-dependent mechanism in the liver. IFN-γ may also be important for the control of viruses in the liver by T cells as Guidotti et al have recently reported that in hepatitis B virus (HBV) transgenic mice, the regulation of the virus antigen in the liver is carried out by CTLs via the production of IFN-γ and TNF-α. However the direct lysis of hepatocytes by the CTLs was minimal (Guidotti et al., 1996).

This dichotomy in the mechanisms used by NK cells in the control of MCMV was also seen in IFN-γR$^{0/0}$ mice made chimeric with C57BL/6 bone marrow cells. NK cell-depleted, MCMV-infected chimeric IFN-γR$^{0/0}$ mice had more virus in the spleen but exhibited similar viral titers in the liver when compared to NK cell-intact B6→IFN-γR$^{0/0}$ mice (Table III-5). These data not only reinforced the results obtained from mice treated with anti-IFN-γ Abs, but they also strengthened our hypothesis that NK cells used different mechanisms to control MCMV in spleen and the liver. The host cells of B6→IFN-γR$^{0/0}$ mice did not have the ability to respond to IFN-γ, and the depletion of NK cells from day 3 MCMV-infected B6→IFN-γR$^{0/0}$ mice increased only the splenic but not the liver titers. These results suggest that the virus in the spleen was controlled by NK cells via a IFN-γ-independent mechanism. It was still possible that the increase in titers in the spleens of B6→IFN-γR$^{0/0}$ mice after NK cell depletion could be explained by an IFN-γ-mediated mechanism, as the spleens of these mice became repopulated by the donor immune cells that would have the ability to respond to IFN-γ. However, IFN-γR$^{0/0}$ mice reconstituted with perforin 0/0 bone marrow cells showed no increase in splenic MCMV titers after NK cell depletion, arguing against that interpretation. Also, spleen cells from normal unreconstituted perforin 0/0 mice should have IFN-γ responsiveness, but after NK cell depletion, titers of MCMV in the spleen were not enhanced (Table III-3). It is interesting to note that the depletion of NK cells in B6→129 chimeric mice resulted in increases in both splenic and liver MCMV titers (Table III-5), while depleting the NK cells in *Cmv-1*^{s}, 129 mice only resulted in marginal increases in MCMV titers in both organs.

This suggests that NK cell-dependent factors other than *Cmv-1* are missing or non-functional in the 129 strain. These factors will include a high cytolytic capability of NK cells and NK cell stimulatory cytokines such as IL-12 that induce the production of IFN-γ.

This report is also the first to indicate that the control of MCMV synthesis in the liver is by NO. Treating mice with L-NMA, a competitive inhibitor of NOS, enhanced MCMV titers in the liver but not in the spleen. This set of data adds further evidence that IFN-γ is more important in the liver, as one of the ways IFN-γ can exert its anti-viral actions is through the induction of NOS to produce NO. These results also support the concept that different mechanisms are utilized to control MCMV as the inhibition of NO production did not affect the control of MCMV synthesis in the spleen. Jacobs et al. showed that the resistance to blood-stage malaria in the spleen but not the liver of C57BL/6 mice is dependent on the expression of NO (Jacobs et al., 1995). However, in a 5 day ectromelia virus infection, control of the virus by NO in both the spleen and the liver has been reported (Karupiah et al., 1993b), suggesting that, depending on the pathogen, the protection by NO may be an organ-dependent phenomenon or a general occurrence.

Contrary to our results, Heise et al have shown that in a 7 day MCMV infection of CB17 SCID mice, IFN-γ depletion increased MCMV titers in both the spleen and liver (Heise and Virgin IV, 1995). The CB17 SCID mice used in their analyses were NK1.1$^-$ and *Cmv-1*^{s}, and this might not allow for the dichotomy in mechanisms used in different organs in the regulation of MCMV by NK1.1$^+$, *Cmv-1*^{r} mice. It is also likely that in this lengthier time of infection, the enhanced titers of virus in the liver would seed the spleen

with more virus, eventually leading to higher titers in that organ. Interestingly, it was also reported in the same paper that the depletion of TNF-α resulted in increases in splenic but not liver MCMV titers (Heise and Virgin IV, 1995).

Just as NK cells may use different mechanisms to control viruses in different organs, their efficacy in certain organs may vary considerably. It is noteworthy that NK cells do not effectively control MCMV replication in the lungs after intra-nasal inoculation of the virus, as the lungs of MCMV-infected beige mice or normal mice depleted of NK cells with antibodies had similar MCMV titers as did normal controls (Bukowski et al., 1984).

This documentation of a *Cmv-1*^{r}, perforin-dependent mechanism controlling MCMV replication in the spleen leads to the suggestion that MCMV-infected splenocytes may be lysed by NK cells. Surprisingly little information has been available concerning the direct cytotoxicity of MCMV-infected targets by NK cells. An early report of MCMV-infected fibroblasts being lysed by NK cells in overnight cytotoxicity assays could be accounted for by the activation of the NK cells in the assays rather than by a selective lysis of MCMV-infected cells (Lee and Keller, 1982). Studies designed to examine the intrinsic sensitivity of MCMV-infected fibroblasts to NK cells in short term assays that precluded additional NK cell activation failed to find any indication of increased sensitivity of these targets to lysis (Bukowski and Welsh, 1985b). Target cells in the spleen may be more sensitive to NK cell-mediated lysis than target cells in the liver. Reports on the natural targets of NK cell cytotoxicity *in vivo* have been limited to lymphocytes, lymphoma cells,

and cells of hematopoietic origin, and most demonstrations of selectivities in NK cell recognition and lysis have used lymphocyte targets (Brutkiewicz and Welsh, 1995). It was thus possible that studies on the NK cell sensitivity of MCMV-infected targets had not used the most relevant target. Early work by Wu et al showed that, 3 days post-MCMV infection, T and B cells isolated from blood constituted the major fraction of cells infected with MCMV (Wu and Ho, 1979). Data presented in Chapter III showed that spleen leukocytes including B and T cells were infected with MCMV 3 days post-infection. Also by 3 days post-MCMV infection, normal mice have a 100-fold more virus in the spleen than do SCID mice (Welsh et al., 1991), suggesting that lymphocytes are major targets of MCMV infection in the spleens, and such targets may be good candidates for NK cell killing. As lymphocytes were potential candidates for NK cell cytotoxicity, attempts were made to see if specific killing of MCMV-infected lymphocytes by NK cells could be observed. Routine ^{51}Cr release assays could not be performed using MCMV-infected splenocytes as targets because only a small percentage of the splenocyte population will be infected by MCMV at a given time. Furthermore, splenocytes are not good targets in the cytotoxicity assays as they spontaneously apoptose *ex vivo*. Instead, splenocytes from 3 day MCMV-infected mice were mixed with poly I:C-treated NK cells or LAK cells for 4 hours, and the cells were plated in an infective center plaque assay. However, inconsistent results were obtained using the infective center plaque assay technique and it is difficult to show specific killing of MCMV-infected targets. A collaboration with Dr. E. Tsuda-Szomolanyi using a molecular approach to this question is in progress. DNA from

MCMV-infected splenocytes incubated with normal or perforin 0/0 NK cells is probed with MCMV-specific DNA probes. The rationale is that if there is any specific killing of MCMV-infected cells by NK cells, MCMV DNA would be degraded and there should be a reduction in the signal picked up by the MCMV-specific DNA probes. A preliminary experiment has suggested that there may be lower levels of high molecular weight MCMV DNA from MCMV-infected splenocytes exposed to normal versus perforin 0/0 NK cells. More experiments are needed to confirm this finding.

The lysis of a virus-infected cell requires that the NK cell bind to and be triggered by the target cell. However, if the virus replication can be inhibited by the cytokines produced by activated NK cells, the effector cell may not have to come in contact with the virus-infected target. Target cells in the spleen may be good targets for NK cell recognition and lysis, but hepatocytes, which constitute the major fraction of liver cells infected by MCMV, may either not stimulate the NK cells appropriately or else not respond to the lytic signals delivered by NK cells. Nevertheless, their sensitivity to NK cell-produced factors such as IFN-γ would render them susceptible to the anti-viral functions of NK cells.

This strategy has a few advantages from the viewpoint of the host. The production of IFN-γ in the liver would allow the anti-viral cytokine to reach a maximum number of infected hepatocytes in the shortest amount of time, and this method of virus control may be more efficient than a direct cytotoxic mechanism. Mentioned earlier, the regulation of HBV in HBV-transgenic mice was carried out by IFN-γ and TNF-α produced by HBV-

specific CTL (Guidotti et al., 1996). These anti-viral cytokines selectively degraded the HBV nucleosapsid particles and their replication genomes and destablized the viral RNA (Guidotti et al., 1996). As direct lysis of hepatocytes by the CTL was sufficiently minimal, the host was spared from adverse immunopathological effects brought on by the destruction of the hepatocytes. The same phenomenon may be happening here with regards to MCMV. Rather than destroying the hepatocytes that are harboring the virus, the production of IFN-γ may also be selectively destroying the virus without causing much damage to the cells themselves.

In the above mentioned experiments, only one time point (3 days post-MCMV) and one virus dose (2×10^4 PFU/mouse) was studied. This snapshot in time and the use of one particular dose of the virus inoculum may not allow us to fully appreciate the potential changes in the abilities of NK cells that may occur during the virus infection. It is highly possible that at different time points post-infection, NK cells may utilize both perforin- and IFN-γ-dependent mechanisms to control MCMV in the spleen and the liver. These changes in the mechanisms used by NK cells to control MCMV throughout the infection could be studied by examining different time points post-MCMV infection and measuring the virus titers in the spleens and livers of the different strains of mice used in my earlier analyses. A potential caveat to these experiments is that NK cell activity starts to wane at day 4 post-infection when the specific T cell response increases. This may be due to the production of transforming growth factor β (TGF-β) by T cells as NK cells are very

sensitive to this cytokine and exposure to TGF-β decreases NK cell activity (Su et al., 1991; Su et al., 1993).

My data on the dichotomy in the mechanisms utilized by NK cells when placed in context with the data provided by other investigators (Guidotti et al., 1996; Kagi et al., 1994b; Jacobs et al., 1995) provided a novel way of viewing the abilities of our immune cells in the regulation of infections. These data strongly suggest that the immune system can preferentially use different mechanisms in different organs to combat infectious diseases. These studies caution us that in the use of immunotherapy to treat diseases, we must pay attention to all the potential mechanisms utilized by the effector cells, as well as the target organ we are treating. For example in the treatment of chronic HBV infection of the liver, the use of immune cells that are capable of producing anti-viral cytokines such as IFN-γ and TNF-α may be more beneficial to the host than the use of cells that have a strong cytolytic potential in resolving the infection. A careful dissection of the different abilities of our immune system in conjunction with the target organ may allow us to design better therapies against various diseases.

Suckling mice less than 2 weeks of age can be used as a model to study the role of NK cells in the regulation of MCMV because NK cells take about 3 weeks from birth to reach maturity. In this model, spleen cells or culture purified LAK cells when transferred into the suckling mice protect the mice from a lethal MCMV infection (Bukowski et al., 1985). Depleting the transferred spleen cells with anti-aGM_1 Abs abrogates this resistance, whereas depleting the T cell population before adoptive transfer has no effect on the

transferred cells' ability to protect against MCMV (Bukowski et al., 1985). In the adult mouse/MCMV model, the regulation of MCMV in the spleen is *Cmv-1*- and perforin-dependent, but in suckling mice my results indicate that this innate resistance gene and perforin do not play a role in the regulation of MCMV by adoptively transferred NK cells. Unlike the adult mouse model, adoptive transfers of spleen cells or LAK cells derived from *Cmv-1*^{r} (C57BL/6) or *Cmv-1*^{s} (129) mice significantly lowered splenic MCMV titers in infected C57BL/6 suckling mice. Furthermore, perforin 0/0 spleen cells or LAK cells are also able to regulate MCMV in the spleens of the suckling mice, indicating that the regulation of MCMV by the transferred cells is perforin-independent (Table IV-1). The mechanism used by the transferred cells is IFN-γ-dependent, as normal C57BL/6 spleen cells when transferred into IFN-γR$^{0/0}$ suckling mice failed to regulate MCMV synthesis in the spleen. These results suggest that in the suckling mouse model the regulation of MCMV does not follow the convention seen in adult mice. Adoptively transferred cells, unlike the NK cells in the adult mouse model, utilized a perforin-independent, IFN-γ-dependent mechanism to control MCMV in the spleens of suckling mice.

It is not surprising that NK cells use different mechanisms to control MCMV in the different mouse models. In the suckling mouse model, both the effector cells and the pathogen are introduced into the peritoneal cavity while in the adult model, the effector cells are part of the system before the virus is introduced. Furthermore in the adult model, there may be an influx of NK cells into the peritoneal cavity from the spleen (Table V-1), while in the suckling mouse model, the adoptively transferred NK cells would have to

traffick into the spleen from the peritoneal cavity to control the virus there. As the adoptively transferred cells are present in the suckling mice prior to the introduction of the virus, it is also possible that the control of MCMV may have occurred within the peritoneal cavity, thereby blocking the migration of the virus to the spleen. Early work by Bukowski et al have shown that culture purified $Lyt2^+$ ($CD8^+$) LAK cells and a T cell clone with NK cell-like activity, when adoptively transferred into the suckling mice protect the mice as effectively as culture purified NK cells (Bukowski et al, 1985; Bukowski et al., 1988). These non-NK cells probably also protected the suckling mice via an IFN-γ-dependent mechanism.

Scalzo et al have shown the importance of *Cmv-1*^{r} in the regulation of MCMV by NK cells in the spleen (Scalzo et al., 1990; Scalzo et al., 1992). In all the experiments performed using adult mice, the presence of the *Cmv-1*^{r} gene is a prerequisite for the regulation of MCMV in the spleens of C57BL/6 mice. This organ-dependent genetic resistance parallels the function of perforin, but the genes for *Cmv-1* and perforin are on different chromosomes. It is interesting to note that one of the genetic susceptibility genes of mice to ectromelia virus has also been mapped within the NK gene complex, close to the *Cmv-1* locus, suggesting that *Cmv-1* or a closely related gene/s may also regulate other viral infections (Delano and Brownstein, 1995).

If all NK cells from C57BL/6 mice express *Cmv-1*, it would suggest that all the NK cells have the ability to utilize different mechanisms to control MCMV. Interestingly, the *Cmv-1* gene locus is believed to be closely linked to the Ly49 NK cell receptor family,

raising the possibility that this MCMV innate resistance gene is an existing or yet undefined member of the Ly49 family (Brown et al., 1997). Another potential way NK cells could utilize different mechanisms to control MCMV in different organs is by using the different subsets of NK cells to home to different organs and control MCMV synthesis in that organ. It is possible that subsets of NK cells that traffick to the spleen may control MCMV in the spleen via a perforin-dependent mechanism and other NK cell subsets that home to the liver may utilize an IFN-γ-dependent mechanism to control the virus there.

There were fewer NK1.1$^+$ cells in the spleen and more NK1.1$^+$ cells in the peritoneal cavity at 3 days post-MCMV infection (Table V-1). However, these changes in NK1.1$^+$ cell numbers in the spleen and peritoneal cavity were not apparant during LCMV infection, where splenic NK1.1$^+$ cell numbers per 10^4 lymphocytes did not fluctuate between uninfected and infected mice (Table V-1). In 3 different experiments, the change in NK1.1$^+$ cell numbers in the peritoneal cavities of LCMV-infected mice was at best erratic (Table V-1). As intraperitoneal MCMV infection resulted in increases in both the absolute number of PEC and also NK1.1$^+$ cells in the peritoneal cavity. These results strongly indicate that there is trafficking of NK cells to the sites of infection.

An examination of the different Ly49 subsets also revealed a change in the distribution of the proportion of NK1.1$^+$ cells after MCMV infection. In the spleen, even though MCMV did not cause any apparant change in the absolute number of splenocytes (data not shown), the virus infection caused an increase in the percentage of Ly49A and Ly49G2 NK cell subsets but a decrease in the percentage of Ly49C and Ly49D NK cell

subsets. Interestingly, these changes in the proportion of NK cell subsets within the spleen are accompanied by decreases in the percentages of the Ly49A NK cell subset and increases in the percentages of Ly49C, D and G2 NK cell subsets in the peritoneal cavity. As there is a consistent pattern in the changes of the Ly49 subsets during MCMV infection but not during LCMV infection, the different Ly49 NK cell subsets may play different roles in the regulation of MCMV *in vivo*. This is the first demonstration that a virus infection can influence the different Ly49 NK cell subsets to shift in proportions between different organs.

Despite the redistribution of the tested Ly49 NK cell subsets during MCMV infection, *in vivo* depletion of any one of the 4 tested Ly49 subsets, or the adoptive transfer of any one of the 4 Ly49 NK cell subsets does not affect the ability of the NK cells to control MCMV. To date, there are 8 cloned Ly49 NK cell receptors but there are only antibodies available to 5 of the receptors. The anti-Ly49D Ab (12A8) used in the above *in vivo* depletion and adoptive transfer studies is cross-reactive with Ly49A NK cell receptor such that the depletion of Ly49D-bearing cells will also cause the depletion of $Ly49A^+$ cells (Mason et al., 1996). Depleting cells with anti-Ly49G2 (4D11) Abs will not only deplete the $Ly49G2^+$ cells but will also inadvertently deplete some $Ly49A^+$ NK cells as well (Mason et al., 1995). This is because approximately 11% of all NK cells express both Ly49A and Ly49G2 NK cell receptors (Mason et al., 1995). However, the cross reactivity of anti-Ly49D Ab and the co-expression of Ly49A and Ly49G2 on some NK cells should not affect our understanding in the ability of the different Ly49 subsets, as the

Ly49A NK cell subset only constitute about 15 - 17% of all NK cells. Furthermore, depletion of the Ly49A NK cell subset or the adoptive transfer of the Ly49A$^+$ or Ly49A$^-$ LAK cells alone did not affect the ability of the NK cells to control MCMV *in vivo*.

Recently the anti-Ly49C Ab (SW5E6) used in my analyses was found to bind not only with Ly49C but also with Ly49I NK cell receptor. Initial studies have indicated that Ly49C$^+$ NK cells mediate the rejection of H-2^d parental bone marrow cells in F1 (b x d) mice (Yu et al, 1996). However, recent antibody binding data show that a new antibody 4LO3311 binds to Ly49C$^+$ cells in both C57BL/6 and BALB/c mice, and amino acid analyses have indicated that the Ly49C NK cell receptor in both strains of mice are identical (Takei et al., 1997). Furthermore, Ly49C NK cell receptors interact with and are inhibited by both H-2^b- and H-2^d-bearing cells. These data suggest that in hybrid resistance, it is the Ly49I$^+$ NK cells that mediate the rejection of H-2^d bone marrow cells (George et al., 1997). Nonetheless, results from the *in vivo* depletion of Ly49C- and Ly49I-bearing cells (using SW5E6, Table V-4), or the adoptive transfer of Ly49C$^+$, Ly49I$^+$ or Ly49C$^-$, Ly49I$^-$ LAK cells (Table V-5) again strongly indicate that the absence or the presence of a particular Ly49 NK cell subset does not affect the ability of NK cells to control MCMV.

The Ly49D and Ly49H NK cell receptors are the only members of the Ly49 multigene family to date that do not contain the ITIM motif in its cytoplasmic tail (Mason et al., 1996; Ryan and Seaman, 1997). Ly49D$^+$ NK cells have been shown to have the ability to lyse tumor cells and Con A blasts of different H-2 haplotypes, and mediate

reverse ADCC through anti-Ly49D Ab on FcγR$^+$ target cells (Mason et al., 1996). These data strongly suggest that Ly49D is a putative activatory receptor, not unlike NK1.1, and therefore may have an important role in the regulation of MCMV. However depleting the Ly49D$^+$ NK cell subset or transferring Ly49D$^-$ NK1.1$^+$ LAK cells into MCMV-infected suckling mice did not affect the NK cells' ability to control MCMV (Table V-4, Table V-5). Taken together, the Ly49 NK cell receptor data provide strong evidence that the none of the 4 Ly49 NK receptors are essential in the regulation of MCMV.

For the suckling mouse model to be useful in testing the importance of Ly49 NK cell receptors in the regulation of MCMV, the NK cells have to come in contact with the target cells. This process is crucial, or the suckling mouse model cannot be used to study the interactions of cell surface molecules and their importance in the control of MCMV. Indeed there is a report that NK cell recognition of targets is a necessary factor contributing to cytokine release (i.e. the release of IFN-γ) (Bellone et al., 1993). However, if cytokines like IL-12 and TNF-α alone can stimulate NK cells to produce anti-viral cytokines (Orange and Biron, 1996a; Orange and Biron, 1996b), this may preclude the need for NK cells to come in contact with the target cell. If the latter case is true, the suckling mouse model will not be useful in the study of cell to cell interactions and the potential roles they may play in the control of MCMV.

The evidence presented in chapter V strongly suggest that *Cmv-1* is probably not one of the tested Ly49 NK cell receptors. However, this evidence does not rule out the possibility that *Cmv-1* may be one of the undefined Ly49 receptors. If *Cmv*-1 is one of the

Ly49 family, it could be either an inhibitory receptor like Ly49A, C or G2, or an activatory receptor like Ly49D. Let's for a moment consider *Cmv-1* as an inhibitory receptor. The inhibitory receptor *Cmv-1* upon ligation with its ligand will send a negative signal to the NK cell, preventing the NK cell from killing its target. If *Cmv-1* is an inhibitory receptor, this receptor has to be found in the BALB/c strain as BALB/c mice are MCMV-sensitive (Scalzo et al., 1990). The MCMV-resistant strain C57BL/6 will either possess a different allelic form of *Cmv-1* that does not recognize the ligand or does not express *Cmv-1* at all. One potential ligand for such an inhibitory receptor is the viral MHC class I homologue of MCMV. This viral class I homologue has been implicated to inhibit NK cell function (Farrell et al., 1997), and if *Cmv-1* is an inhibitory receptor, BALB/c *Cmv-1* may interact with this viral homologue and shut down the NK cell's ability to control the virus. On the other hand, if *Cmv-1* is an activatory receptor like Ly49D or NKRP1, the yet identified *Cmv-1* gene product may be a receptor molecule associated with the cytotoxic function of NK cells. It is possible that *Cmv-1* might encode a receptor that recognizes MCMV-infected cells or even MCMV itself. Such an activatory NK cell receptor has to be found in the C57BL/6 strain but absent or exist in a different allelic form in BALB/c mice. The cloning and identification of the *Cmv-1* gene product will permit us to dissect this mystery.

The interactions between MHC class I molecules and NK cell receptors dictates the functions of the NK cells. This coupled with the fact that some viruses can induce changes in class I molecules has allowed investigators to hypothesize that NK cells may be able to selectively lyse virus-infected targets (Bukowski et al., 1985; Bukowski and Welsh, 1985b;

Storkus and Dawson, 1991; Brutkiewicz et al., 1992; Welsh and Vargas-Cortes, 1992). Virus-induced modulation of MHC class I molecules could be due to a direct virus-induced inhibition in MHC gene transcription, translation, or post-translational processing, or it could be a consequence of viruses interfering with the actions of regulatory cytokines. IFN, which induces the upregulation of MHC antigens, protects targets from NK cell-mediated lysis, and much of this protection correlates with the ability of IFN to upregulate class I MHC antigens (Trinchieri and Santoli, 1978; Welsh et al., 1981; Ljunggren and Karre, 1990; Ljunggren et al., 1990). Cells lacking normal class I MHC expression as a consequence of a β_2m deficiency cannot be protected by IFN, but protection can be restored by transfection with an intact β_2m gene (Ljunggren and Karre, 1990; Ljunggren et al., 1990). It has been proposed that a selective protection of normal but not virus-infected cells would allow NK cells in the host to selectively lyse virus-infected cells and control viral infections without any requirement for specific recognition of virus-infected targets (Trinchieri and Santoli, 1978; Santoli and Koprowski, 1979). This original hypothesis, which was based on early work showing that human fibroblasts infected with certain viruses could not be protected against NK cells by IFN (Trinchieri and Santoli, 1978), received substantial support by a variety of studies in the mouse with MCMV and LCMV (Bukowski et al., 1983; Bukowski et al., 1985; Bukowski and Welsh, 1985a; Bukowski and Welsh, 1985b; Bukowski and Welsh, 1986). These studies established that viral infections induced marked elevations of class I MHC antigens *in vivo* (Bukowski and Welsh, 1986), and that cells (thymocytes) isolated from virus-infected mice resisted lysis by NK cells as they became more susceptible to lysis by CTL (Hansson et al., 1980; Bukowski and Welsh,

1986). MCMV and LCMV were shown to be NK cell-sensitive and -resistant viruses, respectively, *in vivo*, but a selective killing of MCMV-infected cells *in vitro* could not be shown (Bukowski et al., 1983; Bukowski and Welsh, 1985a; Bukowski and Welsh, 1985b; Bukowski and Welsh, 1986). IFN, however, protected LCMV- but not MCMV-infected fibroblasts from lysis by activated NK cells *in vitro* (Bukowski and Welsh, 1985b), a result consistent with the original hypothesis (Trinchieri and Santoli, 1978; Santoli and Koprowski, 1979).

The results in Chapter VI challenge the hypothesis, as the anti-viral functions of NK cells in the β_2m (-/-) mice were similar to those in normal mice. Thus, normal expression of class I molecules *per se*, on either the target cell or the NK cell, is not required for the anti-viral properties of NK cells. The data do not address the question of whether a virus infection must down-regulate class I MHC expression in order to be sensitive to NK cells, because the class I expression is already down-regulated. These results do, however, argue against the concept that a selective IFN-induced protection of uninfected cells via the upregulation of MHC class I molecules focuses the NK cell response onto virus-infected targets which resist IFN-mediated protection. The hypothesis that LCMV is an NK-resistant virus because IFN-induced upregulation of class I MHC antigens protects them from NK cells is also contradicted by the data. These results also challenge the hypothesis that IFN-protection of uninfected cells via the upregulation of class I MHC prevents serious auto-destructive tissue injury during infection. Splenocyte or thymocyte numbers in LCMV-infected β_2m (-/-) mice were not altered by NK cell depletion, indicating that NK cells were not lysing significant numbers of these leukocytes

during infection (Figure VI-5). The cytotoxic capacity of the virus-induced β_2m (-/-) NK cells was relatively weak against any target other than YAC-1 cells, and this may have precluded a significant self-destructive response. Alternatively, by being educated in a β_2m (-/-) environment, the β_2m (-/-) NK cells may have a recognition system distinct from β_2m (+/+) NK cells and may detect subtle differences in the low levels of cell surface MHC class I expression on β_2m (-/-) cells (Hoglund et al., 1991).

NK cells from β_2m (+/+) mice made chimeric with β_2m (-/-) bone marrow cells did not kill β_2m (-/-) Con A blasts, suggesting that the education of NK cells by cells of hematopoietic origin dictates the functions of the mature NK cells (Hoglund et al., 1991). Even though NK cells from β_2m (-/-) mice do not kill β_2m (-/-) Con A blasts, these cells are able to lyse TAP-deficient RMA-S cells more efficiently than the parental RMA cells (Hoglund et al., 1997). In fact, β_2m (-/-) NK cells can distinguish between EL-4.β_2m (-/-) cells (these cells express 20 - 40% of MHC class I molecules compared to the wildtype) and EL-4.β_2m (+/+) by lysing the former (Hoglund et al., 1997). The education of NK cells can be explained by using the Receptor Calibration Model (Sentman et al., 1995; Hoglund et al., 1997). The model makes the assumption that NK cells once triggered by activating receptor/ligand interactions require a certain amount of inhibitory interactions to occur, which will lead to the turning off of the lytic program and release of the effector cells from the target. This model depends on at least 3 factors: a. the concentration of the receptors on the NK cell surface, b. the concentration of MHC class I molecules on the target cell surface and c. the affinity of the interactions between the receptors and MHC

class I molecules. Studies on the Ly49 NK cell receptors have indicated that β_2m (-/-) NK cells have a higher percentage of Ly49A, C and G2 receptors than C57BL/6 mice (Dorfman Jr. et al., 1996; Salcedo et al., 1997). These data suggest that the higher levels of NK cell receptors in β_2m (-/-) mice is to be able to recognize the lower levels of MHC class I molecules. Therefore, β_2m (-/-) NK cells can kill EL-4.β_2m (-/-) cells but not β_2m (-/-) con A blasts, because the latter have higher levels of MHC class I molecules. Alternatively, there may be no calibration of the NK cell receptors, because the class I levels in β_2m (-/-) mice are too low to affect the NK cells. If this is the case, β_2m (-/-) NK cells do not kill β_2m (-/-) Con A blasts because there may be fewer activatory ligands on the cells. These two possibilities can be tested by using NK cells and target cells from TAP$^-$/β_2m (-/-) (undetectable class I molecules), β_2m (-/-) (barely detectable class I) and TAP$^-$ (detectable class I albeit at 90% reduction compared to the wildtype) mice to test if NK cells are calibrated according to the amount of class I molecules found within the education compartment.

β_2m (-/-) adult spleen cells when adoptively transferred into C57BL/6 suckling mice did protect the suckling mice from MCMV, suggesting that the education of NK cells was not essential in the control of MCMV. However the same concerns voiced in the section where the suckling mouse model was used to study Ly49 NK cell receptors apply in this analysis too. Until better experimental approaches are developed, or the question of whether cell to cell contact is necessary for the production of anti-viral cytokines by NK

cells is clarified, the issue of NK cell education in the control of MCMV will remain unresolved.

Thymocytes isolated from poly I:C-treated β_2m (-/-) mice were considerably more sensitive to NK cells than were thymocytes from treated C57BL/6 mice, a result consistent with the hypothesis that IFN-mediated protection *in vivo* was dependent on expression of MHC class I antigens (Figure VI-4). Thymocytes isolated from virus-infected β_2m (-/-) mice were, however, somewhat more resistant to lysis than those from poly I:C-treated mice, even though they did not upregulate MHC class I antigens (Table VI-4). We do not know the explanation for this resistance, but it could involve changes in thymocyte distribution occurring as a consequence of viral infection. The thymocyte number in MCMV-infected C57BL/6 and β_2m (-/-) mice was markedly reduced, and there was a severe depletion in the $CD4^+CD8^+$ subset (Figure V1-5, Brutkiewicz, 1993). In anti-aGM_1-treated, MCMV-infected β_2m (-/-) mice, there was a further loss of the $CD4^+CD8^+$ subset (Figure VI-5). We believe that this severe loss in the double positive thymocyte population was not due to the cytotoxic capacity of the NK cells but was instead due to the increased replication of the virus after NK cell-depletion. Furthermore, glucocorticoids produced by the host during virus infections may eliminate the cortisone-sensitive $CD4^+CD8^+$ thymocyte population, and this thymocyte subpopulation is shown to be most sensitive to NK cell lysis (Hansson et al., 1979). Infection of mice with the NK cell-resistant LCMV did not affect the phenotype or thymocyte number (Figure V1-5). This may be due to the differences in cytopathicity between the 2 viruses; MCMV being highly cytopathic while LCMV is not. Nonetheless, it remains possible

that some protection of the target cells *in vivo* was due to mechanisms other than IFN-mediated upregulation of class I MHC antigens (Routes, 1992). Thus, although MCMV is a highly sensitive virus to NK cells *in vivo*, fibroblast targets infected with MCMV *in vitro* do not show enhanced sensitivity to NK cell-mediated lysis (Bukowski and Welsh, 1985b), and the data presented here are inconsistent with the concept of a selective funneling of NK cells toward tissues not expressing class I MHC.

Other than MHC class I molecules, there undoubtedly are many NK cell ligands that remain undefined and could play a role in NK cell interactions with virus-infected targets. An earlier study with human NK cell clones showed that some clones lysed varicella-zoster virus-, HCMV- or VV-infected targets while others did not, suggesting the possibility of some NK subset selectivity in recognizing virus-infected targets (Mason et al., 1993). Recent studies using cloned human NK cells have shown similar selectivity against human herpes virus 6 (HHV-6)-infected cells (Malnati et al., 1993), but the ability of the NK clones to lyse these infected cells was thought not to depend on MHC class I expression, as there was little difference in class I levels between HHV-6-infected and the uninfected cells (Malnati et al., 1993). These results suggest that other cell surface elements can restrict NK cell recognition. The rat NKR-P1 can bind oligosaccharide ligands (this has yet to be confirmed by other investigators) and the mouse NK receptor Ly49A is a C-type lectin that has a functional carbohydrate recognition domain (Bezouska et al., 1994; Daniels et al., 1994). NK cell receptors may therefore recognize the carbohydrate or sugar motifs on the class I molecules or even on other proteins.

Substantial earlier work had indicated that purified glycoproteins from mumps, measles, influenza, and other viruses can augment the cytolytic activity of NK cells *in vitro* and *in vivo* (Harfast et al., 1980; Casali et al., 1981; Arora et al., 1984; Arora and Houde, 1988); it is not known whether these glycoproteins or their sugar moieties interact with the defined NK receptor molecules.

Cell surface molecules other than MHC class I may act as NK cell-triggering molecules. Costimulatory molecules expressed on the target cells may activate NK cells to lyse the target. NK cells have long been implicated in the outgrowth of B cells transformed by EBV, and EBV has recently been shown to upregulate the costimulatory molecule CD80 (B7-1) on infected cells (Montel et al., 1995). In fact this upregulation of B7-1 molecules on EBV-infected cells enhanced their susceptibility to lysis by a human NK variant cell line that expresses CD28 (Montel et al., 1995). Recently, costimulatory molecules were shown to play an important role in the regulation of parasitic infections in SCID mice. Infection of SCID mice with *T. gondii* resulted in the expression of CD28 on NK cells, accompanied by the production of IFN-γ by the NK cells and increase in NK cell cytolytic activity. *In vivo* administration of CTLA4-Ig, which blocks the interaction between CD28 and B7 into *T. gondii*-infected SCID mice, resulted in a significant increase in parasitic load, demonstrating the importance of this costimulation through B7 in resistance to *T. gondii* (Hunter et al., 1997). Another recent article also suggests that mouse NK cells can be activated by B7-1 (Chambers et al., 1996), but this activation is reported not to occur through B7-1/CD28 or B7-1/CTLA-4 interactions, implicating a

third receptor for B7-1. Interestingly, in this article the presence of MHC class I molecules on the target cells does not inhibit their susceptibility to NK cell killing as long as the targets express B7-1. These results suggest that B7-1 expressed on virus-infected cells may trigger the NK cells to lyse the virus-infected targets, and that the expression of class I molecules is inconsequential to the outcome.

The results described in this thesis have given us a better understanding about the mechanisms utilized by NK cells and the roles played by NK cell receptors and MHC class I molecules in the regulation of MCMV. Listed below are the important observations made in this thesis:

1. In the early regulation of MCMV infections in adult C57BL/6 mice, NK cells utilized different mechanisms to control the virus in the spleen and the liver. In the spleen, a perforin-dependent mechanism was used, while an IFN-γ-dependent, NO-mediated mechanism was used in the liver. This dichotomy in the mechanisms used by NK cells to control MCMV was dependent on the expression of the *Cmv-1*^{r} gene found within the NK gene complex of the mouse.

2. Unlike the regulation of MCMV infection in adult C57BL/6 mice, adoptively transferred NK cells control MCMV in the spleens of suckling mice via an IFN-γ-dependent mechanism.

3. There is a redistribution of NK cell subsets between the spleen and the peritoneal cavity 3 days post-MCMV infection. Per 10^4 lymphocytes, there is an increase in the percentage of $Ly49A^+$ and $Ly49G2^+$ NK cell subsets and a decrease in the percentage of $Ly49C^+$ and $Ly49D^+$ NK cell subsets in the spleen. This is accompanied by a decrease in percentage of $Ly49A^+$ NK cell subset and increases in the percentages of $Ly49C^+$, $Ly49D^+$ and $Ly49G2^+$ NK cell subsets in the peritoneal cavity.

4. Despite the change in the distribution pattern of the Ly49 receptors 3 days after MCMV infection, NK cells in C57BL/6 mice retain the ability to control MCMV in the absence of Ly49A-, Ly49C-, Ly49D- or Ly49G2-bearing NK cells.

5. Normal expression of MHC class I molecules on NK cells or their targets is not required for the anti-viral functions of NK cells against a NK-sensitive virus (MCMV) nor do they protect a NK-resistant virus (LCMV) from the anti-viral activity of NK cells.

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