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Cytomegalovirus Evasion of Natural Killer Cell Immunity

by

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DISSERTATION

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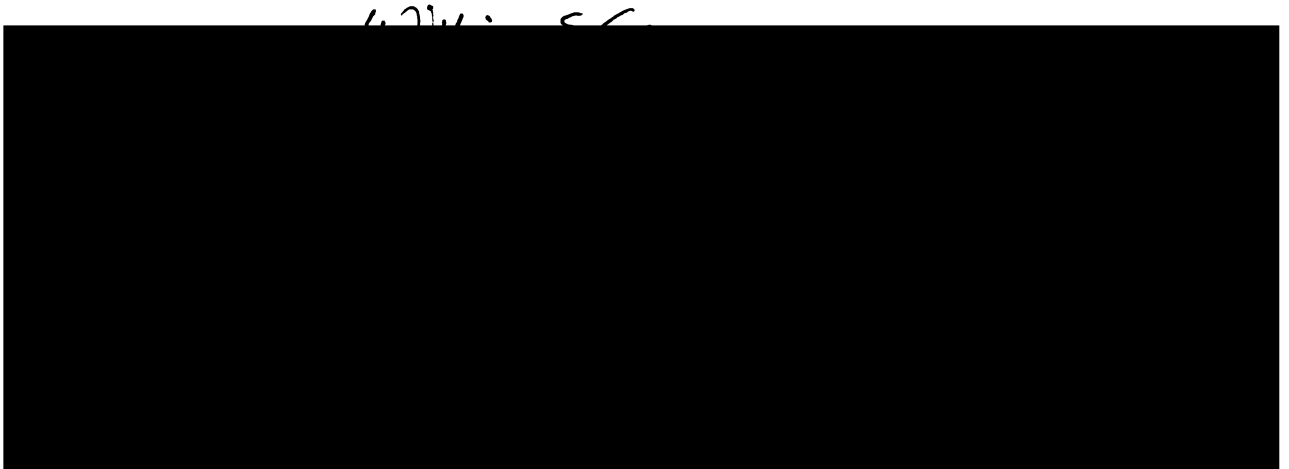

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Contributions of Co-Authors to the Presented Work

Chapter 3 of this dissertation is based on material published in *The Journal of Experimental Medicine* (2003) 197:1245-1253, titled, "NKG2D-Mediated Natural Killer Cell Protection Against Cytomegalovirus Is Impaired by Viral gp40 Modulation of Retinoic Acid Early Inducible 1 Gene Molecules," by copyright permission of The Rockefeller University Press. The following co-authors contributed helpful advice or reagents to this work: Kouetsu Ogasawara, Jessica A. Hamerman, Hisashi Arase, Jeffrey P. Houchins (R&D Systems), Edward S. Mocarski (Stanford University). This work was supervised by Lewis L. Lanier.

Chapter four is based on data from a manuscript that is currently in press at *The Journal of Experimental Medicine*, titled, "The Cytomegalovirus m155 Gene Product Subverts NK cell Antiviral Protection by Disruption of H60-NKG2D Interactions." This work was done in collaboration with the lab of Fenyong Liu at the University of California, Berkeley. The Liu lab provided mutant viruses; in addition, Gerardo Abenes and Sean Umamoto undertook the in vivo MCMV infection experiments. Jeffrey P. Houchins (R&D Systems) provided antibodies against the NKG2D ligands. This work was supervised by Lewis L. Lanier.

Cytomegalovirus Evasion of Natural Killer Cell Immunity

by

Melissa Lodoen

Lewis L. Lanier, PhD

Abstract

Cytomegaloviruses (CMV) are characterized by the ability to persist in individuals despite an active host immune system. This is accomplished through the activity of several gene products committed to evasion of the immune response. Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) are important mediators of host immunity to CMV infection, but they fail to eliminate the virus, and the result is a chronic infection kept under immune control. The ability of CMV to alter the CTL response to the virus has been well studied, and many mechanisms of viral immune evasion have been identified. Viral modulation of NK cell activity, however, is only beginning to be appreciated.

We examined whether CMV infection impacts expression of ligands for a potent activating NK cell receptor, NKG2D, as a means of escape from NK cell immune surveillance. We found that both human CMV and murine CMV infection induced

transcription of ligands for NKG2D. However, these ligands were down-regulated from the surface of virus-infected cells. We subsequently identified and studied two viral proteins that are responsible for the down-regulation of NKG2D ligands on infected cells, and we have defined the precise ligands that are affected. The MCMV m152 gene product specifically down-regulated the cell surface expression of all RAE-1 proteins (RAE-1α, β, δ, ε, γ), but not H60 or MULT-1, the MCMV m155 gene product down-regulated H60, and an unknown gene product down-regulated MULT-1. In addition, we addressed whether viral down-regulation of NKG2D ligands influences the ability of NK cells to control CMV infection. We found that the activity of these immune evasion proteins impaired NKG2D-mediated NK cell antiviral activity, both in vitro and in vivo, by preventing NKG2D from interacting with its ligands. MCMV mutant viruses lacking either m152 or m155 were attenuated in the livers and spleens of BALB/c mice, compared to wild-type virus. However, treatment with neutralizing anti-NKG2D mAb restored virulence of the mutant viruses to wild-type virus levels. Thus, down-regulation of NKG2D ligands by CMV is a powerful mechanism of inhibiting NKG2D-mediated NK cell protection.

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Chapter One

An Introduction to Cytomegalovirus and Natural Killer Cells

The immune response to viral infections is comprised of a complex series of interactions involving immune cells, cytokines and virus-infected cells. The principal lymphocytes in the anti-viral response are natural killer (NK) cells and cytotoxic T lymphocytes (CTL). Both of these cell types respond to viral infection by secretion of inflammatory mediators and by direct cytolysis of infected cells. However, their activities are temporally distinct: NK cells function during the early, innate stage of the immune response, whereas CTL play a critical role during the adaptive phase of the response. Concurrent with the activation of immune cells during infection, viruses express several immune evasion molecules that help the virus elude detection by NK cells and CTL. Thus, studies on antiviral immunity and viral immune evasion strategies contribute to a more thorough understanding of the intricate relationship between pathogens and their hosts.

Cytomegalovirus

Cytomegaloviruses (CMVs) are large, enveloped viruses enclosed in an icosahedral capsid. They are members of the β Herpesvirus family and have double-stranded DNA linear genomes of roughly 230kb, encoding over 200 open reading frames (ORFs) (1, 2). Although the human and murine CMV genomes do not share significant homology by nucleotide sequence, they are colinear in the central region of the genomes (2). HCMV and MCMV also share similar biological characteristics and pathology, making MCMV a commonly used model to study HCMV infection (reviewed in (3)).

CMV is highly prevalent in populations and often infects individuals at a young age. Transmission of CMV occurs through body secretions, such as saliva or breast milk,

from infected individuals. Acute infection in healthy individuals is typically asymptomatic and occurs predominantly in the spleen and liver, with viral dissemination throughout the body (reviewed in (4)). Following the acute stage of infection, CMV transitions into a latent state, during which only a small set of genes is expressed. CMV latency occurs in the acinar cells of the salivary glands, which are poised for secretion when the virus reactivates from latency. Periodic reactivation and shedding of CMV are also asymptomatic in healthy individuals, which allows the virus to remain undetectable to those transmitting or receiving it. Like other members of the Herpesvirus family, CMV persists in infected individuals for life, without causing overt disease. Primary infection or virus reactivation in immunocompromised individuals, however, can lead to severe disease or death, thereby reinforcing the importance of the immune system in controlling the virus. In particular, NK cells are important in the control of both HCMV and MCMV infection.

Like other members of the herpesvirus family, which have large genomes, CMVs are characterized by an impressive array of immunomodulatory mechanisms. Both human and murine CMV encode several glycoproteins that are involved in down-regulation of major histocompatibility (MHC) class I molecules on the surface of infected cells (5, 6). Loss of MHC class I from the cell surface potentially allows escape of virus-infected cells from detection by cytotoxic T lymphocytes (CTL), which rely on signals between MHC class I and the T cell receptor for activation. These viral MHC class I modulators function by a diverse set of mechanisms at distinct stages in MHC class I biosynthesis.

Murine CMV (MCMV) has three gene products, encoded by *m04*, *m06* and *m152*, which inhibit expression of MHC class I on infected cells (7). Interestingly, the products of the *m04*, *m06* and *m152* genes are not homologous to the human CMV (HCMV) class I modulators, US2, US3, US6 and US11. In addition, they down-regulate MHC class I expression by distinctly different mechanisms, suggesting that they evolved independently. gp34, encoded by *m04*, complexes with class I in the ER, is transported to the cell surface, and can inhibit CTL activity in vitro (8, 9). gp48, the product of the *m06* gene, binds to class I molecules in the ER and redirects them to the lysosome for degradation (10). *m152* encodes gp40, which is involved in retention of class I complexes in the ER to Golgi intermediate compartment (ERGIC) (11), and is a potent inhibitor of virus-specific CTL activity in vitro (12). The products of the HCMV *US2* and *US11* genes reverse the translocation of human class I heavy chains from the ER back to the cytosol through the Sec61 channel, ultimately resulting in their degradation by the proteasome (13, 14). In contrast, through a transient association with MHC class I, the US3 gene product prevents forward transport and maturation of MHC class I through retention in the ER (15, 16). The HCMV US6 gene product inhibits antigen presentation by preventing translocation of peptides into the ER for loading on MHC class I (17, 18). Although MHC class I down-regulation can interfere with $CD8^+$ T cell-mediated lysis of virus-infected cells, these cells may be more susceptible to NK cell cytotoxicity, since NK cells preferentially kill cells with low MHC class I expression ("missing self hypothesis") (19).

NK Cells and their Function

NK cells originate in the bone marrow and are derived from a common lymphoid progenitor. They make up roughly 5-10% of the total lymphocyte population and are predominantly found in the blood, spleen and liver. Unlike B and T cells, which express a canonical B or T cell receptor that undergoes extensive rearrangement, NK cells do not express rearranged antigen receptors. Rather, the NK cell surface is decorated with a myriad of activating and inhibitory receptors, and the balance of signals received through these receptors dictates NK cell activity. In terms of receptor repertoire, NK cells are a heterogeneous population, with different NK cell subsets expressing different combinations of receptors (20). However, all NK cells express at least one inhibitory receptor, which engages major histocompatibility class (MHC) I. Because all nucleated cells in the body express MHC class I, and are therefore capable of engaging an NK inhibitory receptor, these receptors may exist to prevent autoimmunity mediated by NK cells. The absence of inhibitory MHC class I on tumor or virus-infected cells, though, can result in NK cell activation. This observation led to the "missing self" hypothesis, which proposed that NK cells can become activated through loss of signaling from inhibitory receptors (19). The ligands for NK cell activating receptors are less well defined than those for inhibitory receptors. Some activating receptors bind to glycoproteins induced by cellular stress, such as transformation or viral infection, whereas others recognize pathogen components that are expressed on the surface of infected cells, and others remain orphan receptors with no defined ligand (21).

Because NK cell activation does not require receptor rearrangement or clonal expansion, NK cells are poised to respond rapidly to infection. NK cells can be activated

by direct engagement of activating receptors on the cell surface or by cytokines produced by other immune cells. NK cell effector functions include the production of inflammatory cytokines at sites of infection and/or direct cytotoxicity of virus-infected cells. The predominant cytokine secreted by NK cells upon activation is IFN-γ, which activates macrophages and T cells, critical effector cells in the antiviral response. NK cells also secrete TNF-α, which can recruit neutrophils and monocytes to the site of infection, and GM-CSF, which activates macrophages. Cytolysis of target cells occurs through the exocytosis of perforin and cytotoxic granules by the NK cell into the extracellular space at the synapse with the target cell (22). Perforin disrupts the plasma membrane of the target cell and may interfere with endosomal trafficking (23). Cytotoxic granules contain granzymes, such as granzyme B, a serine protease that activates a cascade of cystein proteases, called caspases, and leads to apoptosis of the target cell (24, 25). Although perforin may facilitate the delivery of granzymes into the target cell by playing a role in the endosomal pathway, there is also evidence that granzymes can be taken up by target cells via receptor-mediated endocytosis (26). Thus, the primary effector functions of NK cells, i.e. cytokine production and cytotoxicity, help to contain and control viral spread during the initial hours and days of the infection, while the adaptive immune response develops.

NKG2D

NKG2D is an activating receptor expressed on virtually all human and mouse NK cells, as well as human $CD8^+$ T cells and activated mouse $CD8^+$ T cells (27, 28). NKG2D is expressed on the cell surface as a type II disulfide-bonded homodimer. In resting

mouse NK cells, mouse T cells and human NK and T cells, a long isoform of NKG2D forms a multi-subunit complex with a transmembrane adaptor protein called DAP10 (29). The association of NKG2D with DAP10 is mediated by a salt bridge between a positively-charged arginine in the transmembrane domain of NKG2D and a negatively-charged aspartic acid in the transmembrane region of DAP10. DAP10 has a YxxM motif in its cytoplasmic tail, which allows association of the p85 subunit of phosphatidylinositol (PI)-3 kinase and sets off a signaling cascade that results in AKT phosphorylation (29), similar to the pathway used by the co-stimulatory molecules CD28 and ICOS. In activated mouse NK cells, an additional short isoform of NKG2D, generated by alternative RNA splicing, can also associate with the DAP12 adaptor protein (30). DAP12 has immunoreceptor tyrosine-based activation motifs (ITAMs), defined by the sequence $YxxL_{6-8x}YxxL/I$, in its cytoplasmic domain. Phosphorylation of the ITAMs of DAP12 lead to recruitment and activation of Syk and/or ZAP70 tyrosine kinases (31). This short isoform of NKG2D lacks 13 amino acids in the cytoplasmic NH_2 terminus of the protein, and only exists in the mouse (30). In human NK cells, therefore, NKG2D only signals through DAP10. By contrast, in mouse NK cells, NKG2D signals through DAP10 in resting cells, and through both DAP10 and DAP12 in activated NK cells. This difference in signaling capacity has been proposed correlate with an ability of NKG2D to function as a primary stimulatory molecule in mouse cells or as a co-stimulatory molecule in mouse and human cells (30, 32). However, additional studies have shown that human NKG2D efficiently triggers cell-mediated cytotoxicity in the absence of DAP12, indicating that DAP10 is sufficient for NK cell signaling downstream of NKG2D (33, 34).

NKG2D Ligands

Ligands for human NKG2D

Ligands for human and mouse NKG2D all share some homology with MHC class I proteins, although they do not associate with β2-microglobulin or bind peptides. Human NKG2D ligands are comprised of a group of highly polymorphic MHC class I chain-related molecules, MICA and MICB (27), and the UL16-binding proteins, ULBP-1, -2, -3 and -4 (35, 36). The *MICA* gene was identified by searching the HLA-B region of the human MHC for other expressed genes (37). MICA and MICB are expressed on fibroblasts and epithelial cells in the gastrointestinal epithelium and are recognized by intestinal epithelial γδ–TcR$^+$ T cells (38). Expression of *MICA* and *MICB* is regulated by promotor heat shock elements (39), suggesting that these molecules are involved in signaling cellular stress. In addition, human cytomegalovirus (HCMV) infection induced expression of MICA on the surface of infected fibroblasts (40). MIC upregulation on the cell surface allowed engagement of NKG2D on CD28$^-$ CD8$^+$ αβ T cells, which enhanced TCR-dependent cytotoxic and cytokine responses from virus-specific T cells. These findings indicate that NKG2D can function in a co-stimulatory manner on human CD8$^+$ T cells by substituting for CD28 (40).

The ULBP glycoproteins were initially identified by their ability to bind to an HCMV glycoprotein, UL16. Using an expression cloning approach, Cosman and colleagues screened a cDNA library from HSB2, a human T cell line, with a fusion protein generated from the extracellular domain of HCMV UL16 and the Fc domain of human IgG1 (35). Two gene products bound specifically to UL16: MICB and a novel protein named UL16-binding protein-1 (ULBP-1). Three other ULBPs were then

identified by database searching, and all were found to bind NKG2D and to activate NK cell-mediated cytoxicity of targets bearing these ligands (35, 36). The functional significance of the interaction between UL16 and ULBP-1, -2 and MICB was revealed in co-expression studies. The transmembrane and cytoplasmic domains of UL16 localized it to the ER and cis-Golgi, while the ectodomain associated with ULBP-1, -2 and MICB, retaining them intracellularly (41-43). Intracellular retention of MICB, ULBP-1 and -2 by UL16 decreased the susceptibility of cells to NK cell-mediated cytotoxicity (41-43).

Ligands for mouse NKG2D

Similar to the large number of ligands for human NKG2D, murine NKG2D also binds to several ligands. These include a family of retinoic acid early inducible-1 gene products (RAE-1α, β, γ, δ, and ε), a minor histocompatibility antigen called H60 (44, 45), and a murine UL16-binding protein-like transcript-1 (MULT-1) glycoprotein (46, 47). *RAE-1* was initially identified and named because transcripts from *RAE-1α, β,* and *γ* were induced in the F9 embryonic carcinoma line after treatment with retinoic acid (48). Subsequently, the RAE-1 glycoproteins were found to bind to NKG2D. Ectopic expression of RAE-1γ or δ in the MHC class I-positive tumor cell line RMA allowed the rejection of these tumors in vivo (49, 50), indicating that the RAE-1-NKG2D interaction could override MHC class I inhibition, and stressing the importance of the NKG2D receptor in activating NK cell effector functions. The five RAE-1 proteins are highly related to one another (>92% sequence identity) (44) and appear to be differentially expressed in different mouse strains. RAE-1α, β and γ are expressed in BALB/c mice, whereas RAE-1δ and ε are expressed in C57BL/6 mice (Lanier lab, unpublished data).

The RAE-1 transcripts are abundant in mouse embryonic tissue and in many mouse tumor cell lines, but rare in adult tissue (44, 51).

Prior to its identification as an NKG2D ligand, the H60 antigen was known to play a critical role in transplant rejection between C57BL/6 and BALB.B mice, which are BALB/c mice congenic for H-2b. In BALB.B mice, an eight amino acid peptide (LYL8) from the H60 protein is loaded into H-2K^b (52). Although the *H60* gene exists in C57BL/6 mice, it is mutated in the residues that allow loading into MHC class I (Lanier lab, unpublished data). In C57BL/6 mice, the TCR recognizes the LYL8 peptide as the dominant alloantigen, and transplant rejection of C57BL/6 tissue into BALB.B mice is mediated by a CTL response against this H60 peptide (53). H60 was subsequently identified as a ligand for NKG2D in the same expression cloning system that identified the RAE-1 glycoproteins as NKG2D ligands (44, 45). Interestingly, the residues in the LYL8 peptide that allow it to anchor in H-2Kb are the same residues that mediate interaction with NKG2D (54). Thus the H60 protein encoded in C57BL/6 mice plays no functional role in NKG2D activation. In addition, H60 does not appear to be expressed on the surface of cells from C57BL/6 mice (Lanier lab, unpublished data).

The third ligand identified for mouse NKG2D was found by database searching for murine counterparts to the human ULBP glycoproteins. Using the sequences of the ectodomains of ULBP-1, -2 and –3 to search mouse EST databases, Carayannopoulos and colleagues discovered a gene that bore >20% identity to ULBP-3, and which was named murine UL16-binding protein-like transcript-1 (MULT-1) (55). When transfected into Ba/F3 (a mouse pro-B cell line) or RMA (a mouse T cell thymoma) cells, which do not express NKG2D ligands, MULT-1 conferred NKG2D-tetramer staining and

rendered cells susceptible to NK cell lysis, which could be blocked by the addition of recombinant soluble H60 (55). Unlike RAE-1 and H60, MULT-1 appears to be broadly distributed in both lymphoid and non-lymphoid tissues.

Although RAE-1, H60 and MULT-1 are all distantly related to MHC class I molecules (~30% similarity), they are actually fairly divergent on a sequence level (44, 45). They only share ~20% sequence identity at the amino acid level, indicating that NKG2D is relatively promiscuous in its ligand binding. Analysis of the structure of co-crystals of murine NKG2D with RAE-1β indicates that the association is similar to that between human NKG2D and MICA, and it resembles TCR recognition of the α1 and α2 platform of MHC class I (56, 57). However, the α helices of RAE-1 are more closely positioned than those in MHC class I, closing off the groove between the helices and preventing peptide binding. Although the sequences of RAE-1, H60 and MULT-1 are only distantly related, the interface with which they contact NKG2D is essentially identical (57), which may explain the promiscuity of the receptor for its multiple ligands.

Although a growing list of ligands for NKG2D has been defined, the regulation of these molecules remains elusive. Some data suggest that the human NKG2D ligands, such as MICA and MICB, are induced by cellular stress, such as transformation or virus infection (39, 40). Transcripts for the ULBP molecules, however, appear to be broadly expressed in healthy adult tissue (35), suggesting that they are regulated in a different manner than the MIC glycoproteins. There is also little known about the regulation and expression of NKG2D ligands in the mouse. Although the RAE-1 glycoproteins are abundantly expressed in the mouse embryo at day 7 of gestation, transcripts from these genes are undetectable by day 18 and absent from most adult tissues examined (44, 51).

Given that engagement of NKG2D by its ligands can override inhibitory signals delivered by self-MHC class I (49, 50), and considering the critical importance of NK cells in controlling viral infection, we examined whether cytomegalovirus (CMV) impacts expression of the ligands for NKG2D. The research presented in this thesis reveals that CMV infection specifically modulates NKG2D ligand expression, and that down-regulation of these ligands impairs NK cell recognition of virus-infected cells.

NK Cells and CMV Infection

The important contribution of NK cells in anti-viral immunity has been well studied. NK cells play a critical role in controlling both human and murine cytomegalovirus (CMV) infection, functioning early during the innate phase of the immune response to the virus. NK cell deficiency in humans can lead to life-threatening HCMV infection (58), and CMV-resistant mice that are depleted of NK cells become sensitive to MCMV infection (59).

Early studies on NK cells and MCMV revealed that resistance or susceptibility of certain mouse strains to MCMV could be mapped to a precise locus, called *Cmv1*, in the murine genome (60). This locus fell in a region of mouse chromosome 6 that encodes several NK cell receptors and regulates NK cell activity. *Cmv1* was present in CMV-resistant C57BL/6 mice, but absent from CMV-sensitive strains, such as BALB/c and DBA/2 mice. It was later determined that the *Cmv1* locus encoded an activating NK cell receptor of the Ly49 family (61-63). Ly49 receptors are members of the C-type lectin-like superfamily, and can be either activating or inhibitory receptors. *Cmv1* encodes an activating receptor, named Ly49H, which associates with the transmembrane adaptor

protein DAP12 (64). The cytoplasmic domain of DAP12 contains ITAMs, which allow the receptor to signal and to induce cytokine production and cytotoxic activity against target cells. In accordance with these findings, DAP12-deficient mice are more sensitive to MCMV infection than wild-type C57BL/6 mice, because NK cells from the mutant mice lack the adaptor protein necessary for signal transduction downstream of the Ly49H receptor (65).

The identification of Ly49H as the receptor that confers resistance of C57BL/6 mice to MCMV led to efforts to clone the ligand for this receptor. Using reporter cell assays, it was found that the ligand for Ly49H was a viral glycoprotein, called m157, that is expressed on the surface of MCMV-infected cells (66, 67). m157 shares structural homology with MHC class I, and prior to its identification as a ligand for Ly49H, had no known function. The discovery of a viral gene product as a ligand for an activating NK cell receptor raises the question of why the virus would retain such a gene. Viruses have co-evolved with the host immune system for thousands of years, leaving ample opportunity to dispose of genes that could compromise their virulence. Arase et al. (66) provided an explanation for this apparent conundrum by showing that, in addition to its interaction with Ly49H, m157 can also bind to an inhibitory NK cell receptor in a CMV-sensitive strain of mice. An m157-Ig fusion protein stained Ly49I-expressing cells in the 129/J CMV-susceptible strain of mice, in addition to staining Ly49H-expressing cells in a CMV-resistant C57BL/6 strain of mice. These findings suggest that CMV may have retained m157 in its genome to engage inhibitory NK cell receptors in some strains of mice, and that perhaps C57BL/6 mice evolved an activating receptor that could recognize m157 and confer resistance to MCMV. This theory would predict that repeated passage

of MCMV through Ly49H$^+$ C57BL/6 mice would result in loss or mutation of m157. Indeed, recent work has shown that after three passages of wild-type MCMV through C57BL/6 mice, the harvested viruses had mutations in the m157 gene that prevented its interaction with Ly49H (68). Thus, escape variants of m157 occurred in the presence of selective pressure from the host immune system. These findings collectively support the hypothesis that viruses can drive the selection of host immune receptors, and a functional host immune system can select for viruses that contain a repertoire of more highly virulent genes.

Other research on the role of NK cells in CMV infection has focused on viral glycoproteins that are evolutionarily related to MHC class I. Both HCMV and MCMV encode one protein that is homologous to classical MHC class I, associates with β2-microglobulin and binds peptides: HCMV UL18 (69) and MCMV m144 (70). Both proteins were originally thought to serve as decoy ligands for NK cell inhibitory receptors. The initial studies on UL18 supported this idea, showing that UL18 prevented NK cell recognition of HCMV-infected cells (71). Later work, however, revealed that UL18 expression on virus-infected cells may actually increase NK cell activity against these cells, and cells infected with a deletion mutant virus lacking UL18 were less susceptible to NK cell lysis than wild-type virus-infected cells (72). Subsequent studies on UL18 found that it binds to a receptor expressed predominantly on myeloid cells and B cells, called leukocyte immunoglobulin-like receptor 1 (LIR-1), indicating that UL18 may not preferentially target NK cells (73).

MCMV m144 also encodes an MHC class I-like cell surface glycoprotein, which may contribute to MCMV virulence by interfering with NK cell antiviral immunity.

When a deletion mutant virus lacking m144 was injected into mice, its replication during acute infection was reduced when compared to wild-type virus. Depletion of NK cells, however, restored virulence of the m144-deletion mutant virus (70). In addition, it has been shown that ectopic expression of m144 in RMA-S cells, an MHC class I-deficient cell line, can inhibit NK cell cytotoxicity in vitro and tumor clearance in vivo (74). However, these findings have proven controversial, and efforts to find the receptor for m144 have not yielded any candidates, hampering attempts to address the mechanism of its activity.

Another CMV glycoprotein that modulates NK cell recognition of virus-infected cells is HCMV UL40. The leader sequence from UL40 can be loaded into the peptide-binding groove of HLA-E by a mechanism that does not require TAP (75). HLA-E is a non-conventional MHC class I molecule that typically loads the leader peptides of other MHC class I molecules into its peptide binding groove, and it was identified as the ligand for CD94/NKG2A, an inhibitory NK cell receptor (76, 77). Because CD94/NKG2A recognized a molecule that displayed peptides from other MHC class I proteins, it was postulated that HLA-E served as an indicator of normal MHC class I expression in cells. When MHC class I is down-regulated, as in the case of virus infection or transformation, HLA-E expression is reduced on the cell surface, causing a loss of inhibitory signal through CD94/NKG2A. Loading of the UL40 leader peptide into HLA-E, however, can restore HLA-E expression on the surface of virus-infected cells, counteracting virus-induced MHC class I down-regulation (75). In this manner, viral UL40 has co-opted a mechanism used by NK cells to detect stressed cells, in order to evade NK cell recognition.

Thus, viral evasion of NK cells occurs through the concerted activity of multiple immune evasion genes. The work presented in this thesis examines CMV modulation of NK cell activity through virus-induced down-regulation of ligands for NKG2D, a potent NK cell activating receptor. We report that both HCMV and MCMV have gene products that specifically down-regulate ligands for NKG2D, resulting in impaired NK cell recognition of CMV-infected cells.

Chapter Two

HCMV UL16 and Expression of the Human NKG2D Ligands

Introduction

The ligands for human NKG2D are comprised of the MIC molecules, MICA and MICB, and the UL16-binding proteins (ULBP-1, -2, -3, -4). Although the ULBP glycoproteins were cloned by their ability to bind to a HCMV glycoprotein, UL16 (35), the initial studies on these molecules did not define a function or significance to this interaction. In addition, UL16 only bound to ULBP-1, –2 and MICB, but not MICA or ULBP-3 or –4. We decided to determine whether HCMV infection could affect expression of the ligands for NKG2D, and to investigate if the interaction between UL16 and the NKG2D ligands had functional consequences for NK cell recognition of HCMV-infected cells. Since beginning our work on UL16, several papers have been published showing that UL16 binds to and sequesters ULBP-1, -2 and MICB intracellularly in an ER-cis-Golgi compartment, preventing NK cell recognition of these NKG2D ligands (41-43). However, these data were unknown at the time of our studies on UL16.

Results

To examine the importance of the interaction of UL16 with ligands for NKG2D, we first generated cells expressing these molecules. Based on sequence analysis, UL16 appeared to be a transmembrane glycoprotein (Fig. 2.1A). We designed primers specific for the UL16 open reading frame, and performed PCR on viral genomic DNA isolated from HCMV. The UL16 PCR product was cloned into a retroviral vector, pMX-pie, which contains an IRES-EGFP element at the 3' end of the UL16 cDNA. This vector allows the use of green fluorescent protein (GFP) as an indication of cells expressing UL16 cDNA. In addition, an eight amino acid Flag epitope tag was inserted onto the 5' end of the UL16 cDNA, for detection of the protein with an anti-Flag antibody, since we did not have an antibody that specifically recognized UL16 (Fig. 2.1A). Retroviral transduction of the Flag-UL16 construct into a murine pro-B cell line called BaF/3 resulted in the production of GFP^+ cells. However, these cells did not stain positively for Flag on the cell surface, indicating that UL16 was not a cell surface protein (Fig. 2.1B).

Gazing at the UL16 protein sequence revealed that the cytoplasmic domain of UL16 contained a YxxL motif, which is an endocytosis motif (78). To investigate whether this motif functioned to retain UL16 inside cells, we generated two additional constructs: 1) a truncation mutant in which the cytoplasmic tail was truncated at amino acid position 220, leaving the ectodomain, the transmembrane region, and a transmembrane-proximal portion of the cytoplamic tail (called Flag-UL16 trunc); and 2) a Y-F mutant, in which the cytoplasmic tail was left intact, but a single amino acid change was made in the YxxL motif to create FxxL (called Flag-UL16 Y-F). These constructs were also cloned into the pMX-pie vector and retrovirally transduced into

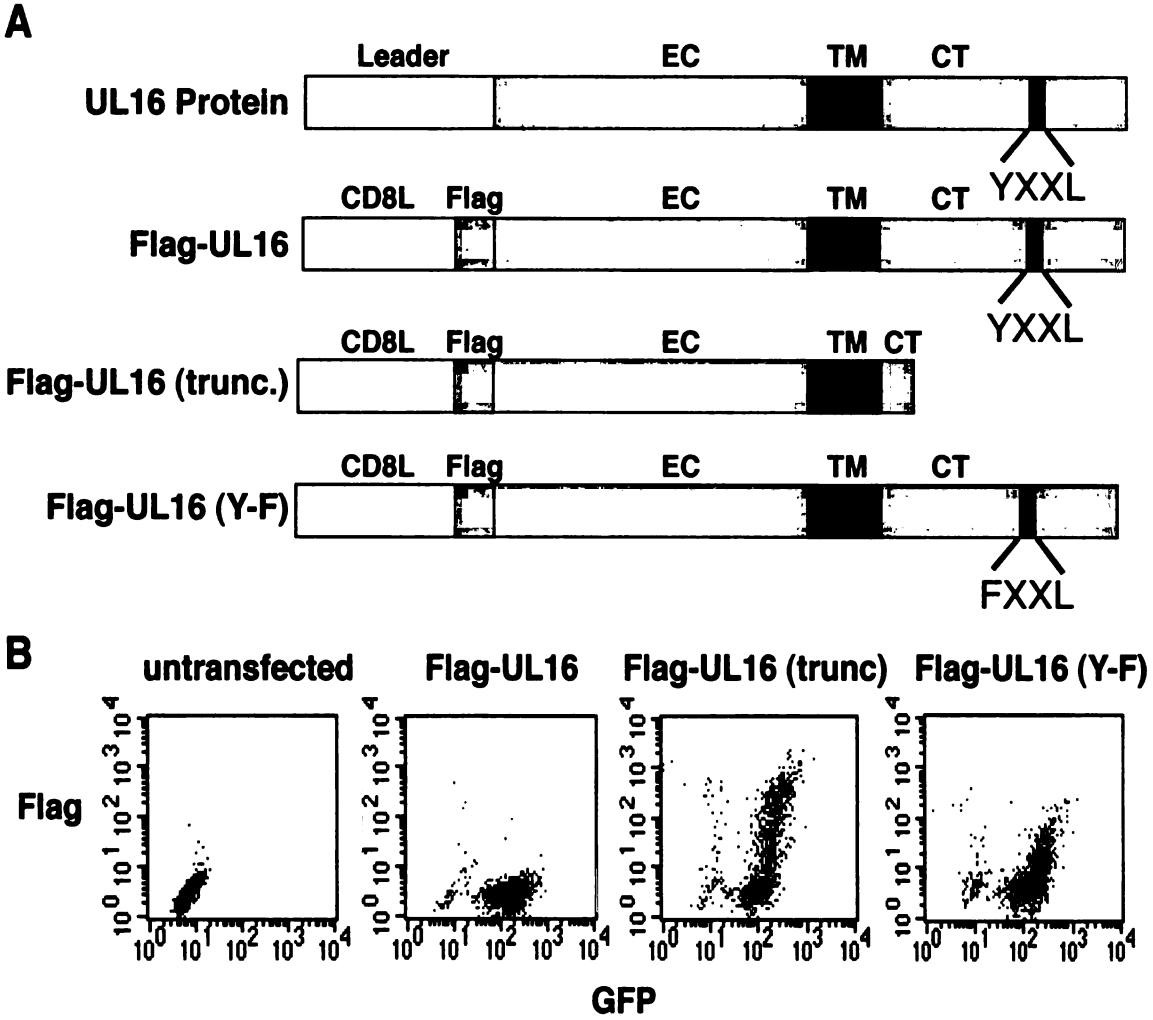

Figure 2.1. Flag-UL16 constructs and expression on the surface of BaF/3 cells. A) Full-length UL16 has a YxxL motif in its cytoplasmic tail. Three UL16 constructs were made, all of which have a Flag epitope tag on the 3' end of the cDNA, following the human CD8a leader sequence. Flag-UL16 retains the full-length cytoplasmic domain of UL16; Flag-UL16 (trunc.) is truncated at position 220; and Flag-UL16 (Y-F) has a single amino acid change that converts the tyrosine of the YxxL motif to a phenylalanine. B) BaF/3 cells were retrovirally transduced with Flag-UL16 constructs in an IRES-EGFP vector (pMX-pie), and cells were stained with anti-Flag M2 mAb. GFP+ cells indicate expression of Flag-UL16. Full-length Flag-UL16 is not expressed on the cell surface, but truncation of the cytoplasmic tail or mutation of the YxxL motif to FxxL allow cell surface expression of Flag-UL16.

BaF/3 cells (Fig. 2.1A). Mutation of the tyrosine residue in the YxxL motif resulted in partial expression of Flag-UL16 on the cell surface, whereas truncation of the cytoplasmic tail led to high levels of Flag-UL16 staining on the cell surface (Fig. 2.1B). These data indicated that the cytoplasmic domain of UL16 plays a role in the intracellular localization of the protein, since mutation of this region resulted in cell surface expression of UL16.

In order to examine the impact of UL16 on expression of the NKG2D ligands, we also generated cells expressing each of the known human NKG2D ligands: ULBP-1, -2, -3, -4 or MICA. cDNAs from each of these genes were cloned into the pMX-pie vector, and each vector was retrovirally transduced into BaF/3 cells. Staining of the transfectants with an NKG2D-Ig fusion protein, consisting of the extracellular domain of human NKG2D fused to the Fc portion of human IgG1, showed that NKG2D ligands were expressed on the surface of the transfectants (Fig. 2.2B). To confirm the published finding that a UL16-Ig fusion protein could stain NKG2D ligands on the cell surface, we also generated a UL16-Ig fusion protein. The UL16-Ig stained the surface of ULBP-1 and –2 transfectants, but not ULBP-3, -4 or MICA transfectants (Fig. 2.2A). These data are consistent with the original published finding that UL16 only bound to ULBP-1, -2 and MICB (35).

Based on the intracellular localization of full-length UL16 in Ba/F3 transfectants (Fig. 2.1B), we hypothesized that UL16 may interact with NKG2D ligands in an intracellular compartment and affect their expression on the cell surface. To test this possibility, we co-transfected Flag-UL16 and ULBP-2 cDNAs into Ba/F3 cells. The Flag-UL16 cDNA was expressed in pMX-pie, so the presence of GFP in cells indicates

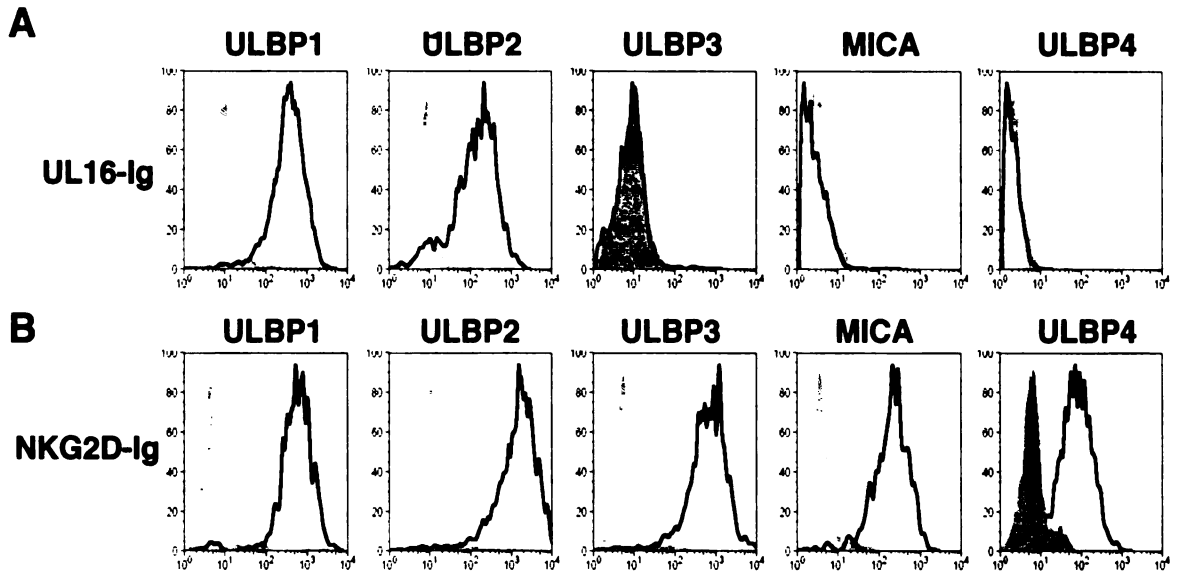

Figure 2.2. UL16-Ig binds to ULBP-1 and –2, but not ULBP-3, -4 or MICA. A) ULBP-1, -2, -3, -4 and MICA cDNAs were cloned into the pMX-pie vector and the vectors were retrovirally transduced into BaF/3 cells. Transfectants were stained with a control Ig (gray histograms) or a UL16-Ig fusion protein (bold histograms). B) Transfectants from A) were also stained with an NKG2D-Ig fusion protein (bold histograms).

UL16 expression. The ULBP-2 cDNA was cloned into a retroviral vector lacking an IRES-EGFP, called pMX-neo. After co-transfection of BaF/3 cells with these constructs, we observed both GFP^+ and GFP^- populations. However, when we stained the cells for ULBP-2 expression, using the NKG2D-Ig fusion protein, we found that ULBP-2 staining on the cell surface was not affected by UL16 expression (Fig. 2.3). This finding was surprising, considering that UL16 bound strongly to the NKG2D ligands, and led us to question whether the ability of UL16 to bind to the NKG2D ligands had any functional consequences for NK cell recognition of HCMV-infected cells.

To address this question, we turned from the transfection system to an in vitro infection system. We first examined whether HCMV infection had an impact on the transcription of NKG2D ligands by using real-time quantitative PCR. We designed primer and probe sets that specifically recognized the five different human NKG2D ligands. The ligands are roughly 50% homologous, so primers and probes could be designed in regions of least similarity. We infected human MRC-5 fibroblast cells with the wild-type AD169 strain of HCMV and 48 hours post-infection harvested total RNA from uninfected or infected cells. We generated cDNA and used it as the template in Taqman PCR experiments with the primer and probe sets specific for each of the NKG2D ligands. We observed that HCMV infection caused a 10-fold increase in MIC transcription (Fig. 2.4). However, it appears that transcription of the ULBP genes is not greatly affected by HCMV infection of MRC-5 cells.

We also examined the effect of HCMV infection on cell surface levels of NKG2D ligands. We used U373 cells, a human glioblastoma cell line that is infectable by HCMV, for these studies. By staining cells with the NKG2D-Ig fusion protein, we

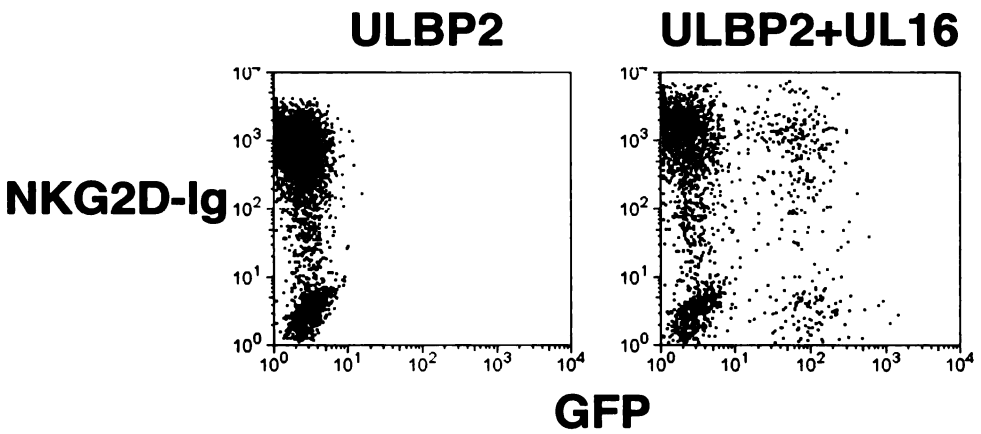

Figure 2.3. UL16 expression does not affect cell surface expression of ULBP-2. BaF/3 cells were transfected with the ULBP-2 cDNA alone, or the ULBP-2 cDNA and the UL16 cDNA. UL16 was encoded on the pMX-pie vector, allowing visualization of UL16-expressing cells by GFP.

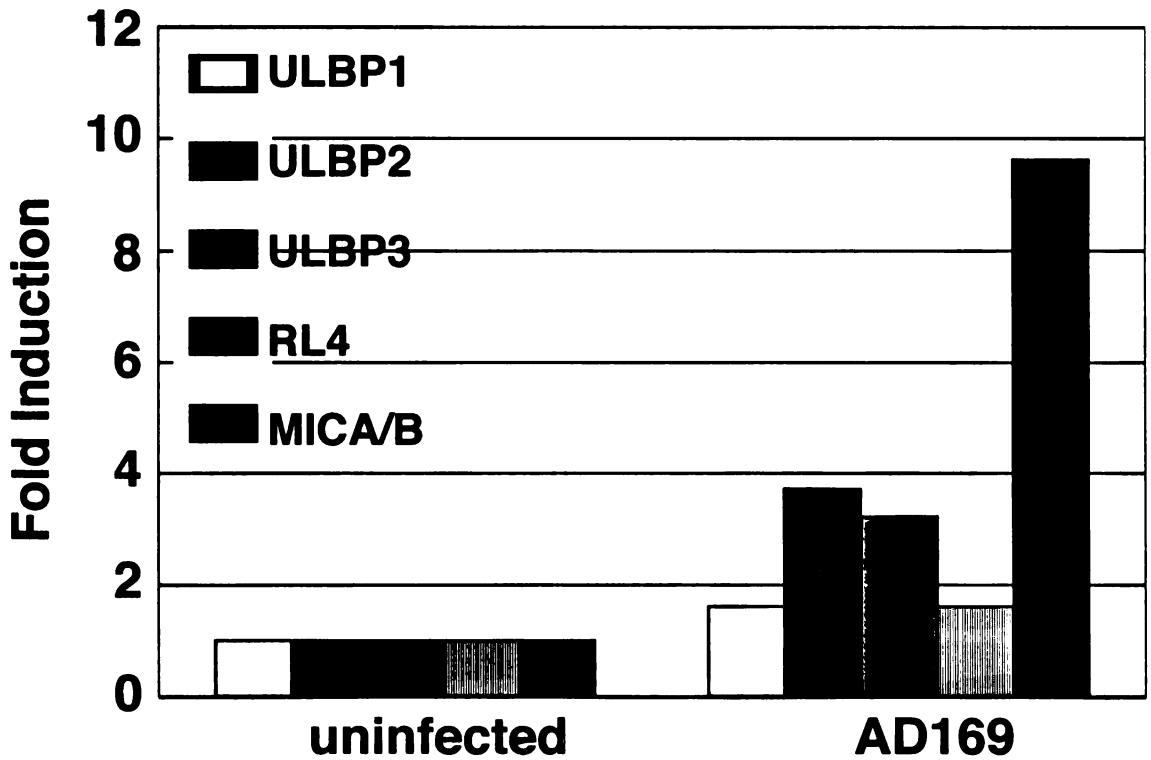

Figure 2.4. Transcriptional induction of MIC by HCMV infection. MRC-5 fibroblasts were infected with the wild-type AD169 strain of HCMV at an MOI of one. 48 hours post-infection, total RNA was harvested, cDNA was synthesized, and TaqMan real-time PCR was performed with primer and probe sets specific for each of the NKG2D ligands. Values from triplicate wells were averaged and normalized to the values obtained from each sample for amplification of the human GAPDH gene. Standard deviations of triplicate wells was <1% of total values.

observed that uninfected U373 cells expressed relatively high levels of NKG2D ligands (Fig. 2.5A). However, infection with the wild-type AD169 strain of HCMV resulted in a marked down-regulation of NKG2D ligands as determined by immunofluorescent staining with NKG2D-Ig and by analysis by flow cytometry (Fig. 2.5A). Interestingly, we found that infection of U373 cells with a deletion mutant virus lacking only the UL16 ORF largely restored NKG2D ligand expression on the cell surface (Fig. 2.5). NKG2D ligands are not restored to the level seen on uninfected cells, suggesting that viral gene products elsewhere in the genome may play a role in the down-regulation of NKG2D ligands. However, it appears that UL16 may be the dominant player. We also stained the infected and uninfected U373 cells with a pan-HLA human MHC class I monoclonal antibody, DX17. DX17 staining is bright on uninfected cells, but is down-regulated on cells infected with either the wild-type virus or the ΔUL16 virus, indicating that infection efficiency was relatively equal (Fig. 2.5B).

To determine if the down-regulation of NKG2D ligands by HCMV infection influenced the ability of NK cells to recognize and kill virus-infected cells, we performed NK cell cytotoxicity assays. We used a human NK leukemia cell line, NKL, as effector cells and chromium-labeled HCMV-infected or uninfected U373 cells as target cells. We found that when NKL cells were co-cultured with infected U373 cells 48 hours post-infection, the NK cells were highly cytotoxic against the HCMV-infected cells, compared with uninfected cells (Fig. 2.6A). Infection of U373 cells with the ΔUL16 virus rendered them slightly more susceptible to NK lysis than cells infected with AD169 virus, perhaps because they have slightly higher levels of NKG2D ligands on the cell surface. Thus, it appears that 48 hours of infection with HCMV increases the sensitivity of U373 cells to

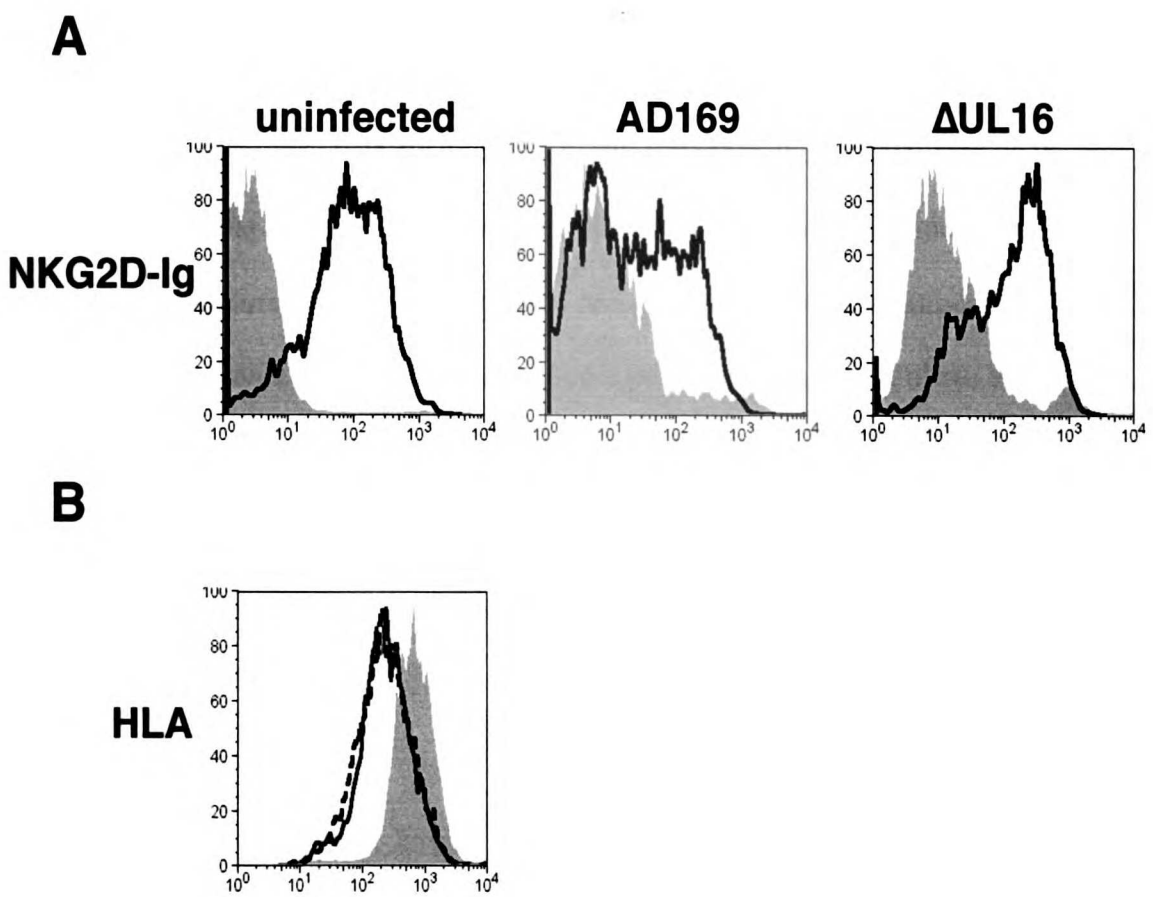

Figure 2.5. Infection of U373 cells with HCMV causes down-regulation of NKG2D ligands from the cell surface. A) U373 cells were infected with the wild-type AD169 strain of HCMV or a ΔUL16 mutant virus and stained with either a control Ig (gray histograms) or an NKG2D-Ig fusion protein (bold histograms). B) Uninfected cells (gray histogram) and cells infected with the AD169 virus (bold histogram) or ΔUL16 virus (dashed histogram) were stained with mouse anti-human MHC class I mAb DX17 mAb.

killing by NKL. Conversely, we observed that U373 cells infected with HCMV for 4 days prior to co-culture with NK cells were highly resistant to NKL killing (Fig. 2.6B), regardless of whether we infected cells with the wild-type AD169 strain or the ΔUL16 mutant strain of MCMV. As a control to verify functional NKL in our assay, we also co-cultured NKL with chromium-labeled BaF/3 cells transfected with ULBP-2. These transfectants were killed at relatively high levels, indicating that the NK cells in the assay were functional (Fig. 2.6B). Therefore, there seems to be a temporal window following HCMV infection during which NK cells can recognize and lyse virus-infected cells. In our assays, it appeared to occur around 48 hours post-infection, with little to no NK cell killing by four days post-infection.

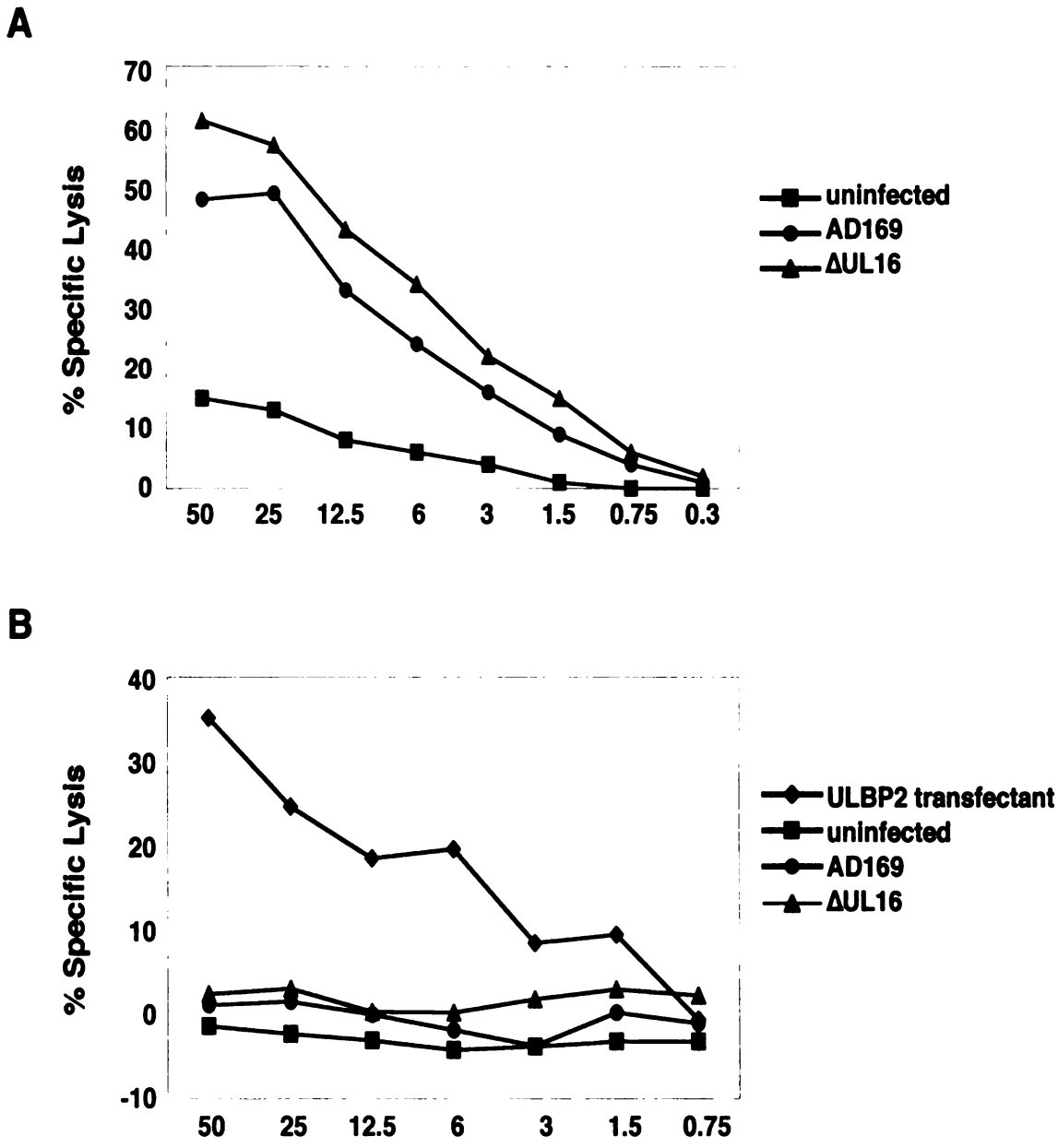

Figure 2.6. Susceptibility of HCMV-infected cells to NKL lysis changes during the course of infection. A) U373 cells were infected with the AD169 or ΔUL16 strain of HCMV for 48 hours before labeling with chromium and co-culture with NKL cells. B) U373 cells were infected as in A) but left for 4 days before labeling and co-culture with NKL cells. Data are shown as the percent specific lysis of the target cells over a range of effector:target (E:T) cell ratios.

Discussion

Based on the data presented in this chapter, it is difficult to ascertain the importance of UL16 in HCMV virulence. Our preliminary studies on UL16 supported data that was published at the time when we began our work: we generated a UL16-Ig fusion protein that bound to cells expressing ULBP-1 and -2 but not ULBP-3, -4 or MICA. However, when we co-transfected UL16 in cells that expressed ULBP-2, one of the NKG2D ligands with which UL16 interacts, ULBP-2 expression was unchanged. These data suggested that although UL16 could interact with ULBP2, it did not affect its expression on the cell surface. We ruled out the possibility that UL16 might associate with ULBP-2 on the cell surface and prevent its association with NKG2D, because we were able to stain ULBP-2 on the surface of UL16-ULBP-2 co-transfectants using an NKG2D-Ig fusion protein. Thus, we inferred from our experiments that UL16 neither retained ULBP-2 inside cells nor hindered its recognition by NKG2D on the cell surface. One experiment we did not try was to co-express UL16 and ULBP-1 or –2 in different cell types to see if the effect of UL16 on ULBP-2 was cell-type dependent. Interestingly, several papers have recently been published on UL16 showing that it functions by retaining ULBP-1, -2 and MICB in an intracellular compartment, preventing their interaction with NKG2D (41-43). Elegant biochemistry and microscopy studies showed that the ectodomain of UL16 was important for the interaction of UL16 with NKG2D ligands, whereas the transmembrane and cytoplasmic domains serve to localize UL16 to an ER-cis-Golgi compartment (43). These data are consistent with experiments we performed in which we mutated the cytoplasmic domain of UL16 and found that it no longer localized intracellularly, but was expressed on the cell surface (Fig. 2.1B).

Unfortunately, our observation that UL16 had no impact on ULBP-2 expression in BaF/3 cells led us away from pursuing studies on the interaction of UL16 with the NKG2D ligands.

Using an in vitro infection system, we were able to examine the consequences of HCMV infection on NKG2D ligand expression. We determined that HCMV infection induced transcription of MIC in fibroblasts, although we did not observe a substantial change in transcription of the ULBP genes following infection. At the protein level, we found that NKG2D ligands were down-regulated from the surface of HCMV-infected cells, but were restored when we infected cells with a deletion mutant virus lacking the UL16 ORF. These data seemed inconsistent with our transfection data. However, the differences in expression system (transfection instead of infection) and cells (BaF/3 instead of U373) could account for the discrepancy. In addition, we observed that after 48 hours of infection, U373 cells were highly sensitive to NKL lysis, but that by 4 days post-infection, they had become resistant. These findings could be explained by gross changes on the surface of cells during the course of virus infection. In addition to down-regulation of NKG2D ligands by HCMV, expression of other cell surface glycoproteins is also likely altered by virus infection, with varying kinetics. Although changes in the composition of cell surface receptors during infection complicates the ability of NK cells to recognize virus-infected cells, it does appear that short-term infection renders cells more sensitive to NK cell cytotoxicity.

Chapter Three

RAE-1 Down-Regulation by MCMV m152

Introduction

The critical role of NK cells in controlling infection by HCMV and MCMV has long been appreciated (58, 59). In Chapter 2 we showed that HCMV infection strongly down-regulated expression of human NKG2D ligands, and that this was largely mediated by the viral glycoprotein UL16. Although there is no homologue of UL16 in the MCMV genome, we hypothesized that MCMV infection may down-regulate ligands for mouse NKG2D, such as the RAE-1 glycoproteins, H60 or MULT-1. Studies by Krmpotic et al. (79) demonstrated that the MCMV m152 gene product, gp40, affects NK cell immunity against MCMV by modulation of ligands for NKG2D. We sought to precisely identify the NKG2D ligands that are down-regulated by MCMV infection. In this study, we have examined viral modulation of RAE-1 and the functional consequences of RAE-1-NKG2D interactions in NK cell responses to MCMV *in vitro* and *in vivo*.

Results

Transcriptional induction of RAE-1 genes by MCMV infection

The products of at least seven different genes have been identified as ligands for mouse NKG2D: RAE-1α, β, γ, δ, ε, H60 (44, 45) and murine UL16-binding protein-like transcript-1 (MULT-1) (55). RAE-1α, β, γ, H60 and MULT-1 are expressed in BALB/c mice, whereas RAE-1δ, ε and MULT-1 are expressed in C57BL/6 mice (unpublished observations). To examine the effect of MCMV infection on transcription of *RAE-1* genes, we analyzed RAE-1 and H60 transcripts from MCMV-infected peritoneal macrophages. Resident peritoneal macrophages from BALB/c and C57BL/6 mice were isolated and infected with the wild-type Smith strain of MCMV or an m152 deletion mutant (Δm152). Total RNA was extracted from infected macrophages 48 hours post-infection, and cDNA was synthesized. cDNA was then used in TaqMan quantitative PCR to analyze RAE-1 and H60 transcripts. TaqMan primer and probe sets were designed to recognize H60 and to distinguish each of the five RAE-1 gene products. We observed that MCMV infection transcriptionally induced RAE-1α, β and γ in peritoneal macrophages from BALB/c mice (Fig. 3.1A), and RAE-1δ and ε in infected C57BL/6 macrophages (Fig. 3.1B). Both the wild-type and mutant viruses induced similar levels of RAE-1 transcripts in infected cells; however, neither virus induced H60 or MULT-1 transcripts (data not shown).

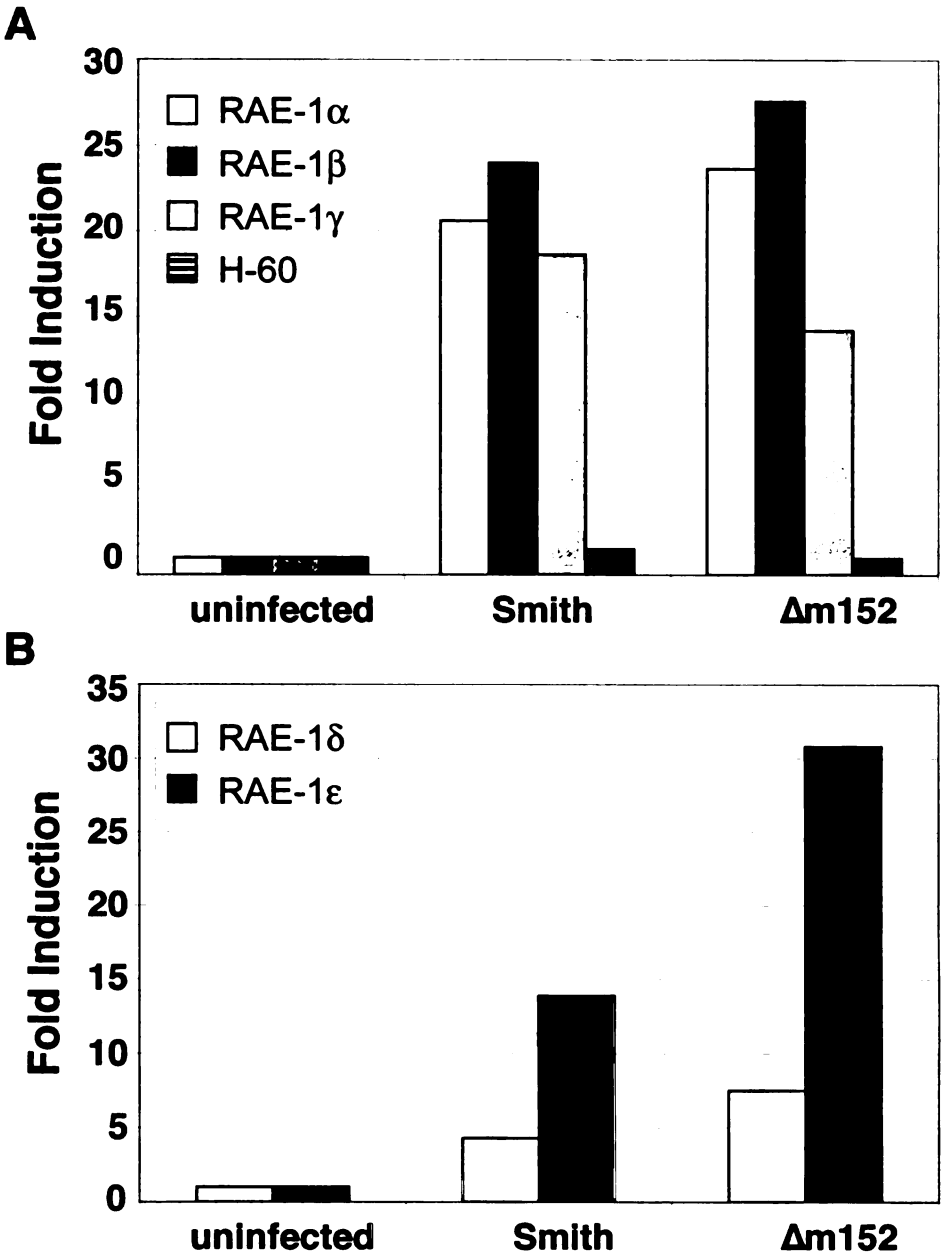

Figure 3.1. RAE-1 transcripts are induced in MCMV-infected peritoneal macrophages. BALB/c and C57BL/6 peritoneal macrophages were left untreated or were infected with Smith or Δm152 (MOI=1). RNA was extracted from cells 48 hours post-infection (hpi) and cDNA was synthesized. TAQMAN quantitative PCR was performed on BALB/c cDNA using primer/probe sets specific for RAE-1α, β, γ or H60 (A), and on C57BL/6 cDNA with primer/probe sets specific for RAE-1δ and ε (B). Signals generated from each sample were normalized to HPRT signals from each set of cDNA. Data are expressed as the fold induction of transcription in infected cells compared to uninfected cells. Standard deviations of triplicate samples were less than 1%. Results shown are representative of three independent experiments.

Down-regulation of NKG2D ligands by MCMV

Given this potent induction of RAE-1 transcription, we examined RAE-1 protein levels on MCMV-infected cells. We infected BALB/c 3T3 cells, an MCMV-permissive cell line that constitutively expresses RAE-1 on the cell surface, with wild-type virus, and 72 hours post-infection, cells were stained for surface expression of NKG2D ligands using two different staining reagents: a mouse NKG2D-Ig fusion protein (44), and CX1, a mAb that recognizes RAE-1α, β and γ (80). Despite observing transcriptional induction of the *RAE-1* genes by MCMV, we found that MCMV infection resulted in dramatic down-regulation of NKG2D ligands, compared with uninfected cells, when analyzed by flow cytometry.

One viral gene product, gp40, encoded by the MCMV *m152* gene, has been shown to down-regulate MHC class I (11), as well as an undefined NKG2D ligand (79). We infected 3T3 cells with wild-type or Δm152 mutant virus, and stained infected cells with the mouse NKG2D-Ig fusion protein or anti-RAE-1 mAb. Interestingly, cells infected with Δm152 down-regulated expression of NKG2D ligands much less efficiently than wild-type virus (Fig. 3.2). This finding is consistent with that of Krmpotic et al. (79), who showed, using an NKG2D tetramer, that Δm152-infected cells maintained expression of NKG2D ligands, compared with strong down-regulation observed in cells infected with wild-type virus. These experiments demonstrate that gp40 modulates expression of the RAE-1 glycoproteins, in addition to down-regulating MHC class I proteins, on the surface of MCMV-infected cells.

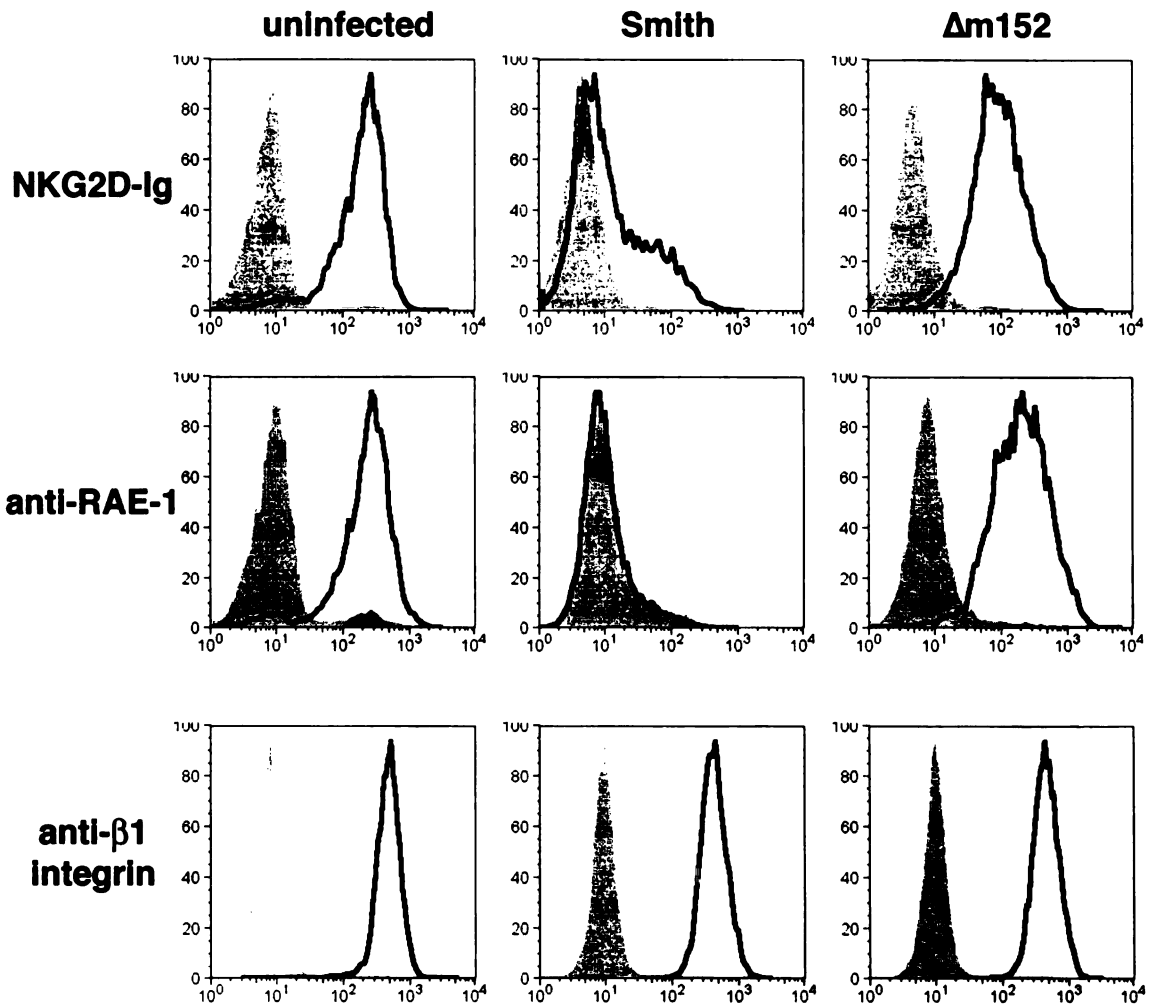

Figure 3.2. MCMV infection results in down-regulation of NKG2D ligands. BALB/c 3T3 cells were infected with wild-type MCMV (Smith) or Δm152 (MOI=1). 72 hpi infected cells were stained with control Ig (cIg), NKG2D-Ig fusion protein, or anti-RAE-1 mAb (CX1). As a control, cells were stained with anti-β1-integrin.

gp40 down-regulates RAE-1α, β, γ, δ, and ε, but not H60

Studies of MHC class I down-regulation by gp40 have revealed an allele-specific preference, with certain MHC class I haplotypes affected more than others (81). To determine whether there are differential effects of gp40 on the five different RAE-1 proteins, we transiently transfected either m152 cDNA or a control empty vector, along with RAE-1α, β, γ, δ, or ε cDNA into human 293T cells. Mouse RAE-1 cDNAs were encoded by a vector that also expressed GFP (IRES-EGFP), whereas m152 cDNA was encoded by a non-GFP vector. 48 hours after transfection of RAE-1 cDNA with or without m152 cDNA, cells were examined for mouse RAE-1 expression by staining with anti-RAE-1 mAbs (CX1 mAb for detection of RAE-1α, β and γ, or 186107 mAb for detection of RAE-1δ and ε). Gating on the GFP^+ population allowed us to analyze only RAE-1-expressing cells. Transfection of RAE-1 cDNA with a control empty vector resulted in high mouse RAE-1 expression as determined by anti-RAE-1 mAb staining (Fig. 3.3A). In contrast, co-transfection of m152 along with any of the RAE-1 cDNAs (α, β, γ, δ or ε) resulted in a substantial down-regulation. All of the RAE-1 proteins were found to be susceptible to the impact of gp40, in particular RAE-1β. As a control, the transfected cells were also stained with an anti-HLA class I mAb. Anti-human HLA staining of human 293T cells was unaffected by m152 transfection (Fig. 3.3B), suggesting that m152 down-regulation of RAE-1 proteins was specific and did not affect human MHC class I expression.

In a similar 293T transfection system, the effect of gp40 on H60 expression was analyzed. Due to the lack of an antibody against H60 at the time of these studies, a Flag epitope tag was inserted onto the 5' terminus of the H60 cDNA and expressed by using

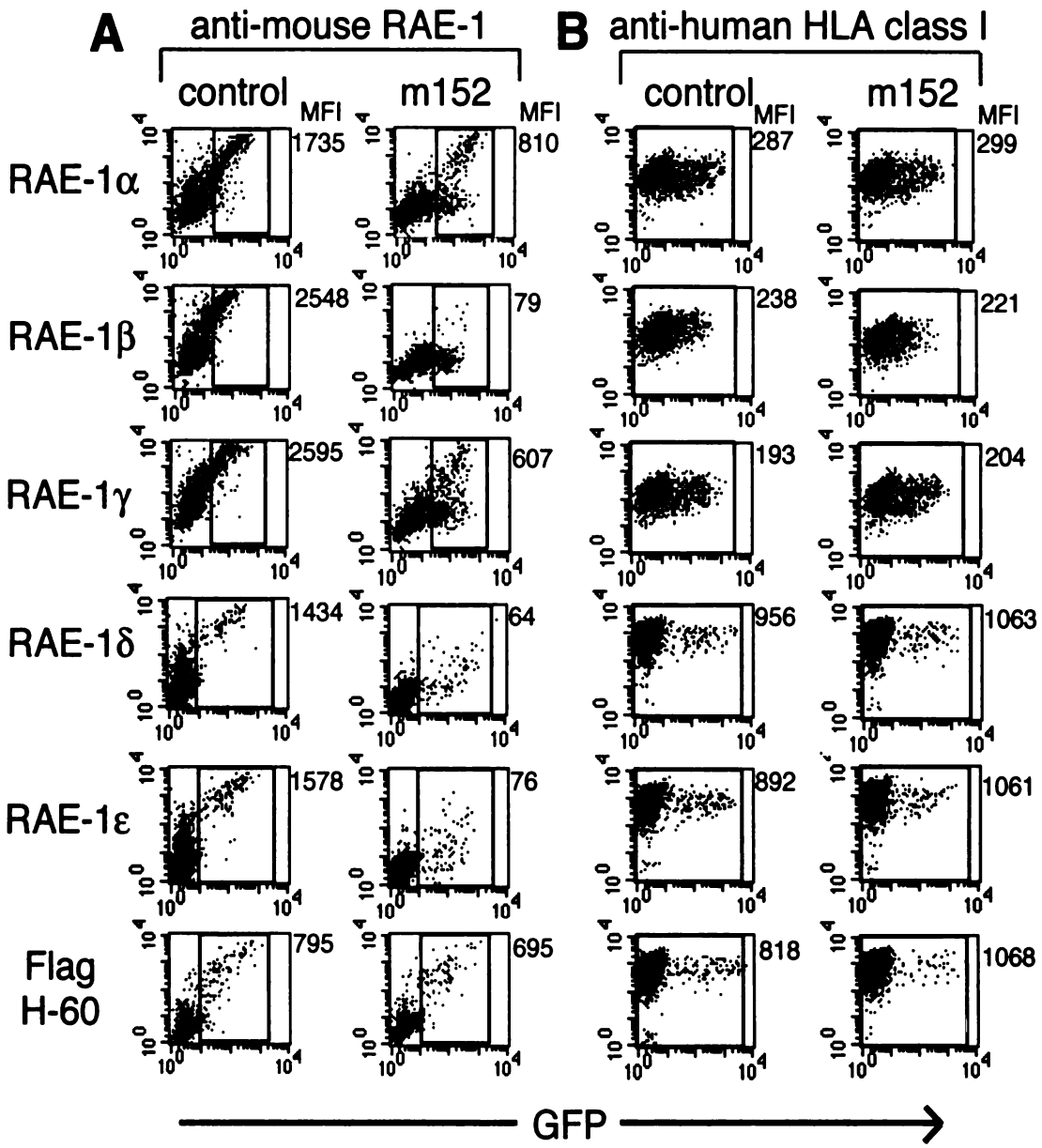

Figure 3.3. gp40 selectively down-regulates RAE-1 proteins but not H60. RAE-1 cDNAs or H60 cDNA (with a 5' Flag epitope tag) were co-transfected with a control empty vector, or with a vector encoding the m152 gene into human 293T cells. RAE-1 and H60 cDNAs were encoded on vectors carrying an IRES-EGFP, whereas a non-GFP vector was used for m152 cDNA and for the control vector. 48 hours post-transfection, cells were stained with control Ig (cIg), biotinylated anti-RAE-1 mAb CX1 (recognizing RAE-1α, β, γ), rat anti-RAE-1 mAb 186107 (recognizing RAE-1 δ, ε) or anti-Flag (A). As a control, cells were stained with anti-human HLA class I mAb DX17 (B). Mean fluorescence intensities (MFI) are shown to the right of each dot plot. MFI in (A) reflect RAE-1 or H60 expression, calculated based on gated, GFP-positive cells. MFI of human MHC class I expression (B) were determined based on the total cell population. Results are representative of similar findings in three independent experiments.

an IRES-EGFP vector. Flag-H60 cDNA was co-transfected with m152 cDNA or with a control vector into 293T cells. Cells were examined for H60 expression by anti-Flag mAb staining 48 hours post-transfection. m152 expression had little or no effect on levels of Flag-H60 (Fig. 3.3A). Co-transfection of m152 and native H60, without the Flag epitope tag, and staining with mouse NKG2D-Ig fusion protein, also indicated that m152 did not effect expression of H60 (data not shown). Thus, while gp40 down-regulates RAE-1α, β, γ, δ and ε, it does not appear to influence H60 expression. We did not examine the effect of gp40 on MULT-1 expression in this assay, because MULT-1 had not yet been identified as a ligand for NKG2D. Subsequent studies, however, showed that gp40 did not affect expression of MULT-1 (data not shown).

MCMV infection induces NKG2D-mediated IFN-γ production by NK cells

In order to evaluate the functional consequences of RAE-1 down-regulation by gp40, we analyzed IFN-γ production by NK cells co-cultured with MCMV-infected cells. For this experiment, TpnT cells (mouse embryonic fibroblasts derived from C57BL/6 mice) were used for infection. These cells stained positively with the NKG2D-Ig fusion protein (Fig. 3.4A), indicating that they constitutively expressed relatively high levels of RAE-1 proteins. TpnT fibroblasts were infected with wild-type or Δm152 mutant virus, and 72 hours post-infection cells were trypsinized and fixed. Infected cells and uninfected control cells were then co-cultured with IL-2-activated C57BL/6 NK cells for 24 hours in the presence or absence of blocking antibodies against mouse NKG2D, mouse Ly49H, or a control mAb. As additional controls, NK cells were cultured alone to determine background production of IFN-γ, or cultured with IL-12 to stimulate maximal IFN-γ production. Supernatants were collected from the co-cultured cells and analyzed

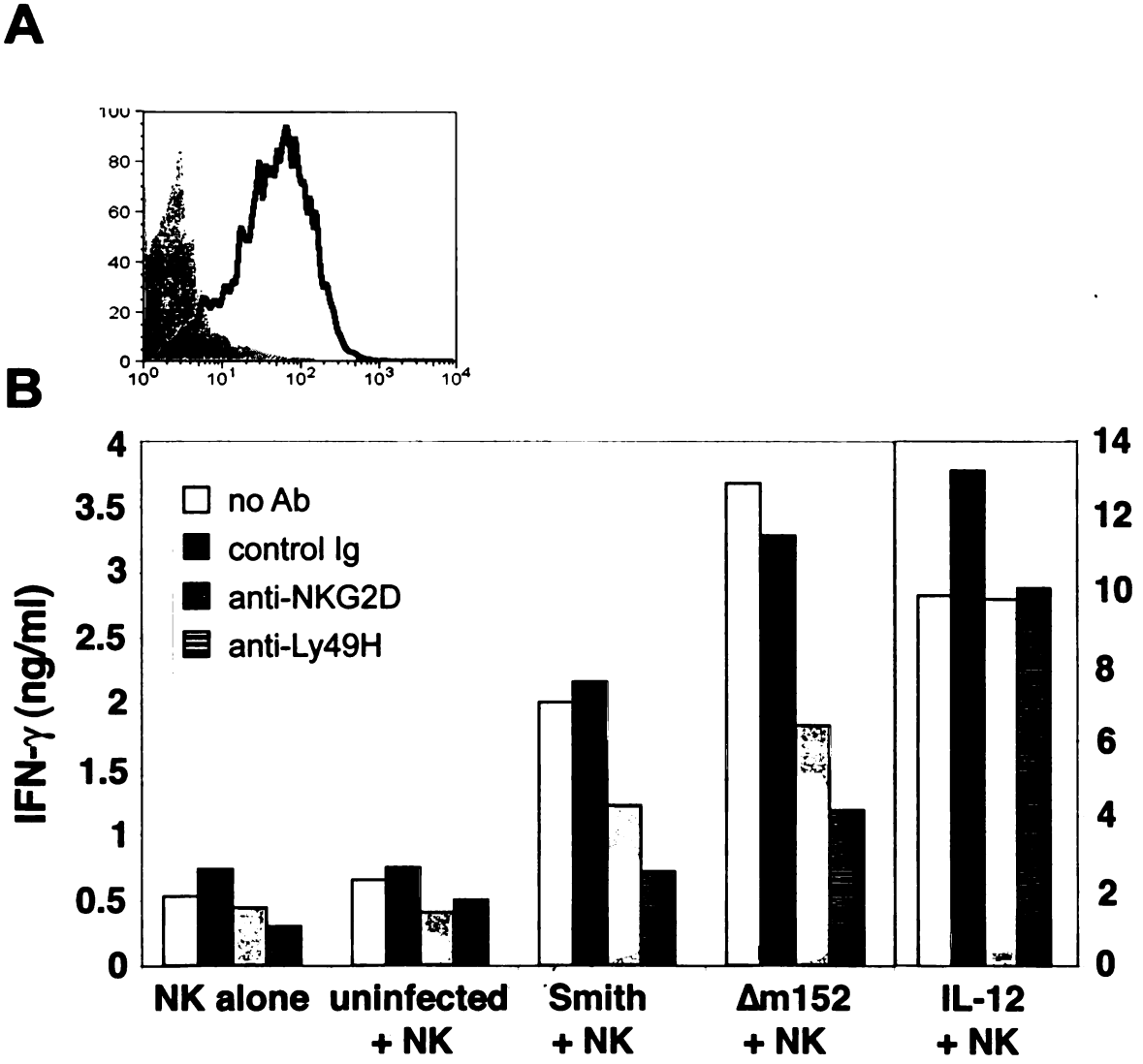

Figure 3.4. MCMV infection co-stimulates NK cell IFN-g production through NKG2D. A) TpnT cells were stained with a cIg (gray histograms) or with NKG2D-Ig fusion protein (bold histograms). B) TpnT cells were infected with Smith or Δm152 virus (MOI=1) and co-cultured with IL-2-activated C57BL/6 NK cells. NK cells were left untreated or treated with control Ig, anti-NKG2D (CX6) or anti-Ly49H (IF8), as indicated. NK cells were also cultured alone or in the presence of IL-12. Standard deviations of triplicate samples were less than 1%.

by ELISA for IFN-γ. Co-culture of NK cells with uninfected cells generated low amounts of IFN-γ, comparable to levels of IFN-γ produced by NK cells alone (Fig. 3.4B). In contrast, co-culture with virus-infected cells, particularly Δm152-infected cells, resulted in high IFN-γ production, which was partially blocked by culturing NK cells with anti-NKG2D mAb. Consistent with prior findings (66), anti-Ly49H mAb also substantially blocked IFN-γ production. Blocking antibodies did not effect IFN-γ production by NK cells cultured with IL-12. These data demonstrate that modulation of RAE-1 molecules by gp40 has functional consequences for NK cell function: the presence of RAE-1 molecules on Δm152-infected cells allows for NKG2D-dependent activation of NK cell IFN-γ production. The ability to block this effect by addition of anti-NKG2D antibody highlights the significance of RAE-1-NKG2D interactions for NK cell activation during virus infection.

m152 contributes to the virulence of MCMV by disrupting NKG2D functions

Based on the *in vitro* experiments described above, gp40-mediated down-regulation of RAE-1 molecules during MCMV infection seemed to impair the ability of NK cells to respond to MCMV-infected cells. In order to examine the physiological relevance of these findings, we evaluated the role of RAE-1-NKG2D interactions during viral infection in MCMV-susceptible BALB/c mice. Because BALB/c mice lack the Ly49H receptor, the importance of NKG2D during MCMV infection could be investigated in the absence of the dominant effects of Ly49H in C57BL/6 mice (62), which may obscure the effects of gp40 (79). Two days prior to infection, BALB/c mice were treated intraperitoneally (i.p.) with a blocking anti-NKG2D mAb (CX5) or an

isotype-matched control antibody. Treatment of mice with anti-NKG2D blocked or modulated expression of the NKG2D receptor, but did not deplete NK cells, as determined by the detection of normal frequencies of $DX5^+CD3^-$ NK cells in the anti-NKG2D mAb-treated mice (80). On the day of infection, NKG2D blocking was verified by staining splenocytes from anti-NKG2D- or control Ig-treated mice with an H60-Ig fusion protein. As shown in Figure 3.5A, treatment of mice with anti-NKG2D resulted in decreased NKG2D expression on $DX5^+CD3^-$ splenocytes. For MCMV infection, $1x10^6$ PFU of Smith or Δm152 virus were injected i.p. into anti-NKG2D- or control Ig-treated mice. Three days post-infection, spleens and livers were harvested from infected mice and homogenized. Virus was extracted from organ homogenates and used in a plaque assay to determine viral titers in each organ. As depicted in Figure 3.5B, in spleens of control Ig-treated mice, Δm152 infection resulted in significantly lower viral titers than Smith infection ($p<0.05$). It is likely that this is due to the expression of RAE-1 on the surface of Δm152-infected cells, which facilitates NK cell-mediated clearance. Consistent with this interpretation, in the anti-NKG2D-treated mice infected with Δm152, viral titers in the spleen and liver were comparable to titers in mice infected with wild-type virus, which down-regulates RAE-1 on infected cells. During infection with wild-type virus, anti-NKG2D mAb treatment consistently resulted in a slight, but not significant, increase in viral titers, as compared to control Ig-treated mice. This is not unexpected, since cells infected with wild-type virus may have very little RAE-1 on the cell surface, due to its down-regulation by gp40. Collectively, these experiments suggest that gp40, encoded by *m152*, may provide a means of MCMV escape from NK cell recognition at early times post-infection.

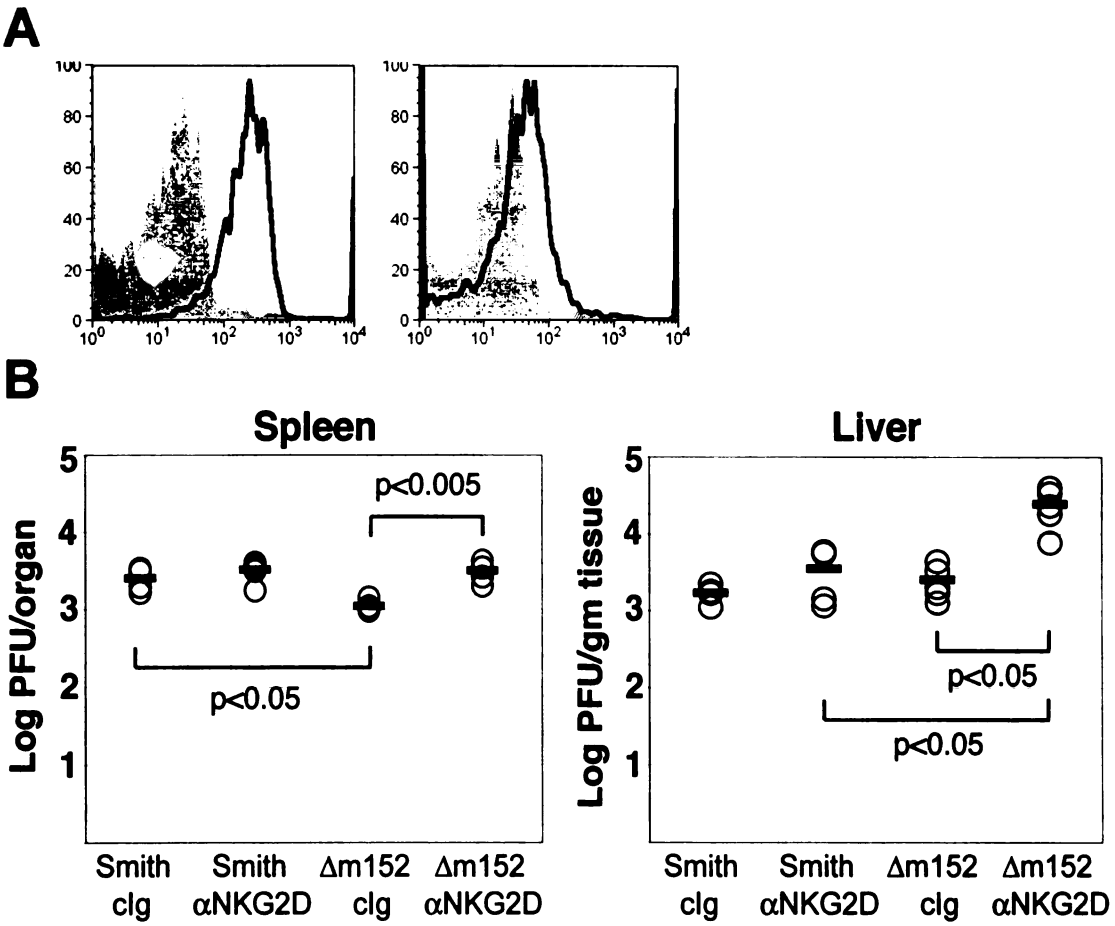

Figure 3.5. Virulence of Δm152 virus is restored by blocking NKG2D in vivo. A) BALB/c mice were injected i.p. with control Ig or anti-NKG2D mAb (CX5). As a control to ensure NKG2D modulation, spleens were harvested from control Ig-treated and anti-NKG2D-treated animals 48 hours after antibody treatment. DX5+CD3- splenocytes were stained with a control Ig (gray histograms) or an H60-Ig fusion protein for NKG2D (bold histograms). B) 48 hours after antibody treatment, mice were infected with 1x106 PFU of Smith or Δm152. 3 days post-infection, mice were sacrificed and spleens and livers were harvested. Plaque assays were performed on organ homogenates to determine viral titers in spleen and liver. Five mice were used per group. Two independent experiments were performed with comparable results; a representative experiment is shown. Statistical analysis was performed using the Student's two-tailed T-test with unequal variance.

Discussion

In this study, we have precisely defined an additional target for the MCMV immunomodulatory gene product gp40. In addition to MHC class I down-regulation (11), gp40 selectively prevents cell surface expression of RAE-1 antigens and impairs NK cell recognition of virus-infected cells. MCMV gp40 does not reduce transcription of the *RAE-1* genes, but appears to prevent expression of RAE-1 glycoproteins on the cell surface by a post-transcriptional mechanism. The ability of gp40 to affect expression of both conventional H-2 class I antigens and the RAE-1 proteins is remarkable, considering that RAE-1 shares only limited amino acid homology with MHC class I (<20%) (44, 45). RAE-1 glycoproteins lack an α3 domain, do not bind peptides, do not associate with β2-microglobulin and are glycosylphosphatidylinositol (GPI)-anchored proteins. Like MHC class I, though, RAE-1 glycoproteins have an extracellular domain containing α1 and α2 helices, which are believed to be the sites of gp40 interaction with MHC class I (82). Further studies will reveal whether gp40 binds to RAE-1 and H-2 in a similar fashion.

Although gp40 impacts all five known RAE-1 molecules, RAE-1α, β, γ, δ and ε, it interestingly does not seem to affect surface expression of H60, another cell surface MHC class I-like glycoprotein with α1 and α2 domains that binds to NKG2D with high affinity (83). While the RAE-1 proteins share a high degree of similarity (94-98% identical), H60 has only ~25% homology to the RAE-1 family (44, 45). It is tempting to speculate that MCMV may have evolved gp40 to bind the RAE-1 proteins, but not H60, because of selective pressure exerted by the immune system. The abundance of RAE-1 RNA in infected cells may have driven the evolution of a viral protein that can specifically prevent cell surface expression of RAE-1 glycoproteins, in addition to

conventional H-2 class I. Since H60 transcription does not appear to be induced by MCMV infection of macrophages, gp40 binding to H60 proteins may confer little selective advantage to the virus.

A recent paper by Krmpotic et al. (79) also demonstrated down-regulation of NKG2D ligands by gp40. In an effort to address whether the susceptibility of BALB/c mice to MCMV is caused by virus-induced subversion of NK cell-mediated immunity, these investigators examined the ability of two MCMV-encoded MHC class I modulators, gp40 and gp48, to regulate NK cell activation. They found that the presence of gp40 during viral infection resulted in diminished NK cell control of MCMV in the lungs of BALB/c, but not C57BL/6 mice. Thus, this study convincingly demonstrated the importance of gp40 in viral pathogenesis *in vivo* in BALB/c mice using a m152-deletion mutant virus (79). Based on prior evidence that BALB/c, but not C57BL/6 mice can express H60 (52), these investigators proposed that gp40 might preferentially affect expression of H60. However, no evidence was provided to verify that H60 is present in MCMV-infected cells or that gp40 can directly modulate H60 expression. In addition to differences in H60 expression, C57BL/6 and BALB/c mice differentially express *RAE-1* genes: RAE-1α, β and γ are expressed in BALB/c but not in C57BL/6 mice, whereas RAE-1δ and ε are expressed in C57BL/6 but not in BALB/c mice. Therefore, the different immune responses to MCMV observed in BALB/c compared to C57BL/6 mice must take into account the expression of different *RAE-1* genes and different activating Ly49 receptors, as well as other background differences, in these two mouse strains.

Immunity to MCMV requires both the action of NK cells and cytotoxic T cells (CTL), both of which express NKG2D. In this regard, it is interesting that gp40 has

evolved to block cell surface expression of conventional MHC class I and RAE-1, a mechanism that may be particularly effective in evading detection by CTL. Our present studies have focused on the early NK cell-mediated events after viral infection. We have shown that transcription of *RAE-1* genes was induced by MCMV infection of primary cells, but that viral gp40 efficiently prevented cell surface expression of the RAE-1 glycoproteins. The physiological importance of this was revealed by comparing the virulence of wild-type and Δm152 virus in MCMV-susceptible BALB/c mice. Consistent with prior findings (79), the m152-deficient virus showed reduced virulence during *in vivo* infection of BALB/c mice, possibly due to the elimination of RAE-1-bearing virus-infected cells by an NKG2D-dependent mechanism. This hypothesis was tested by treating BALB/c mice with anti-NKG2D mAb prior to infection. Treatment of mice with anti-NKG2D mAb restored viral titers of Δm152-infected mice to levels comparable to wild type virus-infected mice. These findings support an important role for m152 in viral evasion of NKG2D-dependent immune surveillance. Blockade of NKG2D in mice infected with wild-type virus showed only modest effects on viral titers early after infection, a result predicted if gp40 efficiently suppresses RAE-1 expression in infected cells.

The ability of gp40 to down-regulate MHC class I has been clearly implicated in evasion of CTL-mediated immunity late after viral infection. However, during the early stages of infection dominated by NK cell immunity, loss of inhibitory MHC class I should render virus-infected cells more susceptible to NK cell attack. In mice infected with m152-deficient virus, which does not efficiently block H-2 expression, however, viral titers in the spleen were in fact lower than in mice infected with wild-type virus at

early times after infection. As noted previously, blockade of NKG2D restored virulence of the m152-deficient virus, suggesting that NKG2D may permit NK cells to recognize and eliminate virus-infected cells, even if MHC class I is expressed on the infected cells. Similarly, recent studies have shown that NK cells are capable of rejecting tumors bearing MHC class I if they express NKG2D ligands (49, 50).

Based on experiments with gene-deficient mice, it was found that a perforin-dependent NK cell-mediated cytotoxicity is critical for control of MCMV in the spleen, but in the liver, NK cell-derived IFN-γ is a predominant mechanism of viral clearance (84). We have shown that C57BL/6 NK cells cultured with wild-type or Δm152-infected cells produced IFN-γ by a mechanism that is at least in part dependent on NKG2D. We have also shown that IFN-γ produced by C57BL/6 NK cells co-cultured with virus-infected cells is largely due to Ly49H-dependent activation, since this effect is blocked by anti-Ly49H antibody. Ly49H, expressed in C57BL/6 but not BALB/c mice, recognizes m157, a MCMV-encoded MHC class I-like glycoprotein that is expressed on the surface of virus-infected cells (66, 85). Engagement of Ly49H by m157 results in NK cell activation and contributes to NK cell protection against MCMV in C57BL/6 mice (61-63). Our *in vitro* results imply that NKG2D may co-stimulate Ly49H-dependent NK cell activation, thereby providing more efficient immunity in MCMV-resistant C57BL/6 mice. However, it appears that Ly49H is the dominant player in this response *in vivo*, since prior studies failed to demonstrate a substantial difference in viral titers in C57BL/6 mice infected with wild-type or m152 deletion mutant viruses (79). Interestingly, we were unable to observe IFN-γ production by BALB/c NK cells *in vitro* in response to MCMV-infected BALB/c-derived fibroblasts (unpublished observations).

These data suggest that Ly49H may be necessary for robust IFN-γ production in response to MCMV infection.

By down-regulating expression of RAE-1 molecules, thereby impairing NK cell recognition of virus-infected cells, gp40 may effectively undermine the early NK cell response to MCMV infection. This mechanism of immune evasion likely contributes to the complex interplay of viral persistence and NK cell protection inherent to herpesvirus infections.

Acknowledgements

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Chapter Four

Down-Modulation of H60 by MCMV m155

Introduction

A remarkable feature of cytomegaloviruses (CMVs) is their ability to persist for the lifetime of the host despite a fully functional immune system. This occurs through the concerted activity of multiple viral immune evasion molecules, termed immunoevasins (86), which selectively target essential components of the immune response to pathogens and undermine immune surveillance mechanisms. Several human and mouse CMV proteins have been identified that modulate MHC class I expression in infected cells and effectively inhibit antigen presentation to cytotoxic T lymphocytes (CTL) (5).

CMV can also evade NK cells, which are critical effector cells in the innate immune response to both MCMV and HCMV (58, 59). A protective role of the NKG2D receptor in the response to CMV has been revealed by examining the effect of CMV infection on NKG2D ligands, several of which are down-regulated during infection. As shown in Chapter 3, RAE-1 proteins are down-regulated during MCMV infection by the *m152* gene product gp40, which functionally impairs NKG2D-mediated NK cell recognition of infected cells (79, 87). Because H60 was not affected by gp40, we set out to determine if MCMV infection also impacts expression of H60 on virus-infected cells.

Results and Discussion

MCMV infection down-regulates H60

Infection of 3T3 cells with MCMV results in strong down-regulation of RAE-1 by gp40, encoded by the *m152* gene (79, 87). However, gp40 did not down-regulate H60, another high affinity ligand for NKG2D. To address whether MCMV affects H60, we infected 3T3 cells, which constitutively express H60 on the cell surface, with MCMV and 48 hours post-infection analyzed cells for H60. We observed a marked decrease in the level of cell-surface H60 after infection (Fig. 4.1).

To identify the MCMV gene product that down-regulates H60, we infected 3T3 cells with a panel of deletion mutant viruses. After infection with a deletion mutant lacking the m150-m165 ORFs (Δm150-165), H60 expression was partially restored (Fig. 4.1). A deletion mutant virus lacking only m152, which we previously showed could not down-regulate the RAE-1 glycoproteins, retained the ability to down-regulate H60. These data indicate that H60 is down-regulated by a gene product within the m150-165 block, but is not affected by m152.

MCMV m155 down-regulates H60 but not RAE-1 or MULT-1

By transiently transfecting human 293T cells with vectors encoding H60 and vectors encoding each of the MCMV ORFs, we were able to examine the effect of individual MCMV gene products on H60 expression. H60 was expressed in a vector containing an IRES-EGFP element, permitting visualization of GFP^+ cells that express H60 upstream of the IRES. We observed that co-transfection of H60 with m155 resulted in a substantial decrease in H60 expressed on the surface (Fig. 4.2). We also considered

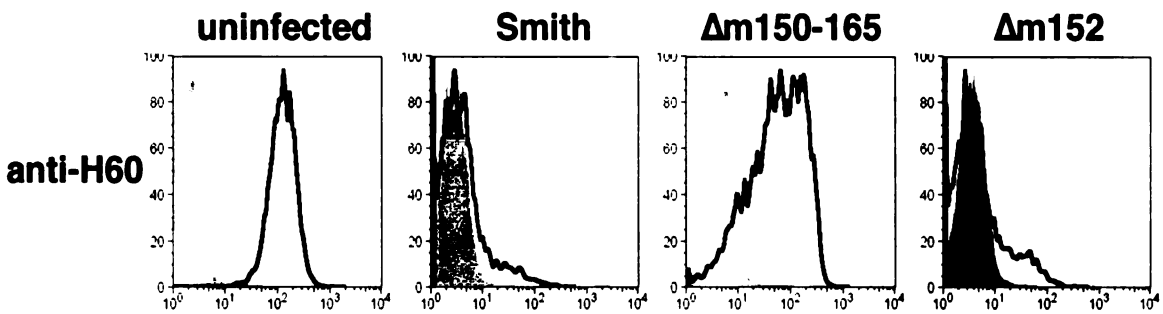

Figure 4.1. MCMV infection down-regulates H60. 3T3 cells were infected with wild-type MCMV (Smith), DMS94.5 (Δm150-165) or ΔMC96.24 (Δm152) viruses at an MOI of two. 48 hr post-infection cells were stained with control IgG2a (dotted histograms) or anti-H60 mAb (bold histograms).

the possibility that m155 may affect other ligands for NKG2D, such as RAE-1 or MULT-1. m155 did not affect of any of the other known NKG2D ligands, since expression of these molecules was not changed by co-transfection with m155 (Fig. 4.2). In addition, m155 did not cause global down-regulation of cell surface receptors, because the level of MHC class I was unaltered on the surface of 293T cells transfected with m155 (not shown).

Zhan et al. (88) previously described an MCMV mutant, Dm155, in which the m155 ORF was disrupted by random insertion of a transposon (Fig. 4.3). This m155-deficient virus demonstrated normal viral replication *in vitro*, but was severely attenuated in SCID mice (89). To assess the effect of m155 on H60 in cells infected with MCMV, we infected 3T3 cells with the Dm155 virus and a revertant virus that restored m155 expression. The Dm155 virus failed to efficiently down-regulate H60, whereas H60 down-regulation was largely restored by infection with the revertant virus (Fig. 4.4). Therefore, m155 plays a significant role in H60 down- regulation.

Because the level of H60 on Dm155-infected cells was not completely restored to the level of H60 on uninfected 3T3 cells, other MCMV gene products may also contribute to H60 down-regulation. To examine the expression of other NKG2D ligands after infection with Dm155 and revertant virus, we stained cells infected with these viruses for RAE-1 and MULT-1. Consistent with the prior finding that MCMV infection down-regulated RAE-1, the revertant virus strongly down-regulated RAE-1 (Fig. 4.4). Interestingly, we also observed down-regulation of MULT-1 after infection with wild-type (not shown) and the revertant virus (Fig. 4.4), which is the first evidence that MULT-1 is also impacted by MCMV. Infection of 3T3 cells with Dm155 did not,

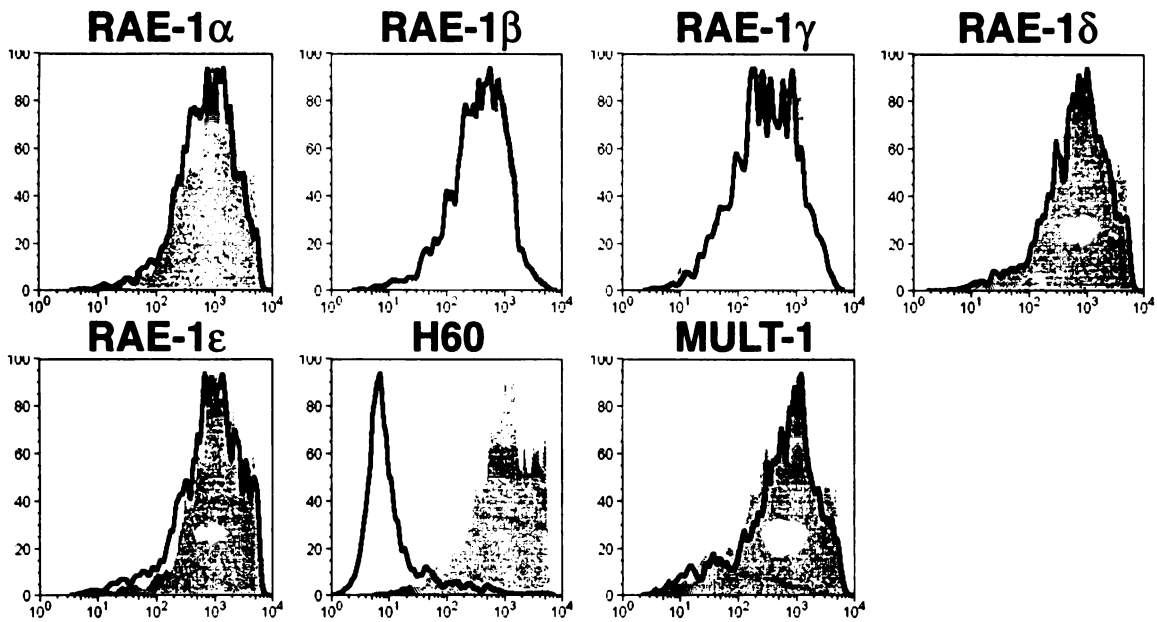

Figure 4.2. m155 down-regulates H60 but not RAE-1 or MULT-1. A) 293T cells were transfected with a vector encoding H60, MULT-1 or RAE-1 and either a control vector (gray histograms) or a vector encoding m155 (bold histograms). 48 hr post-transfection cells were stained with anti-H60, anti-MULT-1 or anti-RAE-1 mAbs. H60, MULT-1 and RAE-1 were encoded on vectors carrying an IRES-EGFP, whereas a non-GFP vector was used for m155 cDNA and for the control vector. Histograms show GFP+ populations.

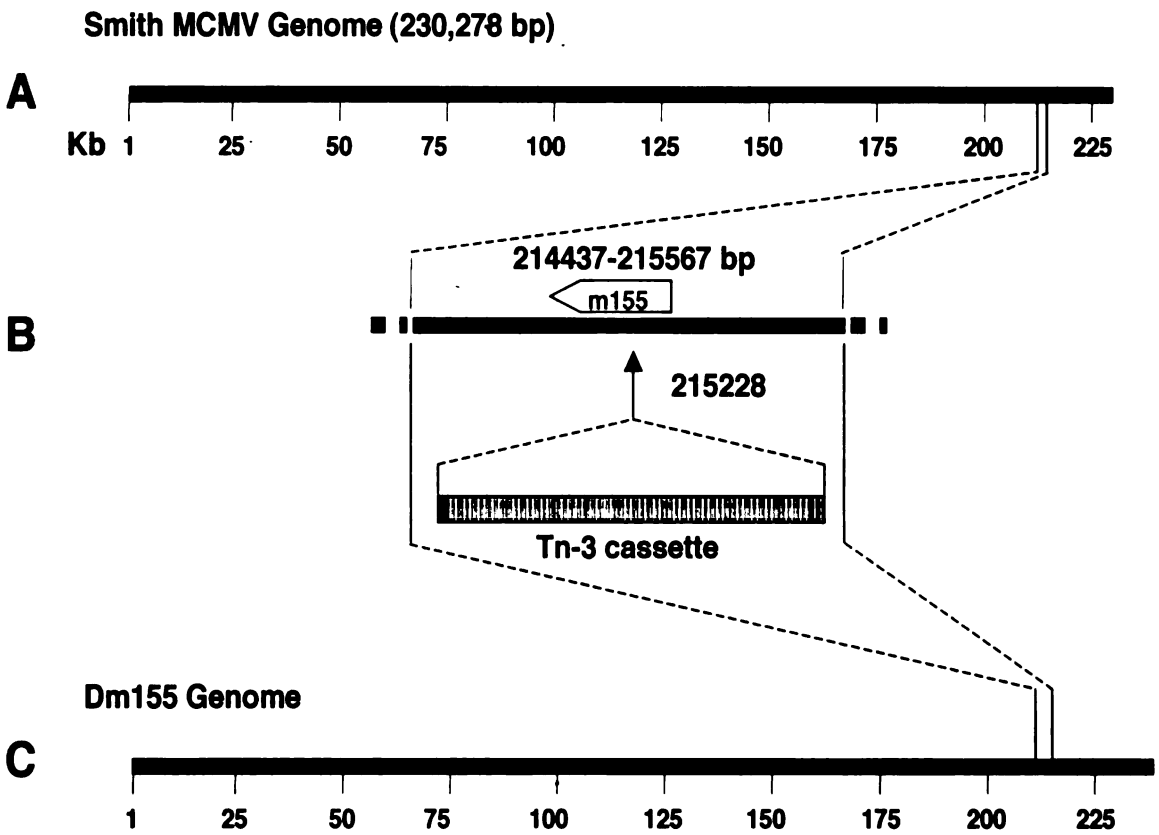

Figure 4.3. Tn-3 mutagenesis of the MCMV m155 gene. The MCMV genome (A) was cut into fragments ranging from 1.6-4 kb by partial restriction with Sau3A, then randomly mutagenized by Tn-3 transposition. The fragment containing the m155 gene (B: nucleotide position 2144437-215567) was verified by sequencing to be transposed at nucleotide 215228. The mutagenized fragment was recombined into the viral genome by homologous recombination to generate the mutant virus Dm155 (C).

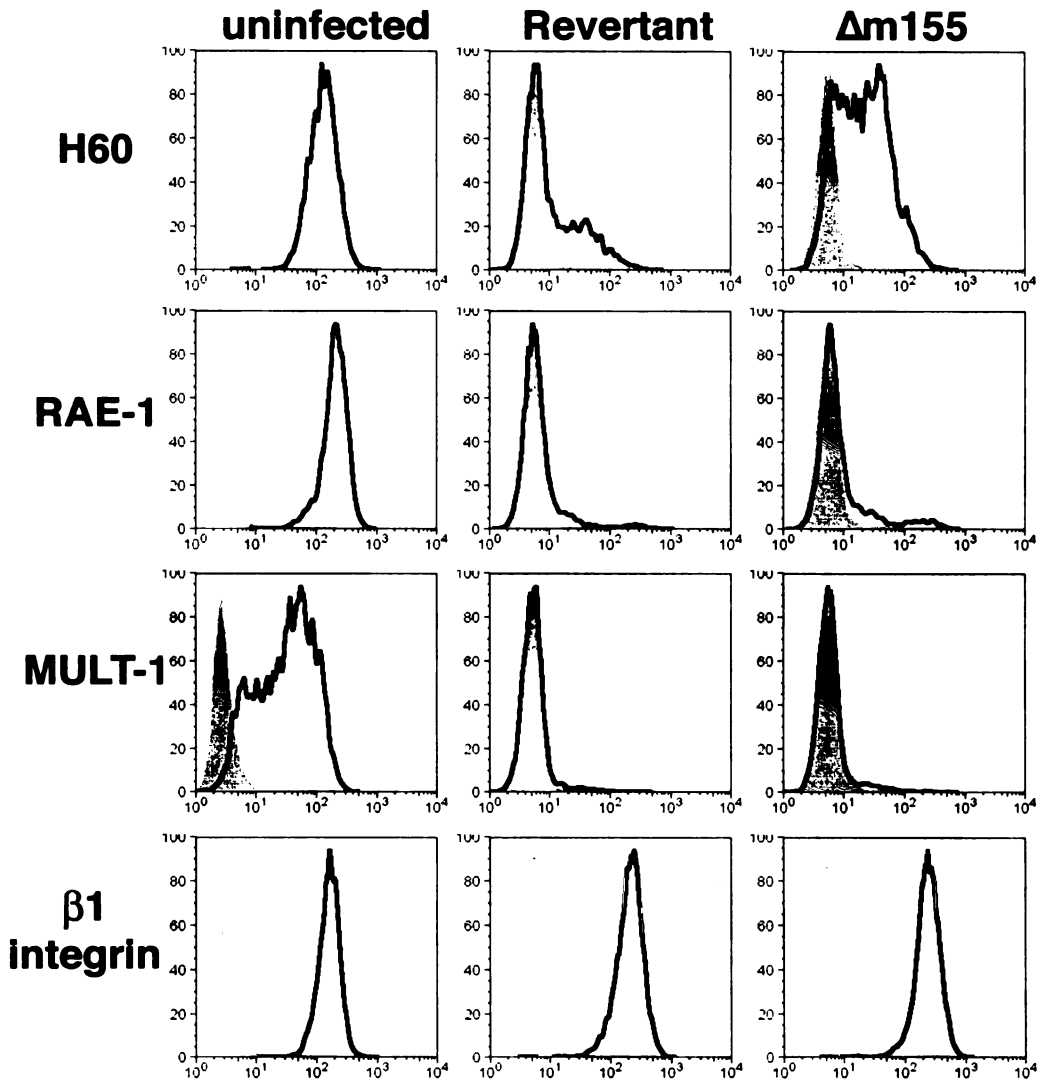

Figure 4.4. Δm155 does not efficiently down-regulate H60. 3T3 cells were infected with Rqm155-Rq155 (the m155 revertant virus) or Dm155 (Δm155) virus at an MOI of two. 48 hr post-infection cells were stained with control IgG2a (gray histograms), anti-H60, anti-RAE-1, anti-MULT-1, or anti-β1-integrin (bold histograms).

however, restore cell surface expression of either RAE-1 or MULT-1, again indicating that m155 does not impact expression of these glycoproteins. To demonstrate that MCMV infection did not cause nonspecific down-regulation of cell surface receptors, we stained the cells for expression of β1-integrin, which was comparable on uninfected and infected cells (Fig. 4.4). Thus, m155 selectively targets H60.

Unlike gp40, which modulates expression of both RAE-1 and MHC class I, m155 does not affect expression of MHC class I. Expression of H-2D^d and H-2K^d on RAW264.7 cells was unaltered by transient transfection with a plasmid encoding m155, implying that m155 does not affect these haplotypes (not shown). This is consistent with the finding that the products of the *m04, m06* and *m152* genes are the only MCMV immunoevasins that substantially impact MHC class I (7).

m155 down-regulates H60 via a proteasome-dependent mechanism

In addressing the mechanism by which m155 down-regulates H60, we considered the possibility that m155 may affect H60 transcription. However, when we analyzed H60 RNA levels in 3T3 cells compared to 3T3 cells stably transfected with m155 (designated CT498 cells), we found a less than 2-fold difference in H60 transcription (Fig. 4.5). This seems insufficient to account for the profound down-regulation of H60 by m155. Further, *m155* encodes a potential membrane glycoprotein, suggesting that it may interact with H60 in a manner similar to the interaction of other CMV proteins with MHC and MHC-like proteins.

Although H60 transcription was not substantially affected by expression of m155, H60 protein was strongly down-regulated from the surface of CT498 cells, compared to

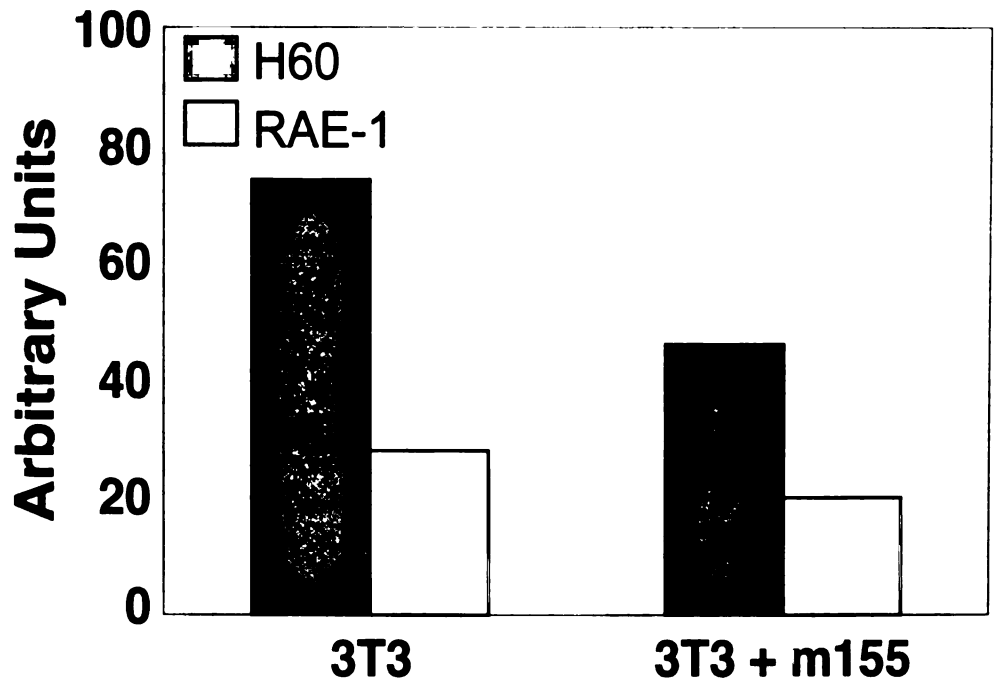

Figure 4.5. m155 does not affect H60 transcription. Total RNA was harvested from BALB/c 3T3 cells and CT498 cells (3T3 cells stably transfected with m155) and cDNA was synthesized. TaqMan quantitative RT-PCR (87) was performed with primer/probe sets recognizing H60 (80) or recognizing all known RAE-1 genes (90). All samples were normalized to the signals generated from HPRT. Standard deviations of triplicate samples were <1%.

untransfected 3T3 cells (Fig. 4.6B). We then hypothesized that m155 may cause degradation of H60. To examine this possibility, we treated 3T3 and CT498 cells with lactacystin, a cell permeable, irreversible proteasome inhibitor, and immunoprecipitated H60 from lysates of treated or untreated cells. As revealed by Western blotting, an ~80 kD band, representing H60, was immunoprecipitated from lysates of 3T3 cells (Fig. 4.6A). No H60 protein was detected when lysates were immunoprecipitated with a control rat IgG2a, indicating that the 80 kD band is specific for H60. In lysates from CT498 cells, the 80 kD band was no longer present, consistent with the absence of H60 from the surface of these cells. However, treatment of CT498 cells with lactacystin restored H60 protein. We also observed restoration of H60 protein in lysates from CT498 cells treated with epoxomicin, another specific inhibitor of the proteasome (not shown).

We also examined whether H60 protein was restored on the cell surface of CT498 cells after treatment with lactacystin or epoxomicin. We observed that treatment with both proteasome inhibitors resulted in high expression of H60 on the surface of CT498 cells (Fig. 4.6B). As a control, we analyzed RAE-1 expression on cells treated with the inhibitors and found that it was essentially unaffected. Thus, specific inhibition of the proteasome restored both intracellular and cell surface H60 in cells expressing m155, suggesting that the m155 gene product down-regulates H60 via a proteasome-dependent mechanism.

It remains unclear how H60 is restored inside cells and on the cell surface by proteasome inhibition. Proteasome inhibitors may prevent direct degradation of H60 by the proteasome, or may serve to stabilize H60 in an indirect manner. The latter

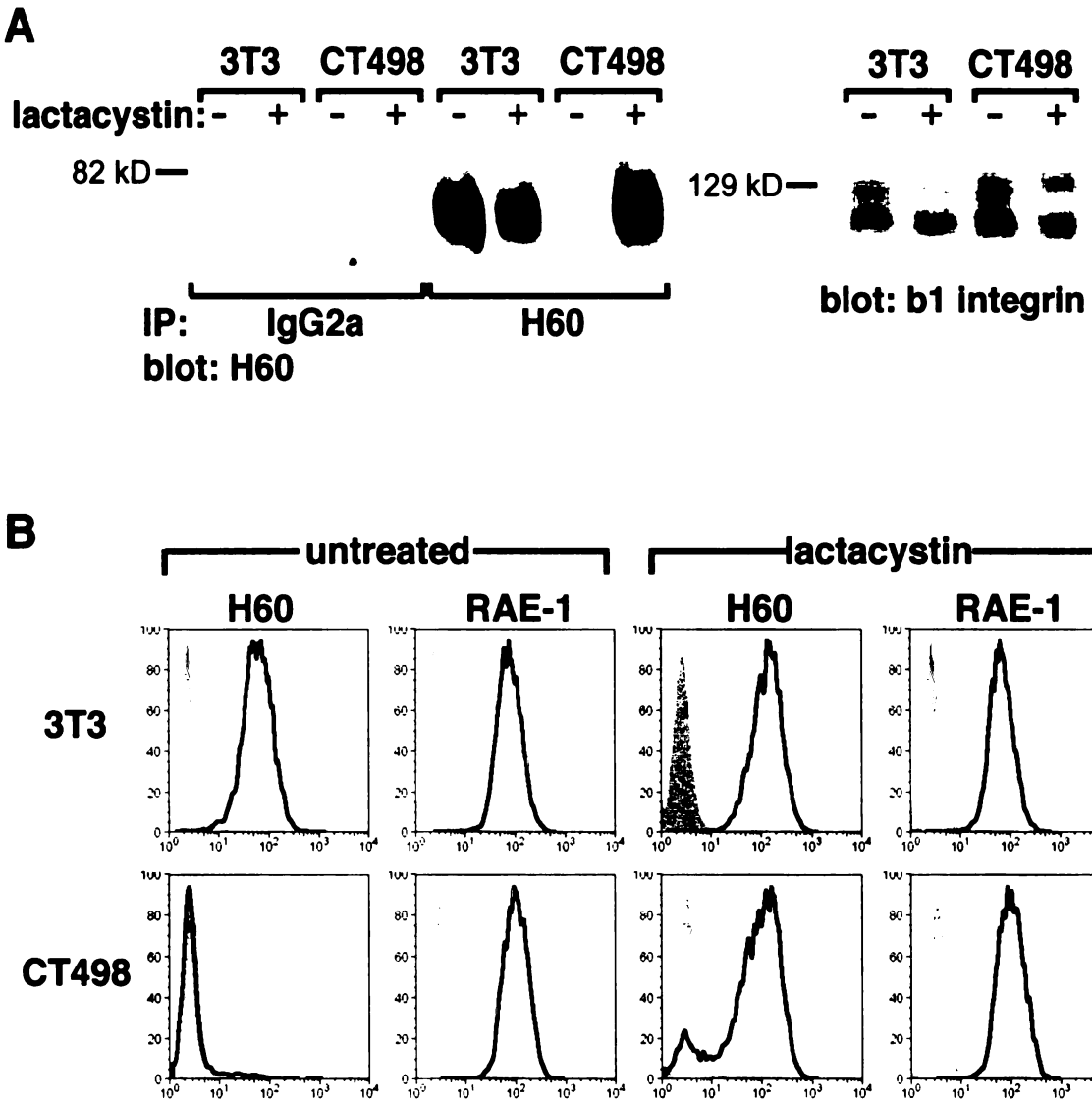

Figure 4.6. Proteasome inhibition reverses m155 down-regulation of H60. A) Lysates were generated from untreated cells or cells treated with 10μM lactacystin for 14 hr and immunoprecipitated with control Ig or anti-H60 mAb. Western blotting was performed for H60 or b1-integrin protein. B) Untreated 3T3 or CT498 cells and 3T3 or CT498 cells treated with 10μM lactacystin or 10 μM epoxomicin for 14 hr were stained with a control Ig (dotted histograms), anti-H60 or anti-RAE-1 mAbs (bold histograms) and propidium iodide (PI). Histograms show PI-negative cells (>95% of total cells).

possibility has been described for the stabilization of MHC class I complexes in cells expressing KSHV K3, which directs internalization of MHC class I from the surface of KSHV-infected cells (91). Because the ubiquitin-proteasome system has been implicated in regulation of the endocytic pathway (91), it may be involved in the down-regulation of H60 from the surface of MCMV-infected cells.

To examine H60 protein during MCMV infection, we infected 3T3 cells with the Dm155 or revertant virus and analyzed H60 protein in lysates from these cells by immunoprecipitation and Western blotting. Consistent with the cell surface down-regulation of H60 by MCMV infection, H60 was undetectable in lysates of 3T3 cells infected with revertant virus (Fig. 4.7). However, H60 protein was partially restored in lysates from cells infected with Dm155 virus. These data indicate that MCMV infection down-regulates H60 protein both from the cell surface and inside cells, and that this effect can be attributed to m155.

NKG2D-mediated protection against MCMV is impaired by m155

The down-regulation of H60 by m155 suggests it may impair NK cell recognition of virus-infected cells. We, therefore, examined the significance of H60 down-regulation during MCMV infection *in vivo*. Prior to infection of BALB/c mice, which express functional H60 (52), we treated mice with a control rat IgG mAb, a neutralizing anti-NKG2D (CX5) mAb or an NK cell-depleting anti-asialo GM1 antiserum. CX5 mAb blocks the binding of NKG2D to its ligands, and it and modulates the NKG2D receptor from the surface of NK cells, but does not deplete NK cells, thereby allowing us to

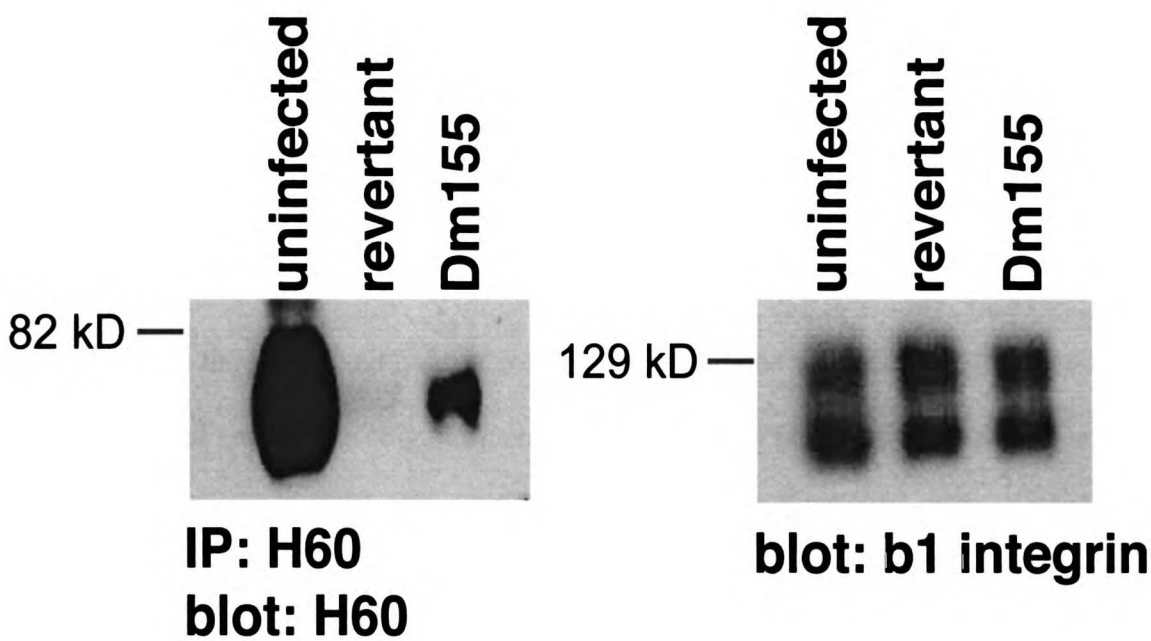

Figure 4.7. H60 protein is absent in MCMV-infected cells. Lysates were generated from uninfected 3T3 cells, 3T3 cells infected with Rvm155 or 3T3 cells infected with Rqm155, and immunoprecipitated with anti-H60 mAb. Western blotting was performed for H60 or b1-integrin protein.

directly examine the role of the NKG2D receptor in the immune response to MCMV. To investigate the total contribution of NK cells to the MCMV immune response, we depleted NK cells by using anti-asialo GM1 antiserum. After antibody treatment, mice were infected i.p. with $1x10^5$ PFU of virus per mouse (Fig. 4.8A) or with $2x10^5$ PFU (Fig. 4.8B) per mouse of wild-type Smith, Dm155 or the m155 revertant virus. Because prior studies have implicated NK cells in the control of MCMV predominantly in the liver and spleen and early during infection, viral titers in these organs were determined on day 3 post-infection.

In both the spleen and liver of control rat IgG-treated mice, the Dm155 virus showed marked attenuation (Fig. 4.8), consistent with prior observations that the Dm155 virus is severely attenuated in growth in multiple organs of SCID mice (89). After treatment of mice with either anti-asialo GM1 or anti-NKG2D, however, viral titers in the spleens of Dm155-infected mice were almost identical to titers of the wild-type and revertant viruses. In the livers of mice treated with anti-NKG2D, titers of Dm155 virus were identical to wild-type and revertant viruses in mice infected with $1x10^5$ PFU (Fig. 4.8A) and slightly higher than titers of wild-type and revertant viruses in mice infected with $2x10^5$ PFU (Fig. 4.8B). Interestingly, in mice depleted of NK cells with anti-asialo GM1, the viral titers in the livers of Dm155-infected mice were slightly higher than viral titers of wild-type and revertant viruses. Collectively, these data indicate that NK cells play a critical role in the control of the Dm155 virus, and that this protection is predominantly mediated by NKG2D. Thus, m155 down-regulation of H60 is an important mechanism of MCMV evasion of NK cell immune surveillance.

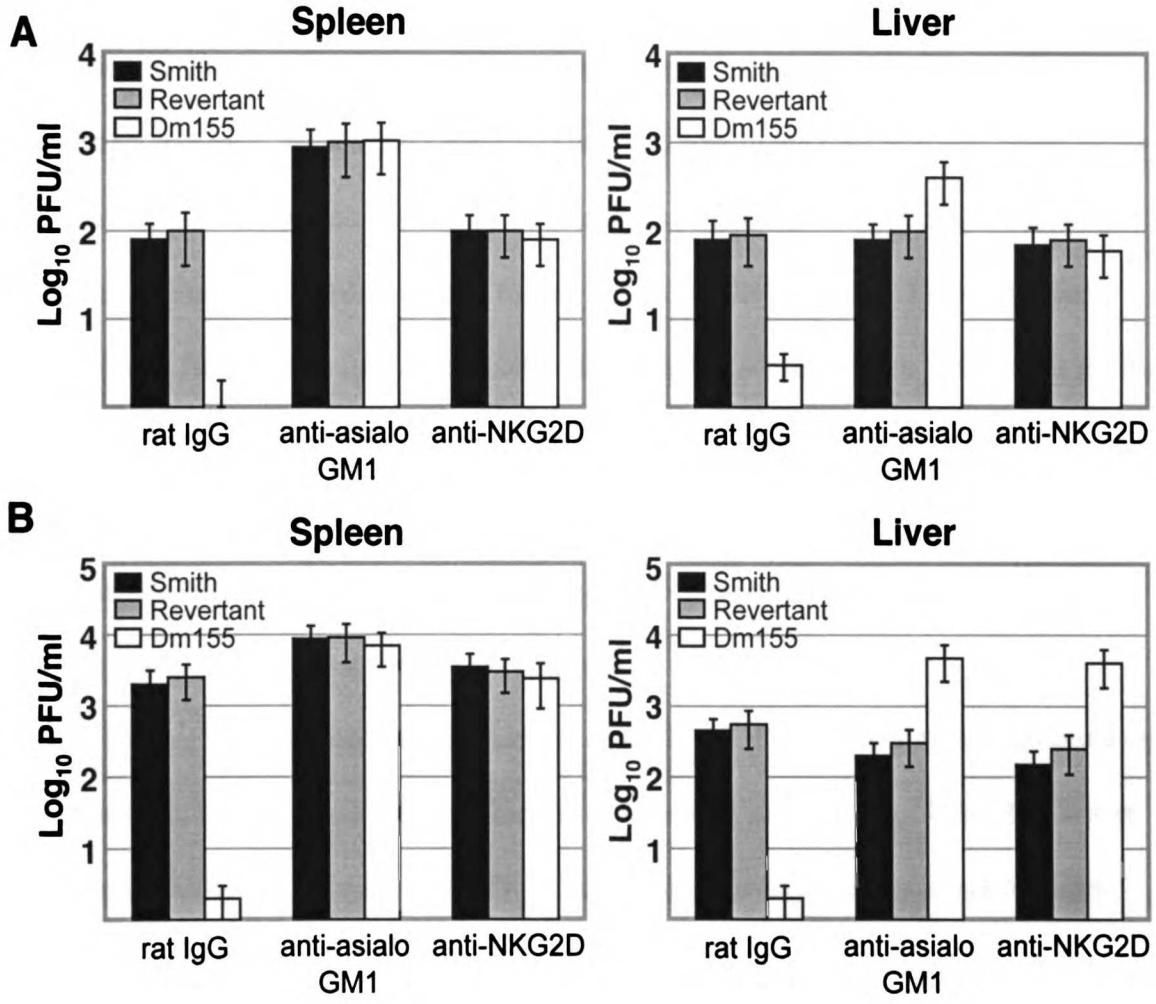

Figure 4.8. Depletion of NK cells or blockade of NKG2D restores virulence of Dm155. A) BALB/c mice were injected i.p. with control rat IgG, anti-asialo GM1 antisera, or anti-NKG2D mAb (CX5). After antibody treatment, mice were infected with 1x105 PFU (A) or 2x105 PFU (B) of Smith, Rqm155 (revertant) or Rvm155 (Dm155). 3 days post-infection, spleens and livers were harvested. Plaque assays were performed on organ homogenates to determine viral titers. In each experiment, three mice were used in each experimental group, and error bars represent the standard deviation of the viral titers from these three mice. Three independent experiments were performed and data from two experiments using 2x105 PFU were combined for presentation in B. The limit of detection was 1 PFU/ml organ homogenate.

An emerging theme in the study of MCMV immune evasion is the discovery of viral mechanisms of modulating NK cell immunity. In addition to encoding several MHC class I modulators, MCMV expresses at least two proteins committed to down-regulation of NKG2D ligands, and these proteins have specificity for different ligands. These data indicate that NKG2D plays a significant role in the host immune response to MCMV and that the virus has evolved mechanisms to counter this pathway. The ability of the m152 and m155 gene products to down-regulate the RAE-1 and H60 proteins, respectively, from the surface of infected cells is clearly advantageous to the virus during MCMV infection, since deletion mutant viruses lacking these genes are less virulent (88, 92). However, virulence of both deletion mutant viruses is restored by treatment of mice with anti-NKG2D mAb. Therefore, blocking NKG2D recognition of its ligands during MCMV infection confers a distinct survival advantage to the virus.

During the course of MCMV infection, the NKG2D ligands may be expressed on different cells types or with different kinetics. Although expression of RAE-1 is low or absent on healthy adult tissues, MCMV infection of peritoneal macrophages strongly induces transcription of the *RAE-1* genes (87). H60 and MULT-1 transcripts, however, were not induced by MCMV infection of macrophages. Given the large number of NKG2D ligands, it is possible that they are differentially regulated in different cell types, thereby providing non-redundant functions. For this reason, perhaps it is not surprising that MCMV encodes multiple immunoevasins that modulate expression of the NKG2D ligands.

Acknowledgements

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Chapter Five

Concluding Remarks

Immune control of a persistent virus, such as CMV, is constantly challenged by viral immune evasion mechanisms. Although host immunity can control the virus, it fails eliminate it, and the virus periodically reactivates and spreads to new individuals. The result is a life-long detente between the host immune system and the virus. NK cells and CTL comprise the primary cellular immune defenses against CMV infection, and both HCMV and MCMV have developed mechanisms of eluding detection by these cells. This thesis presents the identification of a novel set of viral immune evasion genes that function by interfering with NK cell antiviral activity. Specifically, these viral gene products target the ligands for NKG2D, a widely expressed and potent activating NK cell receptor. By down-regulating NKG2D ligands from the surface of virus-infected cells, thereby impairing NK cell activation through the NKG2D receptor, both HCMV and MCMV subvert NK cell antiviral immunity.

Based on our studies, CMV appears to affect the NKG2D ligands at both a transcriptional level and a post-translational level. We observed that HCMV infection induced transcription of MIC in fibroblasts, and MCMV infection induced transcription of all five RAE-1 glycoproteins in resident peritoneal macrophages. We did not observe MCMV induction of H60 or MULT-1 transcripts in resident peritoneal macrophages, but we did not analyze these transcripts in other infected cells, so we cannot rule out the possibility that induction is cell type-dependent. The precise mechanism by which CMV induced transcription of NKG2D ligands remains unknown. The upstream regulatory elements for most of the NKG2D ligands are undefined, except for MICA and MICB, which have heat shock elements in their promotor regions (39). Hamerman et al. (93) have shown transcriptional induction of the RAE-1 genes by microbial products through

a Toll-like receptor (TLR) and MyD88-dependent pathway. To explore the possibility that MCMV also induces NKG2D ligands through a Toll-like receptor (TLR) pathway, we examined transcription of NKG2D ligands in infected cells from wild-type C57BL/6 mice and MyD88-/- mice. In resident peritoneal macrophages from MyD88-/- mice, we observed that LPS did not induce RAE-1 transcripts, while MCMV infection did (Lanier lab, unpublished data). These data indicated that induction of RAE-1 transcripts by MCMV may be distinct from the Toll-like receptor (TLR) pathway. Alternatively, because MyD88 is not the only adaptor protein down-stream of the TLRs, it is also possible that transcriptional induction of the RAE-1 genes by MCMV occurs through a different TLR TIR-domain containing adaptor protein, such as TIRAP, Trif or TRAM (94). We have also examined the effects of type I and type II interferons on induction of RAE-1, to explore the possibility that MCMV may induce RAE-1 indirectly, through interferon secretion by infected cells or bystander cells. Neither IFN-α nor IFN-γ, however, induced transcription of the RAE-1 genes in resident peritoneal macrophages, suggesting that at least for these cells, induction is not mediated through interferon signaling (Lanier lab, unpublished data).

Although we observed transcriptional induction of NKG2D ligands by CMV, this did not translate into increased expression of NKG2D ligands on the cell surface. Rather, CMV infection down-regulated NKG2D ligands in cells that endogenously expressed high levels on the cell surface. By infecting cells with deletion mutant viruses that lack certain viral ORFs and assaying for viruses that did not efficiently down-regulate NKG2D ligands, we were able to map this effect to specific viral gene products. We and others have shown that HCMV and MCMV have evolved at least four different genes

devoted to down-regulating expression of NKG2D ligands. HCMV UL16 retained ULBP-1, -2 and MICB in an intracellular compartment (41-43). The MCMV m152 gene product gp40 down-regulated the RAE-1 gene family from the surface of virus-infected cells (79, 87), the m155 gene product down-regulated H60 (95), and an as yet unidentified gene product down-regulated MULT-1. Considering that the list of NKG2D ligands has continued to grow over the last couple of years, it is possible that other ligands will be identified. Whether CMV will also affect expression of these ligands awaits further study.

One interesting feature of the viral gene products that impact NK cell activity is that many of them have structural homology to classical MHC class I. These include m152 and m155, both of which modulate NKG2D ligand expression, and m157, which is recognized by the Ly49H NK cell receptor. A bioinformatic analysis of all MCMV genes by a structural prediction program has revealed that there are 11 genes in the MCMV genome that have potential MHC-like folds (67). Given that three of these gene products have been identified as NK cell modulators, studies on the remaining eight genes may prove fruitful for deciphering other viral immune evasion mechanisms.

It seems that viral down-regulation of different NKG2D ligands occurs through distinct mechanisms. The HCMV UL16 protein has been shown to sequester ULBP-1, -2 and MICB in an ER-cis-Golgi compartment by interacting with these NKG2D ligands through its ectodomain, while its transmembrane and cytoplasmic tail anchor it in the intracellular compartment (41-43). It remains unclear how the m152 gene product gp40 down-regulates the RAE-1 glycoproteins. Prior research on gp40 revealed that it down-regulated classical MHC class I from the surface of infected cells through a transient

interaction in the ER, which led to a biochemical modification of the class I heavy chains and their retention in an ER to Golgi intermediate compartment (ERGIC) (11, 96). It is possible that gp40 retains the RAE-1 glycoproteins intracellularly by a similar mechanism. The m155 gene product, however, appears to function in a different manner, using a proteasome-dependent mechanism to down-regulate H60. H60 was no longer detectable by IP and Western blotting from either MCMV-infected cells or cells that were transfected with m155, suggesting that m155 caused H60 degradation. Treatment with two different proteasome inhibitors, which function by different mechanisms, resulted in restoration of H60 protein both inside cells and on the cell surface. Thus, rather than using a retention mechanism to down-regulate H60, m155 may cause proteasomal degradation of H60. Given that the proteasome-ubiquitin system can influence the trafficking of molecules in the endocytic pathway (91), it is also possible that m155 interacts with this system to destabilize H60 on the cell surface and causes its endocytosis and degradation via lysosomes.

Discovering the ways in which viruses elude immune surveillance not only sheds light on the complex nature of viral persistence, but also stresses the importance of specific immune control mechanisms. Viruses have evolved with their hosts for many years, developing genes that allow them to adapt to an evolving immune system. In addition, because viruses encode a relatively small number of genes, their genomes encode few superfluous proteins. Therefore, the identification of numerous CMV gene products that interfere with the MHC class I antigen presentation pathway highlights the importance of the TCR-MHC class I interaction in antiviral immune control. Likewise,

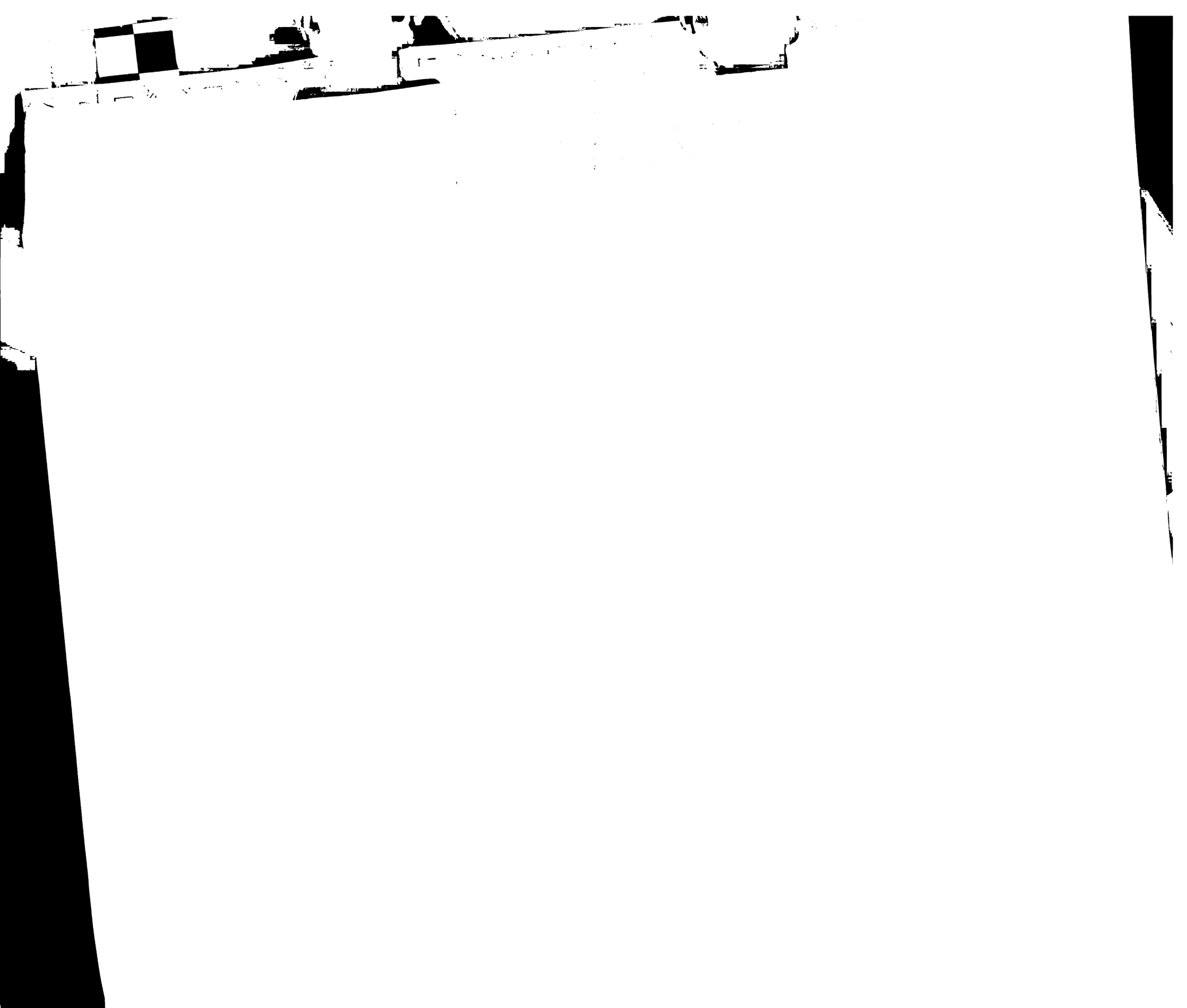
the discovery of several viral gene products that down-regulate NKG2D ligands supports a key role for the NKG2D receptor in NK cell activity during viral infection.

We examined the importance of NKG2D in the immune response to CMV infection both in vitro and in vivo. We found that secretion of IFN-γ by NK cells co-cultured with MCMV-infected cells was partially dependent on NKG2D. Additionally, in vivo infection of BALB/c mice with either Δm152 or Δm155 viruses resulted in reduced viral titers in the livers and spleens of these mice, when compared with the titers obtained from wild-type virus infected mice. However, the titers of both viruses were restored to wild-type levels if an anti-NKG2D antibody, which blocked the NKG2D receptor, was injected into the mice two days prior to infection. These findings indicate that failure of the Δm152 and Δm155 viruses to down-regulate NKG2D ligands results in NKG2D-dependent NK cell activation and clearance of virus-infected cells. They also delineate a clear functional role for NKG2D in antiviral immunity against CMV.

Thus, we have defined a function for the MCMV m152 and m155 gene products in evasion of NK cell immune surveillance. By down-regulating RAE-1 or H60, respectively, from the surface of virus-infected cells, these viral proteins prevent the interaction between the NKG2D receptor and its ligands, and effectively inhibit NK cell recognition and clearance of MCMV-infected cells.

Chapter Six

Materials and Methods



Extraction of viral genomic DNA

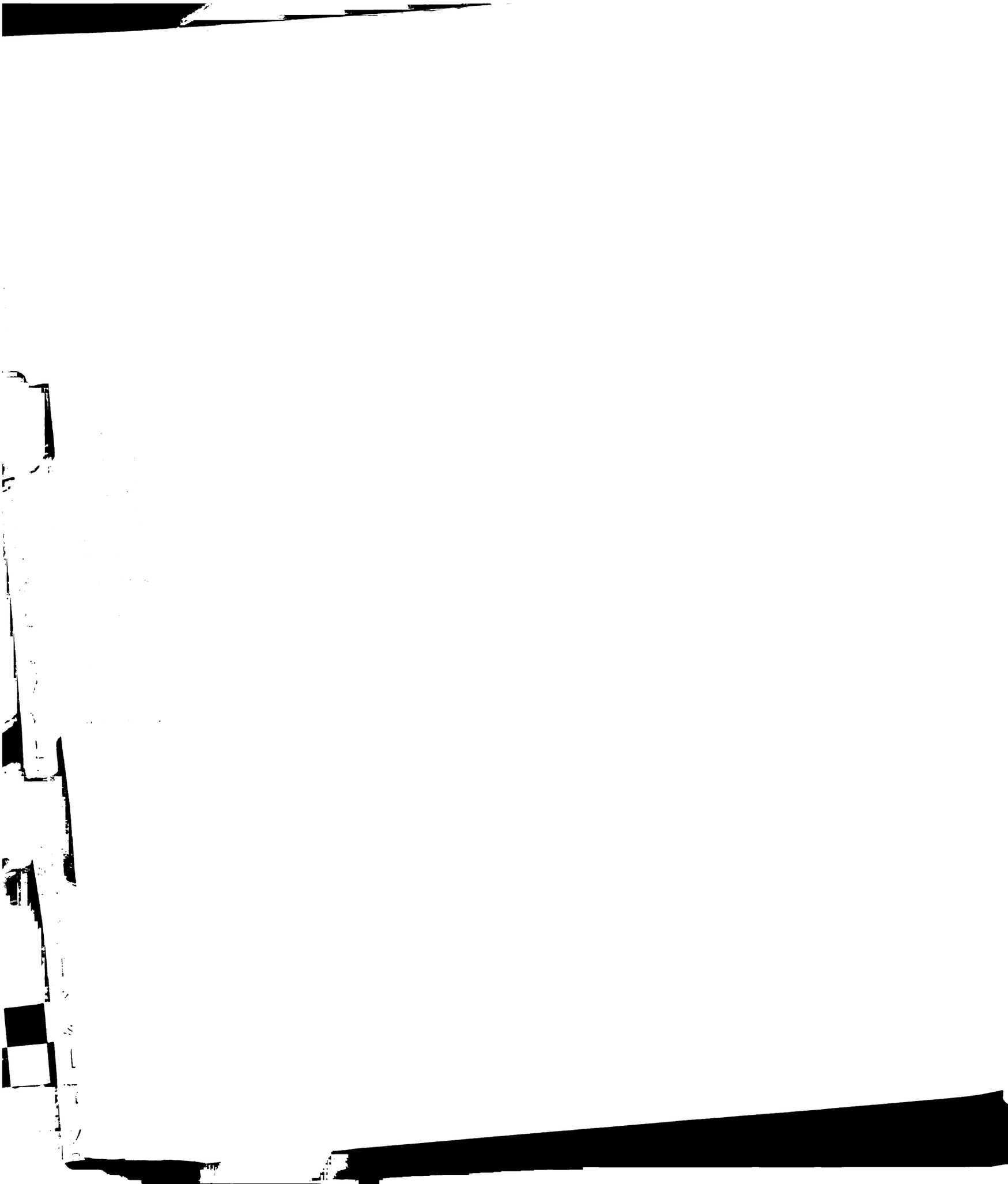
$1x10^6$ virions were incubated in viral lysis buffer (10 mM Tris, 25 mM EDTA, 100 mM NaCl, 1.5% SDS, pH 8.0) for 10 minutes at room temperature. 100 μg protease K was added, and lysates were incubated at 42°C for 2-4 hr. Standard phenol-chloroform extraction and ethanol precipitation were performed, and viral DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Plasmids and constructs

cDNAs of human and mouse NKG2D ligands and cDNAs from CMV open reading frames (ORFs) were cloned into the pMX-pie or pMX-neo retroviral vectors (97). The pMX-pie vector contains an IRES element followed by a cDNA encoding EGFP. The pMX-neo vector lacks the IRES-EGFP element, so cells transfected with this plasmid are not GFP^+.

For cloning, primers containing restriction sites on the 3' end were designed to PCR amplify the ORFs of the cDNAs of interest. PCR products were purified and digested using the appropriate restriction enzymes, and cloned into vectors that were linearized by digestion with the corresponding restriction enzymes. Sequencing was performed for each construct to verify that no mutations were introduced by PCR or cloning procedures.

Because we did not initially have antibodies to detect HCMV UL16 or mouse H60, Flag epitope tags were inserted onto the 5' ends of these cDNAs. When an epitope tag was attached to a cDNA, we replaced the endogenous leader sequence with the human CD8α leader, in order to permit expression. In addition, because the reported



H60 cDNA sequence (52) lacked a stop codon, we engineered a stop codon 5' to the poly-A track. When transfected into 293T cells, this construct allowed stable surface expression of H60.

Fusion protein constructs were generated by cloning the extracellular domain of the gene of interest into a standard type I or type II fusion protein plasmid. This vector contained the mouse SLAM leader, an Xho I site for insertion of the extracellular domain of the fusion protein, and the Fc domain of human IgG1. For type I proteins (i.e. proteins with the extracellular domain on the N-terminal region of the protein), the Xho I restriction enzyme site was inserted between the SLAM leader sequence and the Fc domain of human IgG1. For type II proteins (i.e. the COOH-terminus of the protein is extracellular), the SLAM leader segment and the human Fc region were contiguous, followed by an Xho I restriction enzyme site, and the extracellular domain was inserted at the 3' end after the Fc sequence.

Cells

BALB/c 3T3 cells and MRC-5 cells were obtained from the ATCC (Manassas, VA). BaF/3, 293T cells and TpnT mouse embryonic fibroblasts were generous gifts of Dr. T. Kitamura, University of Tokyo, and Dr. A. Hill, Oregon Health Sciences University, respectively. Dr. P. Leibson, Mayo Clinic College of Medicine, kindly provided NKL cells.

BALB/c and C57BL/6 resident peritoneal macrophages were collected from six-week-old female mice. After harvest, macrophages were plate-adhered for 24 hours, B

cells were removed by washing cells with PBS, and macrophages were infected with MCMV.

NK cells were purified from spleens of C57BL/6 mice. Spleens were harvested, homogenized, and T cells and B cells were depleted: total splenocytes were stained with rat antibodies that specifically recognize mouse CD4 and CD8, then incubated with magnetic beads coated with anti-rat IgG (to bind $CD4^+$ and $CD8^+$ T cells) and anti-mouse IgG (to bind B cells). The magnetic bead-coated T and B cells were then removed from the cell suspension using a bead magnet (Dynal Biotech, Brown Deer, Wisconsin). The remaining cells were positively selected by MACS (Miltenyi Biotech, Auburn, CA) using the DX5 mAb, which recognizes CD49b (VLA-2), an NK cell-associated marker. NK cells were cultured for 7 days in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 50 μM 2-mercaptoethanol and 4000 U/ml recombinant human IL-2, which was provided by the NCI BRB Pre-clinical Repository (Frederick, MD). After 7 days of activation by IL-2, NK cells were used in co-culture experiments with virus-infected cells and assayed for IFN-γ production.

Transfectants

BaF/3 cells were transfected with human NKG2D ligands by retroviral transduction: vectors encoding the human NKG2D ligands in pMX-pie were transfected into Phoenix-A ecotrophic viral packaging cells using Lipofectamine 2000 Reagent (InVitroGen, Carlsbad, CA). After 48 hr the virus was used to infect BaF/3 cells. CT498 transfectants were generated by retroviral transduction of BALB/c 3T3 cells with a plasmid encoding the MCMV m155 cDNA in pMX-neo, using the protocol above.

Transient co-transfection of 293T cells was performed with Lipofectamine 2000 reagent. pMX-pie vector encoding cDNA from RAE-1α, β, γ, δ, ε, H60 or MULT-1 was mixed in a 1:3 ratio with either pMX-neo alone or pMX-neo vector encoding m155 or m152 cDNA. Lipofectamine 2000 was then added to the plasmid DNA, and this mixture was incubated with $5x10^5$ 293T cells at 37°C. 48 hours after transfection, cells were analyzed by immunofluorescent staining and flow cytometry.

Viruses

HCMV AD169 and ΔUL16 (98) were kindly provided by Dr. E. Mocarski, Stanford University.

MCMV Smith, DMS94.5 (Δm150-165) (99) and ΔMC96.24 (Δm152) (92) viruses were a gift of Dr. A. Hill, Oregon Health Sciences University. Rvm155 is an MCMV mutant lacking m155 (Dm155) and Rqm155-Rq155 is the revertant of Rvm155 with a functional m155 gene restored (88, 89).

Tissue culture virus was propagated by infection of BALB/c 3T3 cells with MCMV at a multiplicity of infection (MOI) of 0.001. Cells were incubated with virus at 37°C for 5-7 days, allowing the infection to spread throughout the culture. When 100% of the cells exhibited cytopathic effect (cell swelling and rounding), the supernatent was collected from the cells and centrifuged at 2,000 x g to rid of any cell debris. The supernant was then centrifuged at 11,000 x g to pellet the virus. Viruses were resuspended in DMEM medium with 10% FBS.

Viral titering was performed using a standard plaque assay. BALB/c 3T3 cells plated in 12-well plates were infected with serial dilutions of MCMV. Cells were

incubated with virus at 37°C for 5-7 days, until plaques were visible in the monolayer. Cells were stained with crystal violet dye to allow better visualization of the plaques, and plaques were counted to determine the number of infectious particles in each well.

In vitro viral infections of BALB/c 3T3 cells or TpnT cells were done at a multiplicity of infection (MOI) of one or two.

Mice

BALB/c and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and Charles River (Wilmington, MA), respectively. For *in vivo* experiments, six- to ten-week-old female mice were used.

Immunofluorescent staining and flow cytometry

For all staining, roughly $2x10^5$ cells were stained on ice or at room temperature for 30 minutes with saturating quantities of the reagents. CMV-infected cells were stained 48 hr post-infection, unless otherwise indicated.

Human or mouse NKG2D-Ig fusion proteins were used to detect NKG2D ligands on the cell surface. The fusion proteins were generated from the extracellular domain of human or mouse NKG2D fused to the Fc domain of human IgG1 (44). RAE-1 glycoproteins were detected by staining with biotinylated CX1, a rat monoclonal antibody (mAb) that recognizes mouse RAE-1α, β and γ (80). CX1 reacts strongly with RAE-1γ and weakly with RAE-1α and RAE-1β, but does not bind to RAE-1δ, RAE-1ε, H60 or MULT-1. mAb 186107 was used to detect all RAE-1 glycoproteins, mAb 205310 was used to detect H60, and mAb 237104 was used to detect MULT-1. The

186107, 205310 and 237104 mAbs were generated by immunizing LOU/MWS1 rats with Ig fusion proteins containing the extracellular domains of RAE-1, H60 or MULT-1, respectively. 186107 is a rat mAb that recognizes RAE-1α, β, γ, δ and ε, but not H60 or MULT-1. mAb 205310 is specific for H60 and does not cross-react with MULT-1 or RAE-1, as determined by testing on a panel of BaF/3 transfectants expressing H60, MULT-1 or RAE-1.

The Flag epitope tag on Flag-UL16 and Flag-H60 was detected using the mouse anti-Flag M2 mAb (Sigma, St. Louis, MO). We have previously shown that the Flag epitope tag does not interfere with the binding of mouse NKG2D-Ig to H60 (54). Human MHC class I molecules were detected by staining with DX17, a mouse anti-human pan HLA class I mAb. Mouse β1-integrin (CD29) was detected by staining with HMβ1-1 mAb (PharMingen, San Diego, CA).

PE-conjugated secondary antibodies (Jackson Immunoresearch, Bar Harbor, ME) were used for detection of human Ig fusion protein, mouse and rat mAbs. All samples were treated with propidium iodide to exclude dead cells during analysis. Flow cytometry was performed by using a FACSCalibur with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) or a Guava Personal Cytometer with Guava ViaCount and Guava Express Software (Guava Technologies, Hayward, CA).

TaqMan quantitative RT-PCR

TaqMan quantitative PCR was performed on cDNA generated from RNA of uninfected or CMV-infected MRC-5 cells, BALB/c 3T3 cells or resident peritoneal

macrophages (48 hr after infection). Total RNA was DNAse-treated and cDNA was synthesized by using standard methods. To control for the possibility of amplifying genomic DNA by TaqMan PCR, 25% of total RNA was used in a cDNA synthesis reaction without reverse transcriptase. Samples without reverse transcriptase were used as templates in parallel with samples treated with reverse transcriptase. DNA amplified from samples without reverse transcriptase accounted for less than ten percent of the total signal generated. Primer and probe sets specific for human and mouse NKG2D ligands were used with cDNA for TaqMan PCR. All samples were normalized to the signal generated from a housekeeping gene, either human GAPDH or mouse HPRT. TaqMan PCR was performed by using the ABI PRISM® 7700 Sequence Detection System.

IFN-γ production

The levels of IFN-γ produced by NK cells co-cultured with MCMV-infected cells were determined by a standard ELISA using a PharMingen OptEIA™ Set. TpnT fibroblasts were infected with Smith or Δm152 virus at an MOI of one and incubated at 37°C. 48 hours after infection, cells were trypsinized, fixed with fresh 4% PFA and washed three times with DMEM. Effector NK cells were prepared from C57BL/6 splenocytes and cultured for 7 days in RPMI-1640 medium with 10% FBS, 50 μM 2-mercaptoethanol and 4000 U/ml recombinant human IL-2, which was provided by the NCI BRB Pre-clinical Repository (Frederick, MD). Prior to co-culture with target cells, NK cells were either left untreated or treated for 30 minutes with control Ig, anti-NKG2D mAb (CX6) (80) or anti-Ly49H mAb (1F8), kindly provided by Dr. M. Bennett, University of Texas Southwestern Medical Center. Infected target cells were incubated

with NK cells at a 1:1 ratio for 24 hours at 37°C. Supernatants were harvested and used in ELISA for detection of IFN-γ using a PharMingen OptEIA™ Set. Quantitation of IFN-γ production was determined by averaging triplicate wells and using linear regression from a standard curve.

Proteasome inhibition and immunoprecipitation

BALB/c 3T3 or CT498 cells were left untreated or were treated with 10 μM lactacystin or 10 μM epoxomicin (both from Sigma, St. Louis, MO) for 14 hr. Cells were then either used in flow cytometry or lysed in 1% NP-40 and used for immunoprecipitation. Lysates were also made from uninfected BALB/c 3T3 cells or BALB/c 3T3 cells infected with Rvm155 or Rqm155. H60 was immunoprecipitated with anti-H60 205326 mAb. Samples were separated by 8% SDS-PAGE and analyzed by Western blot analysis. H60 was detected with biotinylated anti-H60 mAb 205310 and β-1 integrin was detected by anti-CD29 mAb 9EG7, followed by peroxidase-conjugated streptavidin or anti-rat IgG, respectively, and ECL developing reagent (Amersham Pharmacia, UK).

In vivo antibody treatment

Two days prior to infection, BALB/c mice were injected intra-peritoneally (i.p.) with either 100 μg rat anti-mouse NKG2D mAb CX5 (80) or 100 μg of control rat IgG (eBioscience, San Diego, CA). In some experiments, an additional group of mice was injected with 100 μg rabbit anti-asialo GM1 polyclonal anti-serum (Wako Chemicals, Richmond, VA) one day before infection and on the day of infection.

The rat anti-mouse NKG2D mAb CX5 blocks binding of NKG2D to the RAE-1, H60 and MULT-1 ligands and inhibits NKG2D-dependent NK cell-mediated cytotoxicity against NKG2D ligand-bearing tumors *in vitro* and *in vivo* (80). When injected *in vivo*, anti-NKG2D mAb CX5 either blocked or modulated NKG2D, but did not deplete NK cells, since the frequency of $DX5^+CD3^-$ NK cells in BALB/c mice and $CD3^-NK1.1^+$ NK cells in C57BL/6 mice was not reduced compared with normal, untreated mice (80). Anti-asialo GM1 was used to deplete NK cells.

As a control to confirm modulation of NKG2D on NK cells from anti-NKG2D-treated mice, spleens from control Ig-treated and anti-NKG2D-treated mice were harvested 48 hr and five days after antibody injection. Expression of NKG2D on $CD3^-$ $DX5^+$ splenocytes was analyzed by staining with either an H60-Ig fusion protein (generated from the extracellular domain of H60 fused to the Fc domain of human IgG1) or CX5 mAb. Samples were analyzed by flow cytometry.

In vivo infection with MCMV Smith and Δm152 viruses

Five BALB/c mice were used in each of four experimental groups. On the day of MCMV infection (48 hours after antibody treatment), $1x10^6$ PFU of Smith or Δm152 virus was resuspended in DMEM without serum and used to i.p. infect mice treated with anti-NKG2D antibody or control Ig. Spleens and livers were harvested from mice 3 days post-infection and transferred to DMEM supplemented with 10% FBS. Liver weights were determined, and organs were homogenized. Tissue homogenates were centrifuged at 3400 rpm to pellet cell debris. Serial dilutions were made of the supernatants, and a standard plaque assay was performed on NIH 3T3 cells to determine viral titers in each

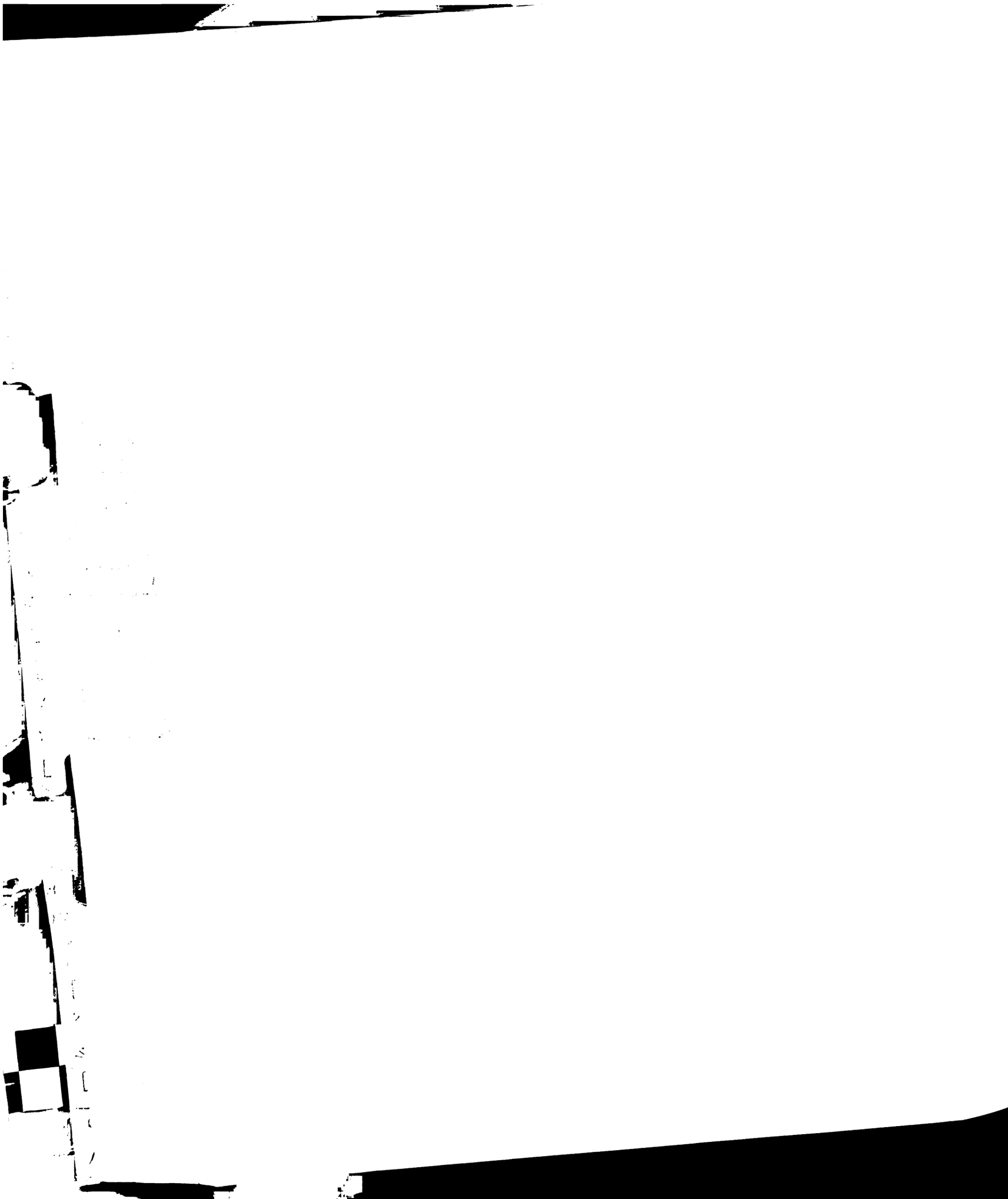
organ. Viral titers for spleen are expressed as plaque-forming units (PFU) per organ, whereas liver viral titers were calculated as PFU per gram tissue.

In vivo infection with MCMV Smith, Δm155 and revertant viruses

Three BALB/c mice were used in each of nine experimental groups. After antibody treatment with control rat IgG, antiNKG2D (CX5) or anti-asialo GM1, mice were infected i.p. with $1x10^5$ PFU or $2x10^5$ PFU of Smith, Rvm155 (Dm155) or Rqm155 (revertant) virus. Spleens and livers were harvested on day 3 post-infection. Organs were homogenized, serial dilutions were made, and a standard plaque assay was performed in triplicate on NIH 3T3 cells. Viral titers are expressed as plaque-forming units (PFU) per ml of tissue homogenate.

Statistics

For *in vivo* MCMV experiments, the Student's two-tailed T-test with unequal variance was used to test the difference between arithmetic mean values of each set of PFUs.



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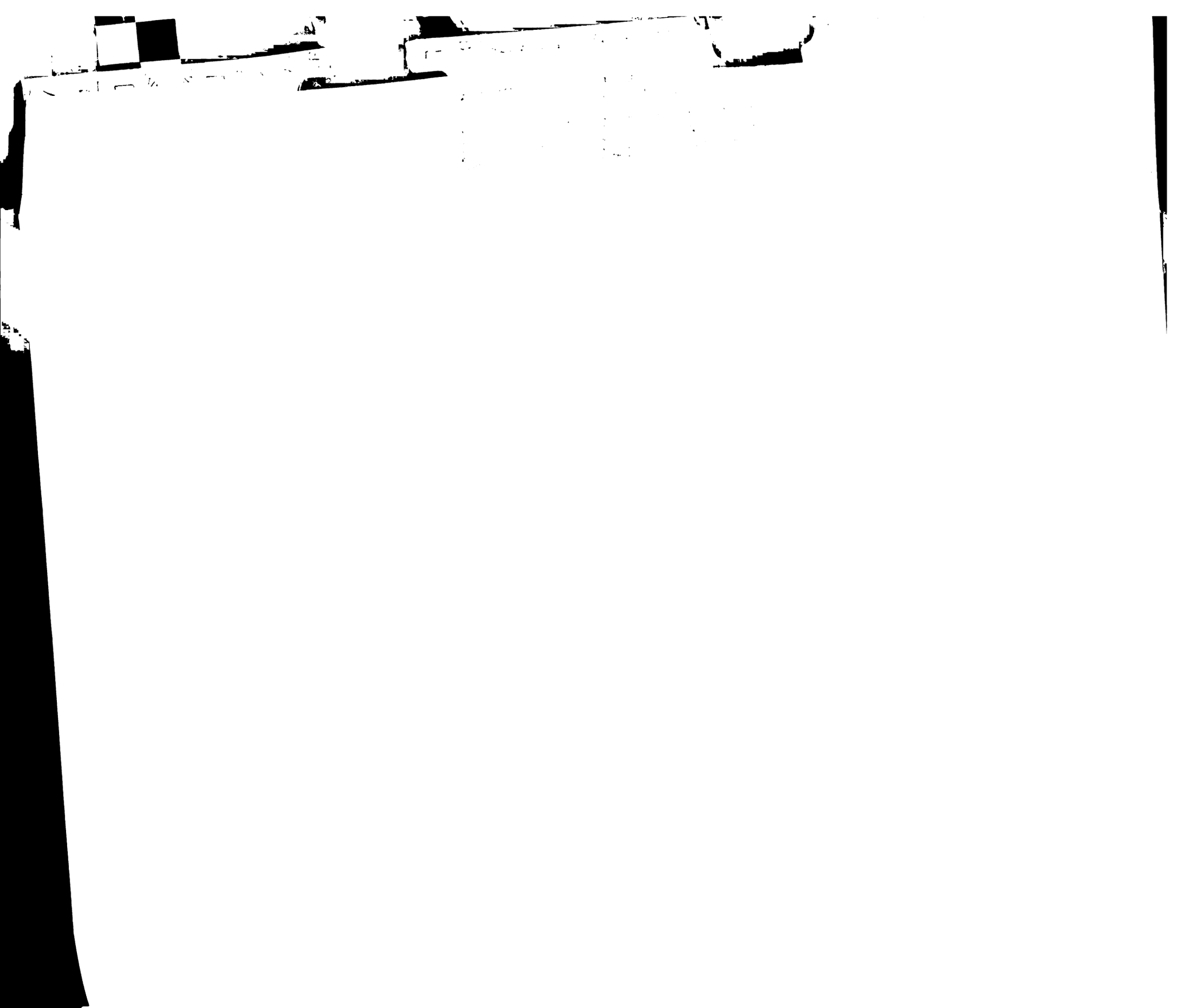

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