Brief Definitive Report

The Cytomegalovirus m155 Gene Product Subverts Natural Killer Cell Antiviral Protection by Disruption of H60–NKG2D Interactions

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Abstract

Natural killer (NK) cells are an important early mediator of host immunity to murine cytomegalovirus (MCMV) infection. However, MCMV has evolved mechanisms to elude recognition and clearance by NK cells. We have identified an MCMV immune evasion protein that impairs NKG2D-mediated NK cell antiviral activity. Infection of BALB/c 3T3 cells with the Smith strain of MCMV resulted in strong down-regulation of H60, a high affinity ligand for NKG2D, from the surface of virus-infected cells. The MCMV m155 protein specifically down-regulated H60 without affecting expression of the other known NKG2D ligands, RAE-1 and MULT-1. Treatment with the proteasome inhibitors lactacystin or epoxomicin reversed m155 down-regulation of H60. An MCMV mutant virus lacking m155 was severely attenuated in BALB/c mice; however, treatment with neutralizing anti-NKG2D monoclonal antibody or with NK-depleting anti-asialo GM1 antisera restored virulence of the mutant virus. Thus, down-regulation of H60 by m155 is a powerful mechanism of inhibiting NKG2D-mediated antiviral function.

Key words: NKG2D • H60 • cytomegalovirus • NK cell

Introduction

A remarkable feature of 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 (1), 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 CTLs (2).

CMV can also evade NK cells, which are an important component of the innate immune response to both murine CMV (MCMV) and human CMV (HCMV; references 3 and 4). 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. In humans, there are two families of ligands for NKG2D, MICA and MICB (5), and the UL16-binding proteins (ULBPs), ULBP-1, ULBP-2, ULBP-3, and ULBP-4 (6). An HCMV glycoprotein, UL16, was recently found to sequester MICB, ULBP-1, and ULBP-2 intracellularly during HCMV infection and decrease susceptibility to NK cell–mediated cytotoxicity (7, 8). Ligands for murine NKG2D include retinoic early inducible-1 gene (RAE-1)α, RAE-1β, RAE-1γ, RAE-1δ, and RAE-1ε, H60 (9, 10), and MULT-1 (11). 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 (12, 13). Because H60 is not affected by gp40, we set out to determine if MCMV infection also impacts expression of H60 on virus-infected cells.

Materials and Methods

Mice. BALB/c mice were purchased from The Jackson Laboratory. Experiments involving mice were conducted using protocols approved by the University of California, Berkeley, Office

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of Animal Care and Use Committee and the University of California, San Francisco, Committee on Animal Research.

Cells and Transfectants. Because the reported H60 cDNA sequence (14) lacked a stop codon, we engineered a stop codon 5′ to the poly-A track. H60, MULT-1, and RAE-1 cDNAs were cloned into the pMX-pie vector, which contains an internal ribosome entry site (IRES) element followed by a cDNA encoding enhanced GFP. In a 1:3 ratio, vectors encoding H60, MULT-1, or RAE-1 were mixed with either pMX-neo alone or pMX-neo vector encoding MCMV m155 cDNA. Transient cotransfection of 293T cells was performed as described previously (13). Cells were analyzed by flow cytometry 48 h after transfection.

BALB/c 3T3 cells were obtained from the American Type Culture Collection. CT498 transfectants were generated by retroviral transduction of BALB/c 3T3 cells. A plasmid encoding MCMV m155 cDNA in pMX-neo was transfected into Phoenix A ecotrophic viral-packaging cells using Lipofectamine 2000 Reagent (Invitrogen). After 48 h, the virus was used to infect BALB/c 3T3 cells.

Viruses. MCMV Smith, DMS94.5 (Δm150-165; reference 15), and ΔMC96.24 (Δm152; reference 16) viruses were provided by A. Hill (Oregon Health Sciences University, Portland, OR). Rvm155 is an MCMV mutant lacking m155 (Dm155; see below, Fig. S1) and Rqm155-Rq155 is the revertant of Rvm155 with a functional m155 gene restored (17, 18).

Flow Cytometry. 3T3 cells were infected with MCMV at a multiplicity of infection (MOI) of 2 and analyzed after 48 h of culture. mAb 186107 was used to detect RAE-1α, RAE-1β, RAE-1γ, RAE-1δ, and RAE-1ε, mAb 205310 was used to detect H60, and mAb 237104 was used to detect MULT-1. The 205310 and 237104 mAbs were generated by immunizing LOU/MWS1 rats with Ig fusion proteins containing the extracellular domains of H60 and MULT-1, respectively. 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 transfectants expressing H60, MULT-1, or RAE-1. Mouse β1 integrin (CD29) was detected with HMβ1-1 mAb (BD Biosciences). All samples were treated with propidium iodide to exclude dead cells during analysis. Flow cytometry was performed with a FACSCalibur (BD Immunocytometry Systems).

Proteasome Inhibitors and Immunoprecipitation. 3T3 or CT498 cells were left untreated or treated with 10 μM lactacystin or 10 μM epoxomicin (both from Sigma-Aldrich) for 14 h. Cells were then either analyzed by flow cytometry or lysed in 1% NP-40 and used for immunoprecipitation. Lysates were also made from uninfected 3T3 cells or 3T3 cells infected with Rvm155 or Rqm155. H60 was immunoprecipitated with anti-H60 mAb 205326. Proteins were separated by 8% SDS-PAGE and analyzed by Western blot analysis. H60 was detected with biotinylated anti-H60 mAb 205310 and β1 integrin was detected by anti-CD29 mAb 9EG7 followed by peroxidase-conjugated streptavidin or anti–rat IgG, respectively, and ECL-developing reagent (Amersham Biosciences).

In Vivo MCMV Infection. 2 d before infection, mice were injected i.p. with either 100 μg of rat anti–mouse NKG2D mAb CX5 (19) or control rat IgG (eBioscience). 1 d before infection and on the day of infection, a group of mice was injected i.p. with 100 μg of anti-asialo GM1 (Wako Chemicals). Mice were infected i.p. with 10^5 PFUs or 2×10^5 PFUs of Smith, Rvm155 (Dm155), or Rqm155 (revertant) virus. Spleens and livers were harvested on day 3 after infection. Organs were homogenized, serial dilutions were made, and a standard plaque assay was performed in triplicate on 3T3 cells. Viral titers are expressed as PFUs per milliliter of tissue homogenate.

When injected in vivo, anti-NKG2D mAb CX5 blocked and/or modulated NKG2D, but did not deplete NK cells (19). Anti-asialo GM1 was used to deplete NK cells. To confirm modulation of NKG2D on NK cells from anti-NKG2D–treated mice, and NK cell depletion in anti-asialo GM1-treated mice, spleens from antibody-treated mice were collected on day 3 after infection and analyzed for NKG2D expression and the percentage of NK cells.

Online Supplemental Material. Construction of the m155 deletion mutant virus is depicted in Fig. S1. The finding that m155 does not substantially affect H60 transcription in shown in Fig. S2. Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20040583/DC1.

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 (12, 13). 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. 48 h after infection, we analyzed cells for H60. We observed a marked decrease in the level of cell surface H60 after infection (Fig. 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 open reading frames (ORFs; Δm150-165), H60 expression was partially restored (Fig. 1). A deletion mutant virus lacking only m152 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

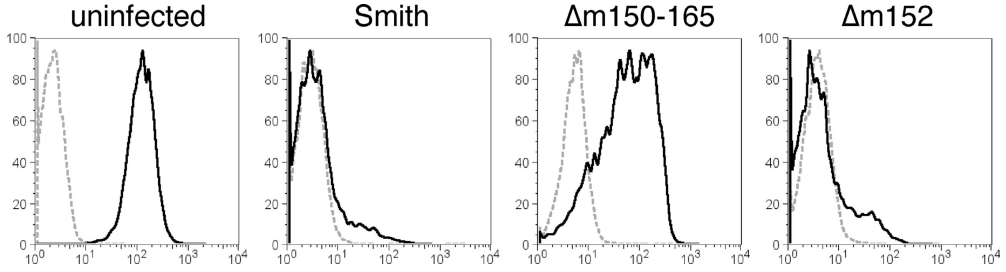

Figure 1. MCMV infection down-regulates H60. 3T3 cells were infected with wild-type MCMV (Smith), DMS94.5 (Δm150-165), or ΔMC96.24 (Δm152) viruses at an MOI of 2. 48 h after infection, cells were stained with control IgG2a (dotted histograms) or anti-H60 mAb (bold histograms).

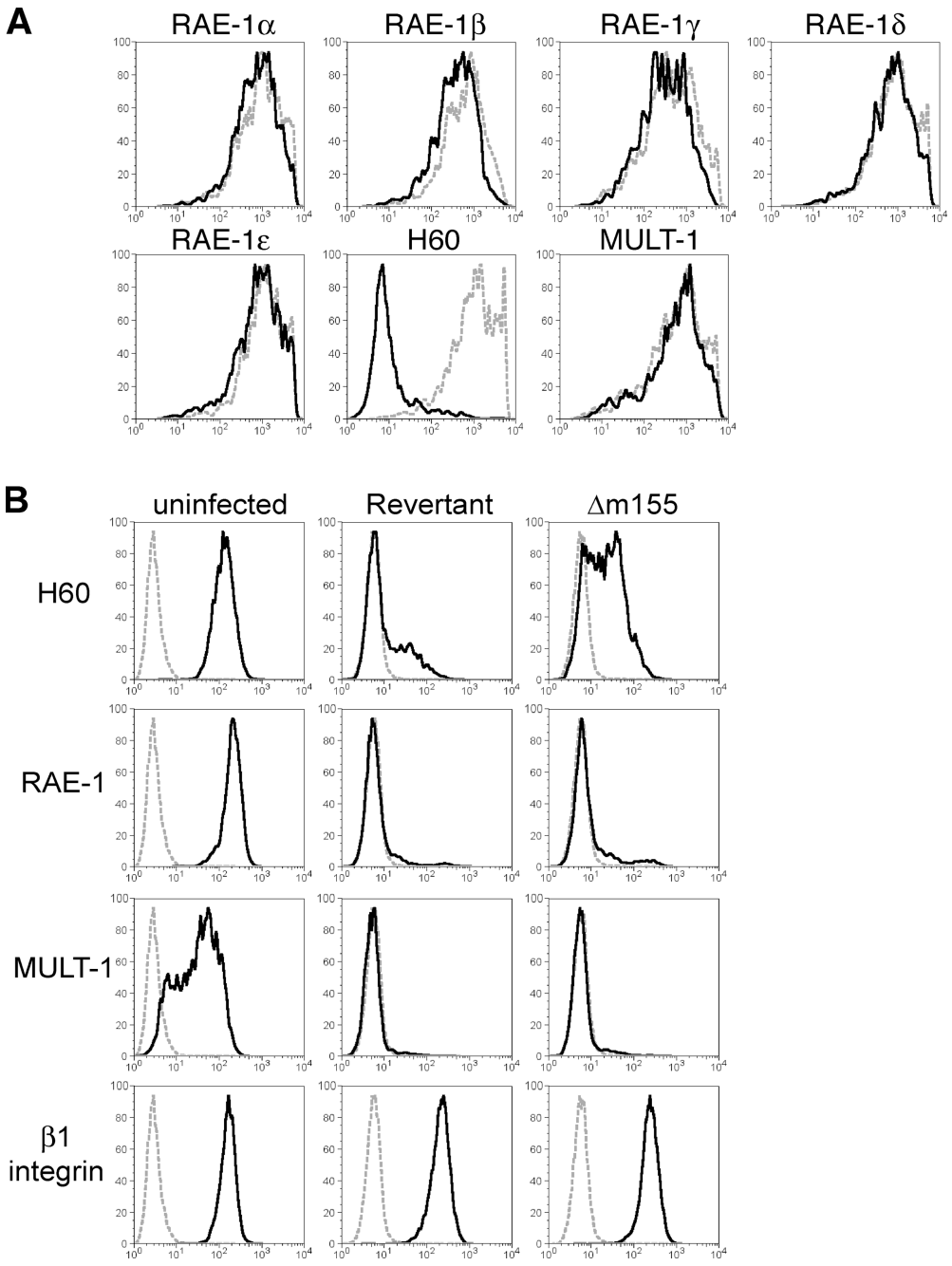

Figure 2. m155 down-regulates H60, but not RAE-1 or MULT-1. (A) 293T cells were transfected with a vector encoding H60, MULT-1, or RAE-1, and either a control vector (dotted histograms) or a vector encoding m155 (bold histograms). 48 h after transfection, cells were stained with anti-H60, anti–MULT-1, or anti–RAE-1 mAbs. H60, MULT-1, and RAE-1 were encoded on vectors carrying an IRES-GFP, whereas a non-GFP vector was used for m155 cDNA and for the control vector. Histograms show GFP^+ populations. Results are representative of three independent experiments. (B) 3T3 cells were infected with Rqm155-Rq155 (the m155 revertant virus) or Dm155 (Δm155) virus at an MOI of 2. 48 h after infection, cells were stained with control IgG2a (dotted histograms), anti-H60, anti–RAE-1, anti–MULT-1, or anti–β1 integrin (bold histograms). This experiment was performed several times with comparable results.

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–enhanced GFP element, permitting visualization of GFP^+ cells that express H60 upstream of the IRES. Cotransfection of H60 with m155 resulted in a substantial decrease in H60 expressed on the surface (Fig. 2 A). We also considered 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 because expression of these molecules was not changed by cotransfection with m155 (Fig. 2 A). 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 depicted).

Zhan et al. (17) previously described an MCMV mutant, Dm155, in which the m155 ORF was disrupted by random insertion of a transposon. This m155-deficient virus demonstrated normal viral replication in vitro, but was severely attenuated in SCID mice (18). 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. 2 B). 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 previous finding that MCMV infection down-regulated RAE-1, the revertant virus strongly down-regulated RAE-1 (Fig. 2 B). Interestingly, we also observed down-regulation of MULT-1 after infection with wild-type (not depicted) and the revertant virus (Fig. 2 B), which is the first evidence that MULT-1 is also impacted by MCMV. Infection of 3T3 cells with Dm155 did not, 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. 2 B). 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 depicted). 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 (20).

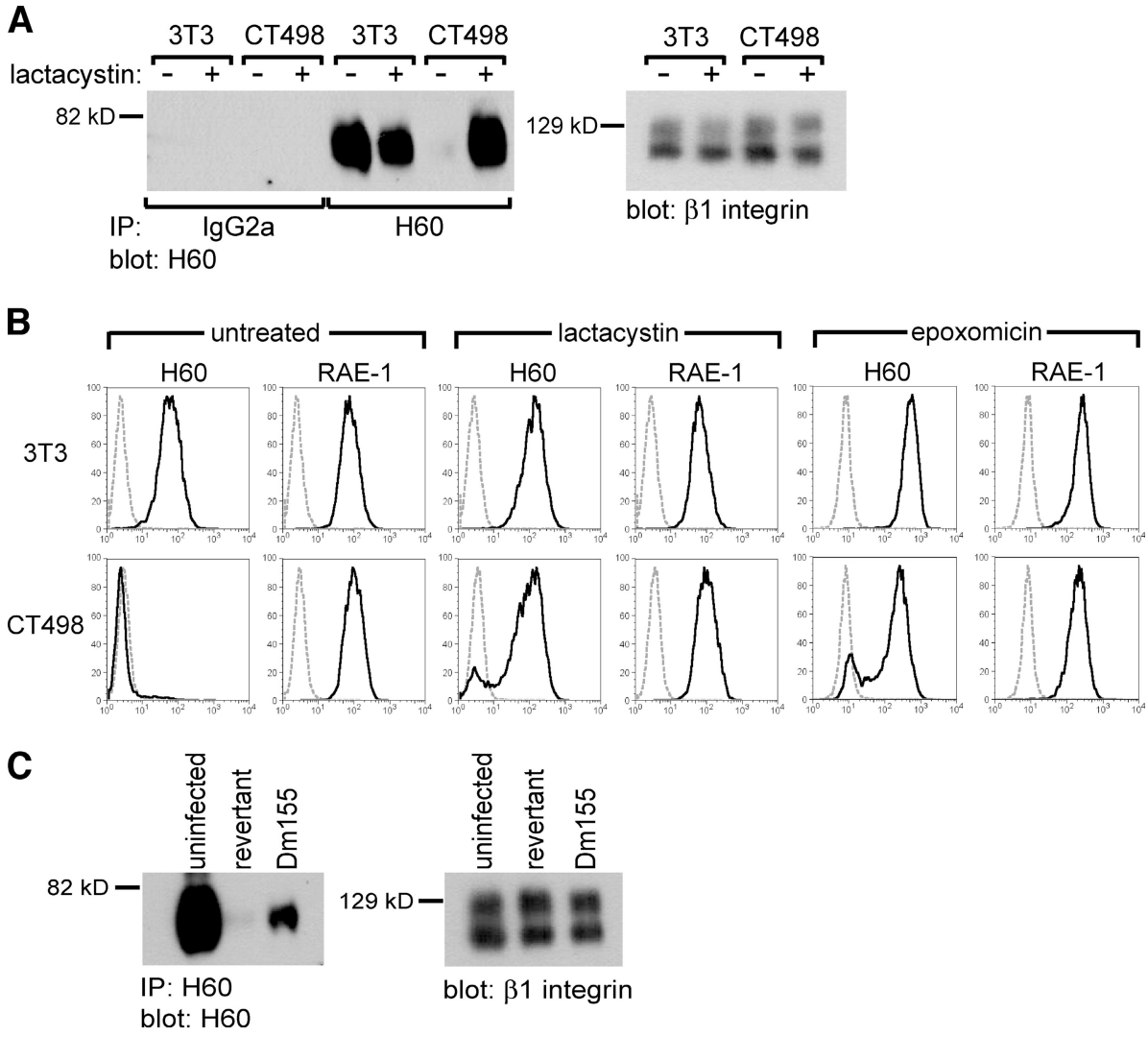

Figure 3. Proteasome inhibition reverses m155 down-regulation of H60. (A) Lysates were generated from untreated cells or cells treated with 10 μM lactacystin for 14 h and immunoprecipitated with control IgG2a or anti-H60 mAb. Western blotting was performed for H60 or β1 integrin protein. (B) Untreated 3T3 or CT498 cells (3T3 cells stably transfected with m155) and 3T3 or CT498 cells treated with 10 μM lactacystin or 10 μM epoxomicin for 14 h were stained with a control IgG2a (dotted histograms), anti-H60, or anti–RAE-1 mAbs (bold histograms) and propidium iodide. Histograms show propidium iodide–negative cells (>95% of total cell population). (C) Lysates were generated from uninfected 3T3 cells, 3T3 cells infected with Rvm155, or 3T3 cells infected with Rqm155, and immunoprecipitated and Western blotted as described in B.

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 with 3T3 cells stably transfected with m155 (designated CT498 cells), we found a less than twofold difference in H60 transcription (Fig. S2, which is available at http://www.jem.org/cgi/content/full/jem.20040583/DC1). This is not surprising, considering that *m155* encodes a potential membrane glycoprotein. Although H60 transcription was not substantially affected by expression of m155, H60 protein was strongly down-regulated from the surface of cells expressing m155 (Figs. 2 A and 3 B). 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. 3 B). 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 depicted).

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 either proteasome inhibitor resulted in high expression of H60 on the surface of CT498 cells (Fig. 3 B). 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 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 (21). Because the ubiquitin–proteasome system has been implicated in regulation of the endocytic pathway (21), it might 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. 3 C). However, H60 protein was partially restored in lysates from cells infected with Dm155 vi-

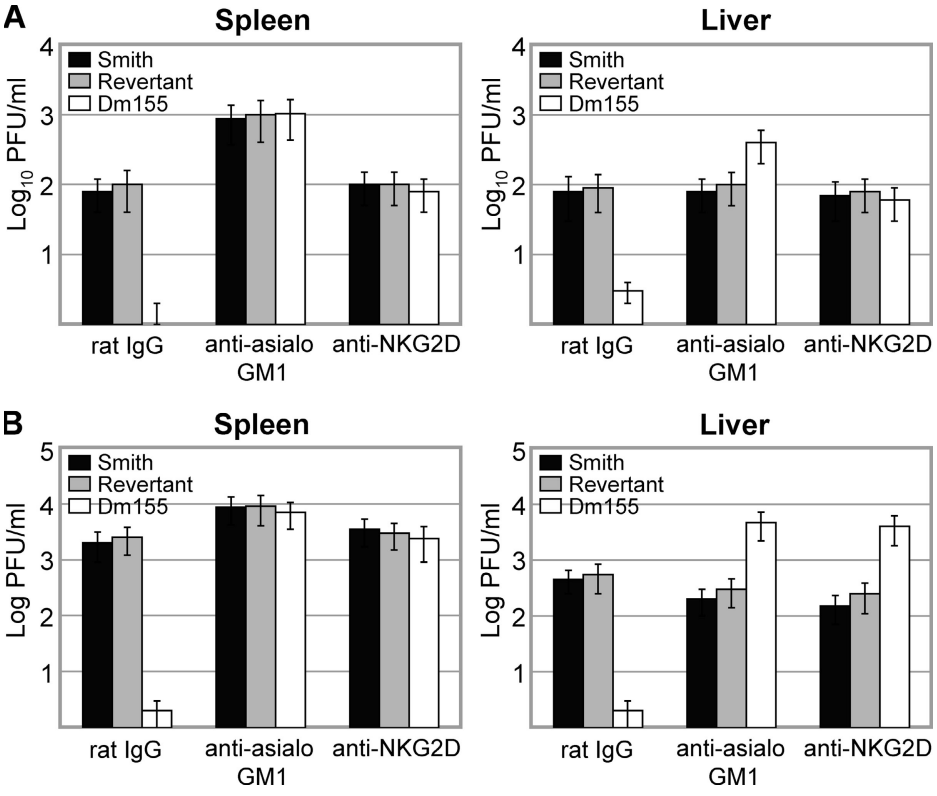

Figure 4. Depletion of NK cells or blockade of NKG2D restores virulence of Dm155. (A) BALB/c mice were injected i.p. with control rat IgG, anti-asialo GM1 antisera, or anti-NKG2D mAb (CX5). After antibody treatment, mice were infected with 10^5 PFUs (A) or 2×10^5 PFUs (B) of Smith, Rqm155 (revertant), or Rvm155 (Dm155). 3 d after 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 2×10^5 PFUs were combined for presentation in B. The limit of detection was 1 PFU/ml organ homogenate.

rus. 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. We hypothesized that NK cell recognition of virus-infected cells may be impaired by m155. Therefore, we examined the significance of H60 down-regulation during MCMV infection in vivo. Before infection of BALB/c mice, which express functional H60 (14), we treated mice with a control rat IgG mAb, a neutralizing anti-NKG2D (CX5) mAb, or an NK cell–depleting anti-asialo GM1 antisera. CX5 mAb blocks the binding of NKG2D to its ligands and modulates the NKG2D receptor from the surface of NK cells, but does not deplete NK cells (22), thereby allowing us to 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 using anti-asialo GM1 antisera. After antibody treatment, mice were infected i.p. with 10^5 PFUs of virus per mouse (Fig. 4 A) or with 2×10^5 PFUs (Fig. 4 B) per mouse of wild-type Smith, Dm155, or the m155 revertant virus. Because previous 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 after infection.

In both the spleen and liver of control rat IgG-treated mice, the Dm155 virus showed marked attenuation (Fig. 4), consistent with previous observations that the Dm155 virus is severely attenuated in growth in multiple organs of SCID mice (18). 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 10^5 PFUs (Fig. 4 A) and slightly higher than titers of wild-type and revertant viruses in mice infected with 2×10^5 PFUs (Fig. 4 B). 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.

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 because deletion mutant viruses lacking these genes are less virulent (16, 17). 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 might 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 (13). 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 nonredundant functions. For this reason, perhaps it is not surprising that MCMV encodes multiple immunoevasins that modulate expression of the NKG2D ligands.

We have defined a function for the MCMV *m155* gene product in evasion of NK cell immune surveillance. By specifically down-regulating H60 from the surface of virus-infected cells, the m155 protein prevents the interaction between H60 and the NKG2D receptor and inhibits NK cell recognition and clearance of infected cells.

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