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

Melissa B. Lodoen, Gerardo Abenes, Sean Umamoto, Jeffrey P. Houchins, Fenyong Liu, and Lewis L. Lanier

1Department of Microbiology and Immunology and the Cancer Research Institute, University of California, San Francisco, San Francisco, CA 94143
2Division of Infectious Diseases, School of Public Health, University of California, Berkeley, Berkeley, CA 94720
3R&D Systems, Minneapolis, MN 55413

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 cytoxicity (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
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 ecotropic 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 AMC96.24 (Δm152; reference 16) viruses were provided by A. Hill (Oregon Health Sciences University, Portland, OR). Rvm155 is an MCMV mutant lacking m155 (Δm155; see below, Fig. S1) and Rqm155-Rq155 is the revertant of Rvm155 (Δm155). H60, MULT-1, or RAE-1. Mouse MWS1 rats with Ig fusion proteins containing the extracellular domains (14) lacked a stop codon, we engineered a stop codon 5' of the m150-165 open reading frames (ORFs; reference 12) and Rqm155-Rq155 is the revertant of Rvm155 below, Fig. S1) and Rqm155-Rq155 is the revertant of Rvm155 (Δm155). The finding that 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 m150-165 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 anti-H60 mAb 205310 and 205326. Proteins were separated by 8% SDS-PAGE and analyzed

**Figure 1.** MCMV infection down-regulates H60. 3T3 cells were infected with wild-type MCMV (Smith), DMS94.5 (Δm150-165), or AMC96.24 (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).
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 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 regulation.

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.
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-2Dd and H-2Kd 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–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.
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.](http://www.jem.org/cgi/content/full/jem.20040583/DC1)
m152 and m155 gene products to down-regulate the host immune response to MCMV and that the virus has evolved mechanisms to counter this pathway. The ability of the m152 and m155 proteins 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.

We thank Ann Hill for generously providing viruses, Ian York for helpful discussion, Chad Borchert for expert assistance in the production of mAbs, and Qiu Zhong and Sarah Clark for their excellent assistance with the animal studies.

This work is supported by National Institutes of Health grants CA89189 to L.L. Lanier and DE14145 to F. Liu. L.L. Lanier is an American Cancer Society Research Professor.

The authors have no conflicting financial interests.

Submitted: 25 March 2004
Accepted: 24 August 2004

References

1. Reddehase, M.J. 2002. Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. Nat. Rev. Immunol. 2:831–844.

2. Tortorella, D., B.E. Gewurz, M.H. Furman, D.J. Schust, and H.L. Ploegh. 2000. Viral subversion of the immune system. Annu. Rev. Immunol. 18:861–926.

3. Bukowski, J.F., J.F. Warner, G. Dennert, and R.M. Welsh. 1985. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. J. Exp. Med. 161:40–52.

4. Biron, C.A., K.S. Byron, and J.L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. N. Engl. J. Med. 320:1731–1735.

5. Bauer, S., V. Groh, J. Wu, A. Steinele, J.H. Phillips, L.L. Lanier, and T. Spies. 1999. Activation of natural killer cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 285:727–730.

6. Cosman, D., J. Mullberg, C.L. Sutherland, W. Chin, R. Armitage, W. Fanslow, M. Kubin, and N.J. Chalupny. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through
the NKG2D receptor. *Immunity.* 14:123–133.

7. Wu, J., N.J. Chalupny, T.J. Manley, S.R. Riddell, D. Cosman, and T. Spies. 2003. Intracellular retention of the MHC class I-related B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein. *J. Immunol.* 170:4196–4200.

8. Dunn, C., N.J. Chalupny, C.L. Sutherland, S. Dosch, P.V. Sivakumar, D.C. Johnson, and D. Cosman. 2003. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytolysis. *J. Exp. Med.* 197:1427–1439.

9. Diefenbach, A., A.M. Jamieson, S.D. Liu, N. Shastri, and D.H. Raulet. 2000. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat. Immunol.* 1:119–126.

10. Cerwenka, A., A.B. Bakker, T. McClanahan, J. Wagner, J. Wu, J.H. Phillips, and L.L. Lanier. 2000. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity.* 12:721–727.

11. Carayannopoulos, L.N., O.V. Naidenko, D.H. Fremont, and W.M. Yokoyama. 2002. Cutting edge: murine UL16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. *J. Immunol.* 169:4079–4083.

12. Krmpotic, A., D.H. Busch, I. Bubic, F. Gebhardt, H. Hengel, M. Hasan, A.A. Scalzo, U.H. Koszinowski, and S. Jonjic. 2002. MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo. *Nat. Immunol.* 3:529–535.

13. Lodoen, M., K. Ogasawara, J.A. Hamerman, H. Arase, J.P. Houchins, E.S. Mocarski, and L.L. Lanier. 2003. NKGD-mediated natural killer cell protein expression against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. *J. Exp. Med.* 197:1245–1253.

14. Malarkannan, S., P.P. Shih, P.A. Eden, T. Hornig, A.R. Zuberi, G. Christianson, D. Roopenian, and N. Shastri. 1998. The molecular and functional characterization of a dominant minor H antigen, H60. *J. Immunol.* 161:3501–3509.

15. Thale, R., U. Szepan, H. Hengel, G. Geginat, P. Lucin, and U.H. Koszinowski. 1995. Identification of the mouse cytomegalovirus genomic region affecting major histocompatibility class I molecule transport. *J. Virol.* 69:6098–6105.

16. Krmpotic, A., M. Messerle, I. Crnkovic-Mertens, B. Polic, S. Jonjic, and U.H. Koszinowski. 1999. The immune evasion function encoded by the mouse cytomegalovirus gene m152 protects the virus against T cell control in vivo. *J. Exp. Med.* 190:1285–1296.

17. Zhan, X., M. Lee, G. Abenes, I. Von Reis, C. Kittinuvorakoon, P. Ross-Macdonald, M. Snyder, and F. Liu. 2000. Mutagenesis of murine cytomegalovirus using a Tn3-based transposon. *Virology.* 266:264–274.

18. Abenes, G., K. Chan, M. Lee, E. Haghjoo, J. Zhu, T. Zhou, X. Zhan, and F. Liu. 2004. Murine cytomegalovirus with a transposon insertion mutation at open reading frame m155 is deficient in growth and virulence in mice. *J. Virol.* 78:6891–6899.

19. Ogasawara, K., J.A. Hamerman, H. Hsin, S. Chikuma, H. Bour-Jordan, T. Chen, T. Pertel, C. Carmaud, J.A. Bluestone, and L.L. Lanier. 2003. Impairment of NK cell function by NKG2D modulation in NOD mice. *Immunity.* 18:41–51.

20. Wagner, M., A. Gutermann, J. Podlech, M.J. Reddehase, and U.H. Koszinowski. 2002. Major histocompatibility complex class I allele-specific competitive and cooperative interactions between immune evasion proteins of cytomegalovirus. *J. Exp. Med.* 196:805–816.

21. Lorenzo, M.E., J.U. Jung, and H.L. Ploegh. 2002. Kaposi’s sarcoma-associated herpesvirus K3 utilizes the ubiquitin-proteasome system in routing class I major histocompatibility complexes to late endocytic compartments. *J. Virol.* 76:5322–5331.

22. Ogasawara, K., J.A. Hamerman, L.R. Ehrlich, H. Bour-Jordan, P. Santamara, J.A. Bluestone, and L.L. Lanier. 2004. NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity.* 20:757–767.