

Expression of m157, a Murine Cytomegalovirus-Encoded Putative Major Histocompatibility Class I (MHC-I)-Like Protein, Is Independent of Viral Regulation of Host MHC-I

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A murine cytomegalovirus (MCMV)-encoded protein, m157, has a putative major histocompatibility complex class I (MHC-I) structure and is recognized by the Ly49H NK cell activation receptor. Using a monoclonal antibody against m157, in this study we directly demonstrated that m157 is a cell surface-expressed glycosylphosphatidylinositol-anchored protein with early viral gene kinetics. Beta-2 microglobulin and TAP1 (transporter associated with antigen processing 1) were not required for its expression. MCMV-encoded proteins that down-regulate MHC-I did not affect the expression of m157. Thus, m157 is expressed on infected cells in a manner independent of viral regulation of host MHC-I.

Infection with human cytomegalovirus can cause severe and fatal disease in neonates and immunocompromised individuals (9, 25). Although cytomegalovirus infection is species trophic, murine cytomegalovirus (MCMV) has pathophysiologic similarities with the human disease, allowing for study of immune control of viral infection by the host. Early innate immune responses to MCMV infections result in NK cell activation (3). Resistance to MCMV infection is controlled by an autosomal dominant locus termed *Cmv1*, which encodes the NK cell activation receptor Ly49H (4). The ligand for the Ly49H receptor was identified as the MCMV-encoded protein m157 (2, 23). Based on computer modeling, we know that m157 has a tertiary structure similar to that of MHC-I-like molecules that serve as ligands for other NK cell receptors, including the Ly49 family of receptors (23). The expression of m157 on MCMV-infected cells results in the activation of Ly49H⁺ NK cells. In the absence of the Ly49H gene, mice are genetically susceptible and MCMV growth remains unchecked, resulting in high titers of the virus in the spleen and eventual death (4). Moreover, an m157 deletion virus is more virulent in genetically resistant strains, demonstrating increased viral titers at 3 days following infection (5). Thus, the importance of the m157-Ly49H interaction in NK cell control of MCMV infection was previously established, using both indirect methods (2, 23) and an m157 deletion virus (5), but little is known about m157 expression during MCMV infection.

Herein we directly determined expression of m157 with a monoclonal antibody (MAb), 6H121, that specifically recognizes m157 and displays no apparent reactivity with host molecules or other viral molecules. We demonstrated that m157 is

expressed on the cell surfaces of MCMV-infected cells with early viral gene kinetics. In addition, we demonstrated that it is expressed as a glycosylphosphatidylinositol (GPI)-anchored protein. The interaction of m157 with Ly49H resulted in the down-regulation of Ly49H on the surface of the NK cell. Finally, viral proteins known to regulate the cell surface expression of MHC-I molecules on MCMV-infected cells did not alter the expression of m157.

Detection of native m157 on the cell surfaces of MCMV-infected cells. Previous work, using FLAG-tagged forms of m157, has shown that this modified form of m157 can be expressed on the surfaces of transfectants (2). In addition, m157 expression can be indirectly tested by use of Ly49H reporter cells (2, 23). To directly demonstrate that native m157 protein is expressed on the cell surface, we generated an immunoglobulin G2b MAb by immunizing BALB/c mice with the m157-transfected pro-B cell line (BaF3/m157). After screening approximately 700 hybridoma clones, we identified 27 by their ability to stain BaF3/m157 cells but not the parental cell line (BaF3). After cloning by limiting dilution, we selected the MAb from clone 6H121 for further analysis of m157. The purified MAb specifically stained the BaF3/m157 cells (Fig. 1A). Furthermore, the staining was in a punctate pattern on the cell surface, as assessed by fluorescence microscopy (Fig. 1B). The MAb 6H121 did not stain the parental BaF3 cells by either approach. In addition, MAb 6H121 did not stain uninfected spleen or thymus cell suspensions, as determined by flow cytometry analysis (data not shown). Finally, transient transfection of a fibroblast cell line with an expression plasmid containing the m157 cDNA resulted in specific staining of the cells with MAb 6H121 (data not shown). Taken together, these data demonstrate that the MAb 6H121 specifically recognizes m157 on the cell surface and does not react with host molecules on hematopoietic cells.

Having demonstrated that m157 could be specifically detected on transfected cells, we next determined if m157 expression was directly detectable on the cell surfaces of MCMV-

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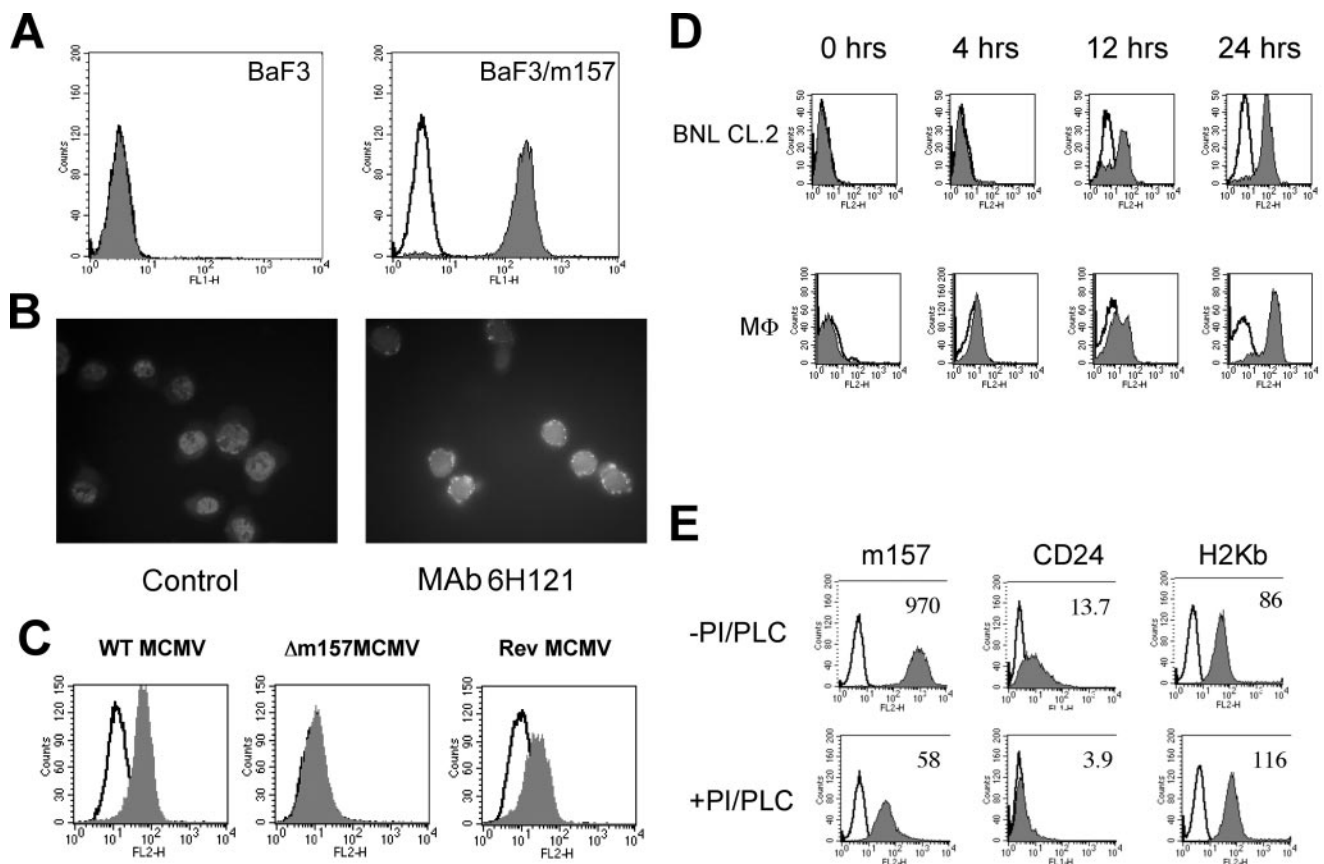


FIG. 1. m157 is a GPI-anchored protein expressed on the cell surfaces of MCMV-infected cells. (A) Stable m157 transfectants (BaF3/m157) and the parental cell line (BaF3) were stained with 10 $\mu\text{g/ml}$ of biotin-conjugated MAb 6H121 (filled curves) or biotin-conjugated control MAb (open curve) followed by 2.5 $\mu\text{g/ml}$ of phycoerythrin-conjugated streptavidin. (B) Immunofluorescence was performed on BaF3/m157 cells stained with 10 $\mu\text{g/ml}$ of biotin-conjugated MAb 6H121 (MAb 6H121) or biotin-conjugated isotype control MAb (control) followed by 5 $\mu\text{g/ml}$ of Alexa 488-conjugated streptavidin. The cells were stained with DAPI (4',6'-diamidino-2-phenylindole) to visualize the nucleus. Magnification, $\times 400$. (C) BNL CL.2 liver cells were infected at a multiplicity of infection (MOI) of 3 to 5, as previously described (23). Cells were infected with either wild-type MCMV (WT MCMV), m157 deletion mutant MCMV ($\Delta\text{m157MCMV}$) or an m157-revertant virus (Rev MCMV). Twenty-four hours postinfection, the cells were assessed for cell surface expression of m157 as described for panel A. (D) Primary bone marrow macrophages were isolated as previously described (23). BNL CL.2 cells and primary bone marrow macrophages (M Φ) were infected with MCMV at an MOI of 5. At various times postinfection, the cells were assessed for cell surface expression of m157 as described for panel A. Open curves represent results for staining with isotype control MAb, and closed curves represent results for staining with the αm157 MAb. (E) One million RMA-m157 cells were incubated in the presence (+PI/PLC) or absence (-PI/PLC) of 1.8 μg of GLYKO PI-PLC (ProZyme) according to the manufacturer's protocol. Following incubation, the cells were stained for cell surface expression of m157, CD24, and H2K^b. The numbers represent the mean fluorescence intensity (MFI) of the respective antibody staining. A representative histogram is shown in each panel.

infected cells. BNL CL.2 cells (a murine hepatocyte cell line) infected for 24 h with MCMV expressed m157 on the cell surface (Fig. 1C). Mock-infected cells did not stain with the 6H121 MAb (data not shown). To confirm that MAb 6H121 was recognizing m157 and not another virally encoded or induced protein, we repeated these experiments with an m157 deletion virus (5) and showed no shift with the MAb 6H121. When cells were infected with the m157 revertant virus (5), a shift was once again seen upon staining with MAb 6H121 (Fig. 1C). The cells were also stained with a monoclonal antibody that recognizes immediate-early protein 1 in MCMV-infected cells, indicating that all cells were infected to a similar degree by the three different viruses (data not shown). Thus, m157, not another virally encoded or induced protein, is recognized by the MAb 6H121, demonstrating directly that native m157 protein is expressed on the cell surfaces of MCMV-infected cells.

Previous work has demonstrated that, following MCMV infections, titers of the virus in the spleen were markedly higher in genetically sensitive (BALB/c) mice than in resistant (C57BL/6) strains possessing the Ly49H gene. In the liver, however, similar titers were seen in both sensitive and resistant strains of mice, suggesting that Ly49H⁺ NK cells play a major role in the control of virus in the spleen but less so in the liver (4). One explanation for this observation is that m157 is not expressed on hepatocytes and is thus not presented to the Ly49H⁺ NK cells in the liver. However, both BNL CL.2 and primary bone marrow macrophages expressed detectable m157 on cell surfaces by 12 h (Fig. 1D). By 24 h, all of the infected cells expressed m157 on their cell surfaces (Fig. 1D and data not shown). Thus, m157 is expressed on hepatocytes, suggesting that a difference in m157 expression in hepatocytes cannot explain the discrepancy between hepatic and splenic viral titers in Ly49H-dependent responses.

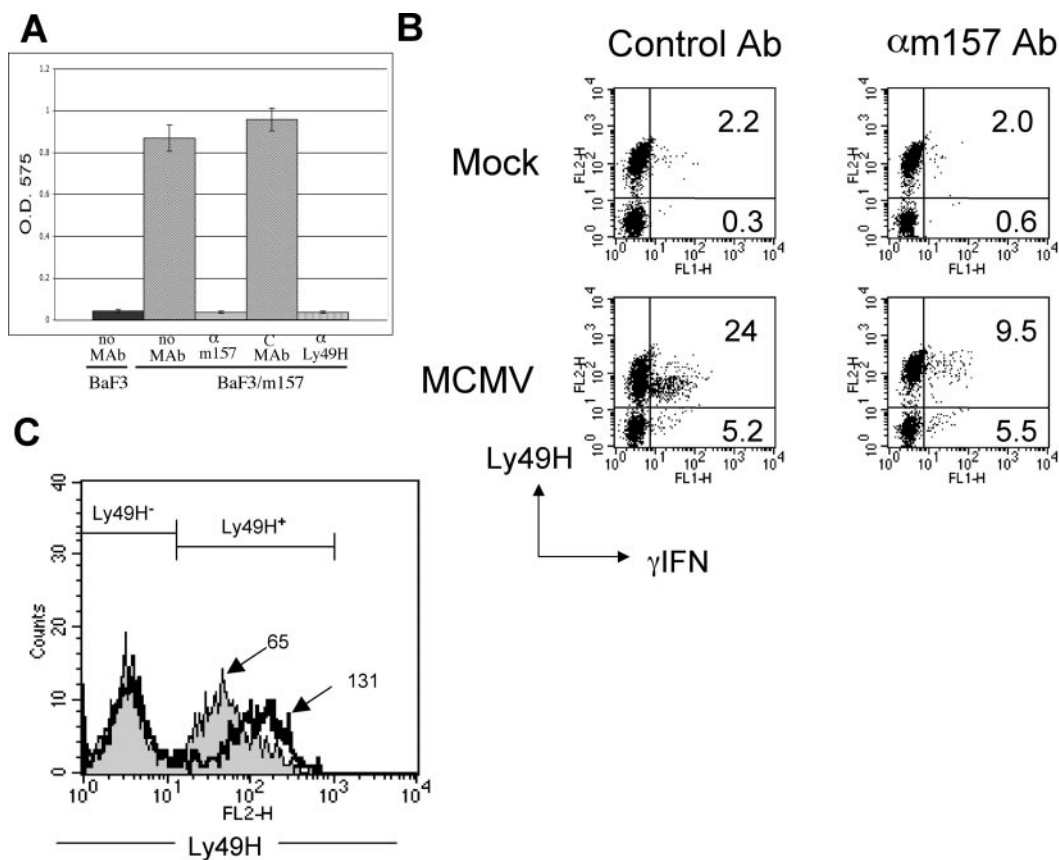


FIG. 2. Blocking the m157-Ly49H interaction inhibits γ -IFN production and the down-regulation of Ly49H receptor. (A) Chlorophenol red β -D-galactosidase analysis of the HD12 reporter line after coculture with BaF3 or BaF3/m157 cells in the presence or absence of 100 μ g/ml α m157 MAb, isotype control MAb, or α Ly49H MAb. Data are expressed as the optical density at 575 nm, which indicates the amount of β -galactosidase activity. The data are presented as the average \pm the standard deviation of the results of an experiment done in triplicate. (B) C57BL/6 and B6.RAG1^{-/-} mice were obtained from the Jackson Laboratory, maintained under specific pathogen-free conditions, and used at 8 to 12 weeks of age. Animal studies were approved by the Animal Studies Committee at Washington University (St. Louis, MO). Primary bone marrow macrophages were isolated from C57BL/6 mice as previously described (23) and infected as described for Fig. 1. Mock-infected or MCMV-infected primary bone marrow macrophages were incubated with 100 μ g/ml of α -m157 Fab fragment (α m157 Ab) or isotype control Fab fragment (control Ab) prior to stimulation of fresh B6.RAG1^{-/-} splenocytes. Fab fragments of the MAbs were used to avoid the possibility of antibody-dependent cellular cytotoxicity. Fab fragments were generated by digestion of approximately 5 mg of MAb 6H121 or control MAb with immobilized pepsin (Pierce) in 20 mM of sodium acetate buffer, pH 4.0. Digestion was stopped by removal of the immobilized pepsin through centrifugation. The supernatant was neutralized with Tris, pH 8.0, and then run over a protein A column. Fab was collected as the flow through and dialyzed into phosphate-buffered saline. Purity of the Fab fragment was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Affinity of the Fab for m157 was demonstrated by its ability to block MAb 6H121 staining of BaF3/m157 cells. Following stimulation, intracellular γ -IFN expression was assessed in the Ly49H⁺ and Ly49H⁻ populations as previously described (23). The numbers represent the percentage of Ly49H⁺ and Ly49H⁻ cells expressing γ -IFN. Gated NK1.1⁺, CD3⁻ cells are shown. (C) Ly49H expression on NK cells following stimulation with MCMV-infected primary bone marrow macrophages as described for panel B. Results for blocking with the α m157 Fab fragment (open histogram) or the isotype control Fab fragment (shaded histogram) are shown. The numbers represent the MFI of the Ly49H⁺ population of NK cells. A representative fluorescence-activated cell sorter analysis or histogram is shown in each panel.

m157 is expressed on the cell surface as a GPI-anchored protein. The m157 open reading frame sequence has a stop codon before the putative hydrophobic transmembrane domain (23), yet it is expressed on the cell surface, suggesting that it is attached with a GPI anchor. Consistent with this possibility, there was a marked decrease in m157 expression when we treated the m157 stable transfectant, RMAm157 cells, with phosphatidylinositol phospholipase C, an enzyme that releases most GPI-anchored proteins from the cell surface (Fig. 1E). CD24, a known GPI-anchored protein (24), also showed a decrease in cell surface expression following enzymatic treatment, while H2K^b (a transmembrane MHC-I molecule) did not. Thus, m157 is indeed a GPI-anchored protein.

Ly49H expression on NK cells is decreased following interaction with MCMV-infected target cells. Previously published data, based on the use of the m157 deletion virus, demonstrated that the m157 protein is crucial for the activation of Ly49H⁺ NK cells (5), but it was not possible to thoroughly evaluate the Ly49H⁺ NK cell population response to cells infected with wild-type MCMV. To better assess the interaction of m157 with Ly49H, we first sought to determine if α m157 MAb blocks the interaction of m157 with its receptor, Ly49H. The Ly49H reporter cells (HD12) were stimulated with BaF3m157 cells in the presence or absence of the α m157 MAb or an isotype control MAb (23). The α m157 MAb resulted in the specific inhibition of Ly49H-dependent β -galactosidase

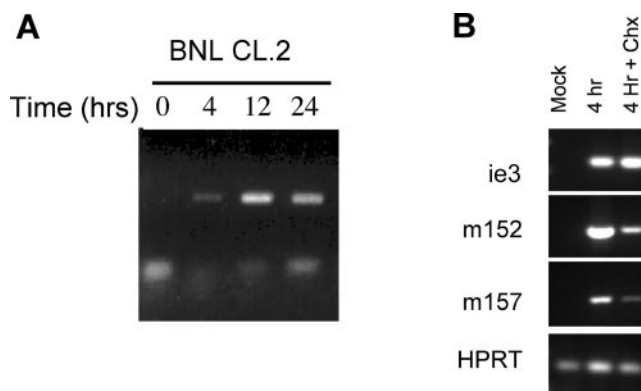


FIG. 3. m157 is expressed as an early viral gene. (A) BNL CL.2 cells were infected as described for Fig. 1, with MCMV at MOI of 5. Total RNA was isolated at the times indicated after infection, using TRIZOL reagent (Life Technologies) according to the manufacturer's protocol. The RNA was treated with DNase I (Invitrogen) per the manufacturer's protocol and reverse transcribed with oligo (dT) primers using the Advantage RT-for-PCR kit (BD Biosciences). The reverse-transcribed products were then amplified using specific primer sets. The amplified products were separated on a 2% agarose gel and visualized by ethidium bromide staining. Primers m157-F (5'-TTACC GTTACAAGAGGTGGCG-3') and m157-R (5'-TCGCGTTCATGT ATGCGAGTT-3') were designed to amplify a 459-bp product within the MCMV m157 gene. (B) BNL CL.2 cells were mock infected or infected for 4 h in the presence or absence of 200 μ g/ml of cycloheximide (Chx). RT-PCR was performed as described above, and the presence of *ie3* (the immediate early gene), m152 (the early gene), m157, and HPRT (control) transcripts was assessed. Primers used to amplify *ie3* (299-bp fragment) and HPRT (163-bp fragment) were previously described (1). Primers m152-F (5'-AAGCCGGACGGCC GTAGTC-3') and m152-R (5'-GTACCATCCCGTCGGGAAG-3') were designed to amplify a 508-bp product within the MCMV m152 gene. PCRs were performed under the following conditions: 1 cycle at 94°C for 3 min; 30 cycles of 30 sec at 94°C, 30 sec at the corresponding annealing temperature, and 30 sec at 72°C; and 1 cycle at 72°C for 10 min. Annealing temperatures for the *ie3*, m152, and HPRT primers was 58°C. Annealing temperature for the m157 primers was 62°C. Specific PCR-amplified products were not detected in control reactions, which lacked reverse transcriptase during the RNA reverse transcription reaction (data not shown).

production by the HD12 reporter cells. This inhibition was similar to the inhibition seen when the reporter cells were incubated with α Ly49H MAb. Thus, the α m157 MAb completely blocks ligand recognition by Ly49H reporter cells.

To further characterize the interaction of m157 with Ly49H, we analyzed the activation of the Ly49H⁺ subset of NK cells by MCMV-infected cells. The production of gamma interferon (γ -IFN) by Ly49H⁺ NK cells was specifically inhibited by preincubation of MCMV-infected cells with the α m157 Fab fragment (Fig. 2B). A down-regulation of the Ly49H molecule on the cell surface of the NK cell also occurs upon exposure to m157 transfectants (23), but it was not possible to determine if this phenomenon was entirely m157 dependent when wild-type MCMV-infected cells were used. Here we show that the down-regulation of the Ly49H receptor on Ly49H⁺ NK cells was blocked when splenocytes were stimulated with MCMV-infected cells in the presence of the α m157 Fab fragment, comparable to that of mock-infected cells (Fig. 2B and C). These findings suggest that the NK cell may become less responsive to further activation by decreasing the number of Ly49H re-

ceptors on the cell surface, thus limiting its response and potentially minimizing "collateral damage" to the host.

m157 is expressed in an early pattern following MCMV infection. In order to determine a time course for m157 expression, BNL CL.2 cells were infected with MCMV and assessed for the expression of m157 transcripts by reverse transcriptase PCR (RT-PCR). For selective expression of immediate-early transcripts, the cultures were incubated from 30 min prior to infection to 4 h postinfection in the presence of 200 μ g/ml of cycloheximide. Infected BNL CL.2 cells demonstrated expression of m157 mRNA by 4 h, and it could still be detected at 24 h postinfection (Fig. 3A). In addition, expression decreased in the presence of cycloheximide. This is similar to the pattern seen with m152, which has previously been shown to be an early gene (14). In contrast to m157 and m152, the pattern of *ie3*, a known immediate-early gene (1, 17), showed no inhibition of expression by cycloheximide (Fig. 3B). Similar results were seen in an MCMV-infected fibroblast cell line (data not shown). Finally, the kinetics of expression by transcript analysis parallels protein expression by flow cytometry (Fig. 1D).

Molecules regulating MHC-I expression do not affect the cell surface expression of m157. Based on computer algorithms, m157 has a putative structure that resembles MHC-I molecules and contains putative α 1, α 2, and α 3 domains (2, 23). The stable expression of MHC-I on the cell surface requires noncovalent association of the α 3 domain of MHC-I with beta-2 microglobulin (β ₂M) (18) and the appropriate loading of peptide onto the MHC-I molecule in the endoplasmic reticulum by the transporter associated with antigen processing (TAP) (26). Accordingly, both β ₂M-deficient and TAP-1-deficient mice show defects in MHC-I expression (26, 28). Twenty-four hours after MCMV infection, primary bone marrow macrophages from wild-type and β ₂M^{-/-} mice expressed similar levels of m157 on the cell surface (Fig. 4A). Similarly, both wild-type and TAP1^{-/-} primary bone marrow macrophages expressed similar levels of m157 on their cell surfaces following infection with MCMV (Fig. 4B). β ₂M^{-/-} and TAP1^{-/-} cells could be differentiated from wild-type macrophages by the inability to express MHC-I on their cell surfaces (data not shown). Thus, although m157 has a predicted tertiary structure related to MHC-I, it does not require either β ₂M or TAP1 for expression on the cell surface.

MCMV has developed a number of mechanisms to subvert immune control. One mechanism is through the interference of antigen presentation by the MHC-I pathway. Three MCMV proteins (m04, m06, and m152) are responsible for the down-regulation of MHC-I following MCMV infection (11, 15, 19). In addition, the m152 glycoprotein has been shown to be involved in the down-regulation of the RAE-1 (retinoic acid early inducible 1) gene products that serve as ligands for the activating NK cell receptor NKG2D and that share structural homology with MHC-I proteins (6–8, 20). As m157 also represents a ligand for an activating receptor (Ly49H) and has a presumed tertiary structure similar to that of MHC-I, it is possible that m157 expression on the cell surface occurred under similar control mechanisms.

To determine if these three viral proteins are involved in the regulation of m157, primary bone marrow macrophages were infected with either wild-type MCMV or a mutant MCMV virus with deletions in the m04, m06, and m152 genes (a triple

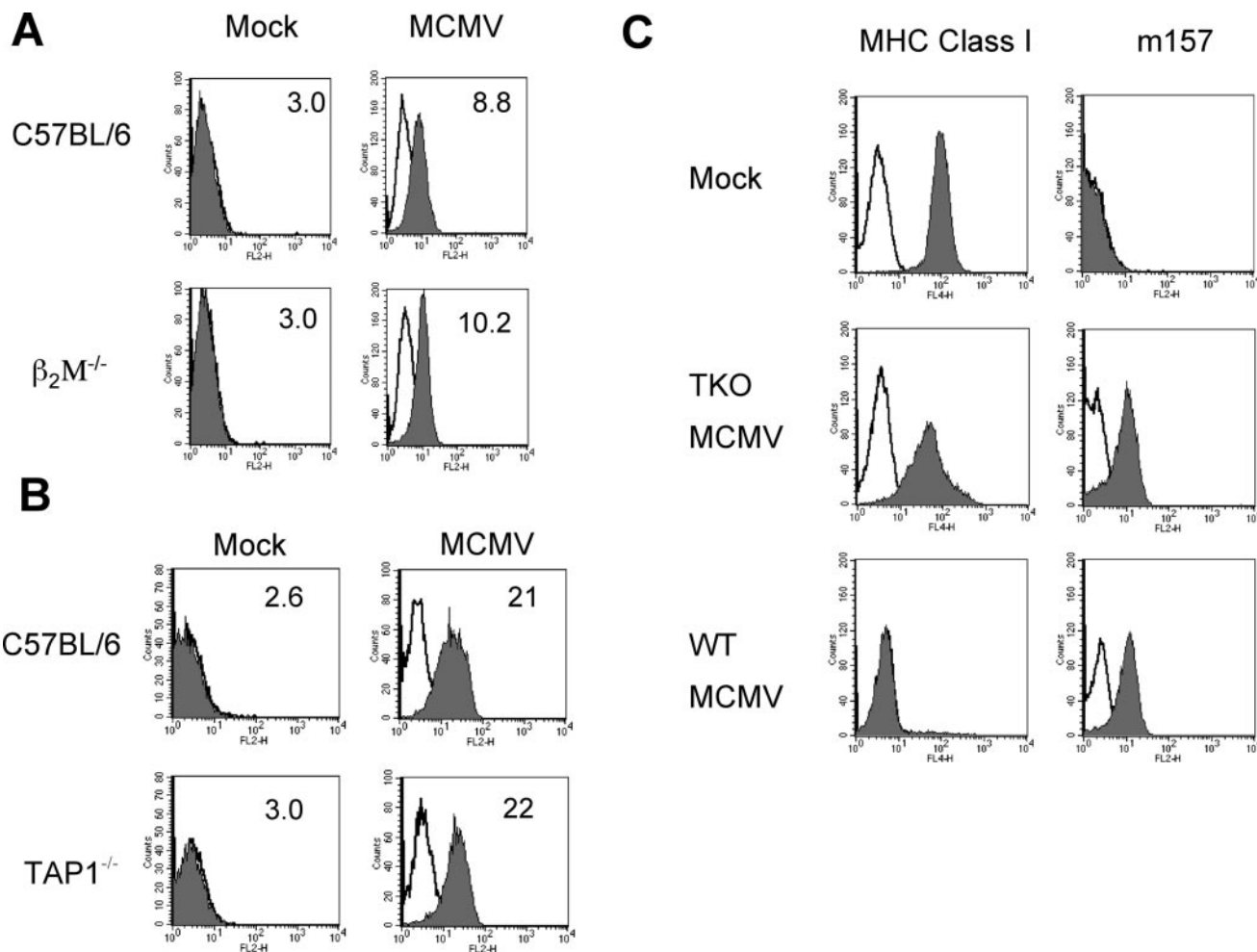


FIG. 4. Molecules regulating MHC-I expression do not affect the cell surface expression of m157. (A) Primary bone marrow macrophages were derived from C57BL/6 mice or B6. $\beta_2M^{-/-}$ mice (The Jackson Laboratory). Following mock infection or infection with MCMV, C57BL/6 and $\beta_2M^{-/-}$ primary bone marrow macrophages were stained for the presence of m157 on the cell surface as described for Fig. 1. Results of isotype control MAb staining is shown in opened curves and $\alpha m157$ MAb staining is shown in closed curves. Numbers represent the MFI of the m157 stained cells. (B) This experiment was carried out identically to that for 4A except that primary bone marrow macrophages (TAP1^{-/-}) were derived from B6.TAP1^{-/-} mice (The Jackson Laboratory) instead of from $\beta_2M^{-/-}$ mice. (C) Primary C57BL/6 bone marrow macrophages were mock infected, infected with a triple-knockout MCMV, or infected with wild-type (WT) MCMV. Twenty-four hours postinfection, the cells were stained for H2K^b/H2D^b (MHC Class I), m157 (closed curves), or isotype control MAbs (open curves). The mouse anti-mouse H-2K^b/H2-D^b (clone 28-8-6) was obtained from Pharmingen and conjugated to allophycocyanin as previously described (13). A representative histogram is shown in each panel.

knockout [TKO] mutant). Infection of primary bone marrow macrophages with either wild-type or TKO MCMV resulted in the expression of similar levels of m157 on the cell surface. When the same cells were assessed for cell surface expression of MHC-I molecules, cells infected with wild-type MCMV clearly had decreased expression of MHC-I on the cell surface. By contrast, MHC-I expression of TKO MCMV-infected cells was similar to that seen on mock-infected cells (Fig. 4C). Thus, viral proteins, which lead to the decreased surface expression of MHC-I molecules, do not alter the expression of m157. Therefore, MCMV has apparently retained m157 expression by sparing it from its mechanisms to down-regulate host molecules with similar putative structures.

Our current data supports the hypothesis that m157 is deliberately expressed by the virus for potential interaction with the host. We found that m157 is expressed early after infection

by a wide variety of cells and is not subject to viral regulation that affects other NK cell receptor ligands, such as MHC-I and NKG2D ligands. It is interesting that m157 is expressed on MCMV-infected cells with early, rather than late, kinetics, preceding the production of infectious virions. From the virus point of view, the early expression of m157 should be detrimental to establishing infection. However, NK cells are not usually present at the normal portal of MCMV infection, such as the mucosal linings of the oropharynx. Infection may thus be established, but perhaps dissemination is controlled, because secondary infection of hematopoietic cells would be readily detected by NK cells in the spleen, where they are poised, in the red pulp and near the marginal sinus, to eradicate virus-infected cells. If so, then m157 may aid the virus by preventing overwhelming viral dissemination, which would result in the death of the host.

It is clear, however, that escape mutants with m157 defects develop when innate immune pressure from Ly49H⁺ NK cells is present as the primary control mechanism (10, 27), begging the question of why m157 is retained at all. Although studies are still ongoing, most laboratory-adapted strains of *Mus musculus* do not have an intact Ly49H gene. Indeed, at least one other strain (129/SvJ) has an inhibitory receptor that binds m157 (5), suggesting that m157 may subserve immune evasion by inhibiting NK cells. The modestly decreased virulence of the m157 deletion virus in BALB/c animals (5) that have no NK cell receptor for m157 also suggests that m157 may have another immune evasion mechanism. Alternatively, signal transduction through GPI-anchored proteins may offer a replication advantage to the virus. For example, NS1 (dengue virus non-structural protein 1), a GPI-anchored protein, is a virally encoded protein that has been shown to be capable of signal transduction (16). Moreover, cross-linking of a number of GPI-anchored proteins, including DAF (decay-accelerating factor), Ly-6, and Thy-1, has been shown to result in the activation of T cells (12, 21, 22, 24). In a similar fashion, m157 might serve as a signaling molecule on MCMV-infected cells. Nevertheless, these findings highlight the continuing “arms race” between MCMV and the host innate immune response.

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