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Functional Consequences of Natural Sequence Variation of Murine Cytomegalovirus m157 for Ly49 Receptor Specificity and NK Cell Activation

Alexandra J. Corbett, Jerome D. Coudert, Catherine A. Forbes, and Anthony A. Scalzo

The Ly49H activating receptor on C57BL/6 (B6) NK cells plays a key role in early resistance to murine cytomegalovirus (MCMV) infection through specific recognition of the MCMV-encoded MHC class I-like molecule m157 expressed on infected cells. The m157 molecule is also recognized by the Ly49I inhibitory receptor from the 129/J mouse strain. The m157 gene is highly sequence variable among MCMV isolates, with many m157 variants unable to bind Ly49H<sup>B6</sup>. In this study, we have sought to define if m157 variability leads to a wider spectrum of interactions with other Ly49 molecules and if this modifies host susceptibility to MCMV. We have identified novel m157–Ly49 receptor interactions, involving Ly49C inhibitory receptors from B6, BALB/c, and NZB mice, as well as the Ly49<sup>HNZB</sup> activation receptor. Using an MCMV recombinant virus in which m157<sup>G1F</sup> was replaced with m157<sup>K181</sup>, which interacts with both Ly49<sup>H</sup>B6 and Ly49<sup>C</sup>B6, we show that the m157<sup>G1F</sup>–Ly49C interactions cause no apparent attenuating effect on viral clearance in B6 mice. Hence, when m157 can bind both inhibitory and activation NK cell receptors, the outcome is still activation. Thus, these data indicate that whereas m157 variants predominately interact with inhibitory Ly49 receptors, these interactions do not profoundly interfere with early NK cell responses. The Journal of Immunology, 2011, 186: 1713–1722.

Natural killer cells are important in limiting early CMV infection. The ability of NK cells to control viral infections is tightly regulated by the inhibitory and activating cell-surface receptors that they express (1). Activating NK cell receptors, including activating forms of killer cell Ig-like receptors (KIRs) in humans, activating Ly49 receptors in mice, and NKG2D in both species, positively recognize tumor and virus-infected cells. The Ly49H NK cell activation receptor was shown to be responsible for Cmv1-mediated resistance to murine cytomegalovirus (MCMV) infection (2–4). Ly49H from the C57BL/6 mouse strain binds specifically to m157, an MCMV-encoded protein with an MHC class I-like structure (5, 6). NK cells also possess inhibitory receptors specific for MHC class I that permit discrimination of normal healthy cells from those that are potentially disease causing, such as virus-infected cells, as these cells often express reduced levels of MHC class I. In mice, these receptors include members of the Ly49 family and CD94/NKG2A (reviewed in Refs. 7, 8). Different individuals within populations (or different inbred mouse strains) express distinct NK gene haplotypes, which may result in varying levels of antiviral immunity. Although the Ly49 receptors have been mapped and relatively well characterized for strains such as B6 (9, 10), BALB/c (11, 12), 129/J (13, 14), and NOD (15), the Ly49 gene expression and allelic relationships between other inbred strains and in wild mouse populations are not completely understood. In addition to Ly49H<sup>B6</sup>, Arase et al. (5) showed that m157 is capable of binding to the Ly49I inhibitory NK cell receptor from 129/J mice. This suggested that m157 evolved to serve as an NK cell immunoevasion protein that delivers inhibitory signals to NK cell subsets expressing Ly49I or Ly49I-like inhibitory receptors (5) and that the activating Ly49H receptor might have later evolved from a Ly49I-like receptor to combat virus infection (5, 16, 17). However, studies to date have been limited to the laboratory MCMV strains Smith and K181, which have an identical m157 sequence, and thus far only B6 and 129/J NK cell receptors have been examined.

The structure of the m157 molecule from the laboratory MCMV strain Smith has recently been elucidated (18). However, MCMV isolates from wild mice vary greatly in their m157 sequences (19). We have previously analyzed m157 variation in a panel of 40 MCMV isolates (20) (A.J. Corbett and A.A. Scalzo, unpublished observations). Among these isolates, only two had sequences identical to K181 and Smith (19). The remaining isolates fell into two sequence clusters. Cluster 1 consisted of 15 isolates with sequences showing 80.4–87.9% identity to m157<sup>Smith</sup>. Cluster 2 consisted of 23 isolates with sequences 63.9–68.2% identical to...
m157\textsuperscript{Smith}. These data demonstrate that m157\textsuperscript{Smith}-like sequences are uncommon in viral isolates from wild mice and suggest that m157 variants may have functions distinct from those in laboratory strains. Analysis of viral isolates encoding m157 variants revealed that, unlike the laboratory strains, many can replicate to high titers in MCMV “resistant” B6 (Ly49\textsuperscript{H+c/Mcmv\textsuperscript{I}I}) mice, indicating that the concept of resistance is context dependent (19).

If m157 primarily evolved to interact with inhibitory NK cell receptors, then we hypothesized that m157 variation may lead to differential binding to variable Ly49 receptors. We analyzed the binding of m157 variants to NK cells and Ly49 receptors from a range of inbred mouse strains and show that, as well as providing a target for B6 NK cell activation through Ly49\textsuperscript{H6\textsuperscript{B6}} recognition, m157 variants bind to a range of inhibitory Ly49 receptors in the NZB, BALB/c, and B6 mouse strains. In most reports to date, the inhibitory signals mediated through Ly49 receptors appear to override Ly49 activation signals and block NK cell activation and inhibitory signals mediated through Ly49 receptors appear to be required for optimal surface expression of activating Ly49 receptors, BWZ-HD12 reporter cells (6) and the BWZ-HI B6 and BWZ-HI129 chimeric reporter cell lines (kindly provided by W. M. Yokoyama, Washington University). In this study, we have identified an m157 variant (m157\textsuperscript{GIF}) that binds both activation (Ly49\textsuperscript{H6\textsuperscript{B6}}) and inhibitory (Ly49\textsuperscript{C\textsuperscript{B6}}) receptors, and we hypothesized that the interaction with the inhibitory receptor might override signals delivered by Ly49H. We have constructed a recombinant m157\textsuperscript{K181G+G161K} gene-swap MCMV and used this to analyze the functional relevance of these interactions in vivo in a model in which both activating and inhibitory NK cell receptors are engaged by the same viral ligand.

**Materials and Methods**

**Animals**

Inbred B6, BALB/c, SJL/J, and 129 \times 1/5V/Si mice and outbred A/Rs/c mice were obtained from the Animal Resources Centre (Perth, Western Australia, Australia) and NZB mice from the Walter and Eliza Hall Institute animal facility (Reew, Victoria, Australia). BALB.C-T8 congeneric mice (23) were bred at the University of Western Australia Animal Care Unit (Perth, Western Australia, Australia). Female mice aged 3 or 8 wk were used for all experiments. All animal experiments were performed in pathogen-free conditions after approval by the Animal Experimentation and Ethics Committee of the University of Western Australia under the guidelines of the National Health and Medical Research Council of Australia.

**Cells and cell lines**

BWZ.36, RMA, Chinese hamster ovary (CHO), and CHO-Ly49 cells (24) were grown in RPMI 1640 containing 10% FCS (Invitrogen), glutamine, sodium pyruvate, penicillin, gentamicin, and 2-mercaptoethanol (R10). BWZ-HD12 reporter cells (6) and the BWZ-HI\textsuperscript{H6} and BWZ-HI \textsuperscript{H2} chimeric reporter cell lines (kindly provided by W. M. Yokoyama, Washington University, St. Louis, MO), which express the Ly49\textsuperscript{H6\textsuperscript{B6}} transmembrane and cytoplasmic domains fused to the extracellular domains of Ly49\textsuperscript{B6} and Ly49\textsuperscript{H2} respectively, were maintained in R10 containing hygromycin (Sigma). COS-7 cells and Platinum-E cells were grown in DMEM (Invitrogen) containing 10% FCS, glutamine, sodium pyruvate, penicillin, and gentamicin. Primary mouse embryonic fibroblasts (MEFs) were produced by trypsin dispersion of 15- to 17-d-old embryos from A/Rs/c mice as previously described (25). MEFs and M210B4 cells were maintained in MEM 10% neonatal calf serum (Invitrogen). Immediately prior to infection, the culture medium was replaced with media supplemented with 2% FCS. Lymphokine-activated killers (LAKs) were produced as previously described (26).

**Abs**

Goat anti-human IgG (Fc fraction), goat anti-mouse IgG, and goat anti-rat IgG were purchased from Jackson ImmunoResearch Laboratories. Streptavidin–HRP, anti-CD49b (DX5), anti-NK1.1 (PK136), anti-Ly49C/I (5E6), anti–IFN-γ, rat IgG1 isotype control, anti-CD3ε (145.2C11), anti-CD69, anti-KLRL1, and anti-mlgG1 were purchased from BD Pharmingen. Purified preparations of 3D10 (anti-Ly49H1), A1 (anti-Ly49A), 5E6 (anti-Ly49C/I), LGL-1 (anti-Ly49G1 and G2), and 4E5 (anti-Ly49D) Abs were kindly provided by W. M. Yokoyama (Washington University), and anti-Ly49C 4LO3311 was kindly provided by S. Lemieux (Institut National de Recherche Scientifique, Laval, Quebec, Canada).

**Production of m157–Fc soluble fusion proteins**

The CP197 construct containing the Smith m157–Fc behind the CD150L sequence cloned in the CDMS vector (5) was kindly provided by Prof. L. L. Lanier (University of California, San Francisco, CA). Soluble m157–Fc fusion proteins for wild-type MCMV isolates were constructed by replacing the Smith m157 sequence in this vector with that of other strains. Each m157 open reading frame (minus the predicted leader sequence and truncated before the GPI anchor motif) was PCR amplified using primers containing the XhoI recognition sequence (see Supplemental Table I) and cloned into CP197 using the XhoI site, such that m157 was in-frame with the Fc portion of human IgG1. Soluble fusion proteins were expressed by transient transfection in COS-7 cells using FuGene 6 transfection reagent (Roche Diagnostics). Supernatants were typically collected at days 3, 5, and 7 after transfection, pooled, and concentrated using spin concentrators (Amicon). To detect soluble m157–Fc fusion proteins by Western blot, both reducing and nonreducing SDS-PAGE were performed on 4–20% polyacrylamide gels (Invitrogen) alongside prestained markers (SeeBlue Plus 2; Novex). Proteins were then transferred to nitrocellulose (Bio-Rad & Schill) and the soluble m157–Fc proteins detected using biotinylated anti-human IgG (Fc fraction) (Jackson Immunoresearch Laboratories), followed by streptavidin–HRP (Amersham Biosciences) and ECL reagents (Amersham Biosciences).

**Generation of BWZ transfectants**

For the construction of the DAP12-FLAG-pEY plasmid, the DAP12 open reading frame, minus the predicted signal sequence, was PCR amplified using forward primer 5’-TAATGGATCCAGTGACACTTCTCAAG-3’ and reverse primer 5’-TATTCTAGATATGATCTTGATATG-3’ and sequenced. The resultant PCR product was digested with BamHI and XbaI and cloned into the pEF-SCMVFlag vector (19). BWZ.36 cells were transfected with DAP12-FLAG-pEY by electroporation using a Bio-Rad Gene Pulser, and stable cells were selected with puromycin (Sigma). BWZ HD12 reporter cells were transduced with the pmX-s-ires-Ly49Gm6\textsuperscript{H6\textsuperscript{B6}} construct to generate BWZ HD12C cells. Clones expressing a range of levels of Ly49\textsuperscript{H6\textsuperscript{B6}} and Ly49\textsuperscript{Gm6\textsuperscript{H6\textsuperscript{B6}}} were selected after dilution cloning, including clones that expressed essentially equal levels of these two receptors.

**RT-PCR amplification and cloning of CB19 and BALB/c Ly49 genes, and generation of retroviruses**

mRNA was purified from NZB and BALB/c LAKs or sorted splenocytes using the micro poly(A)Pure RNA isolation kit (Ambion). First-strand cDNA synthesis was performed using oligo-dT\textsubscript{17}-adapter primer [5’-GACTCGACGACATGCA(T\textsubscript{1}7)-3’] (27) and AMV reverse transcriptase (Promega). Ly49 constructs were amplified by RT-PCR using primers designed based on published Ly49 sequences from the Genbank database (see Supplemental Table II) using HotStar HiFidelity Polymerase (Qiagen) and cloned into the pMX-s-ires-GFP vector (28) (kindly provided by Toshiho Kitamura, University of Tokyo, Tokyo), using the XhoI and NotI restriction sites in the 5’ multimeroning site. For a positive control, Ly49\textsuperscript{H6\textsuperscript{B6}} [PCR amplified from the pmX-s-Ly49H DAP12 vector (6)] was cloned into the BamHI site. To confirm the sequence of constructs, all clones were sequenced with both forward and reverse primers using the Applied Biosystems Dye Terminator Version 3.1 sequencing kit. Sequences obtained for Ly49 clones were compared with the published Ly49 sequences (GenBank). Sequence alignments (ClustalW) and translations were performed using the Bioedit Sequence Alignment Editor suite of programs (Insis Pharmaceuticals). Transmembrane region predictions were performed using the HMMTOP version 2.0 (29, 30) server. Two residues differ from the published sequence (Genbank accession no. MMU49867), possibly due to differences in the colonies of NZB mice maintained in different animal facilities. A full-length sequence was obtained for Ly49\textsuperscript{H2\textsuperscript{B6}}, a member of the activating arm of the Ly49 receptor family. This was identical to the (incomplete) published sequence for Ly49\textsuperscript{H2\textsuperscript{B6}} (GenBank accession no. AY33897), except for one residue (D5E) that resulted from the use of a B6 sequence-based primer. This residue is close to the N terminus and thus unlikely to affect ligand binding. Because the DAP12 primer is required for optimal surface expression of activating Ly49 receptors, BWZ-DAP12 cells were transduced with the retroviral plasmids. Briefly, Platinum-E cells were transfected with 2 μg plasmid DNA using FuGene 6.
transfection reagent (Roche Diagnostics). After 48 h, supernatant was aspirated and replaced with 700 µl fresh medium. At 72 h, retrovirus-containing supernatant was filtered and used to transduce BWZ-DAP12 (two rounds of infections with supernatant in the presence of polybrene [Sigma]). Cells were analyzed for m157–Fc binding by flow cytometry after 2–3 d.

Virus stocks

The MCMV laboratory strains used were Smith (originally obtained from E. S. Mocarski, Stanford University) and K181-Perth (K181). Origins of wild-derived isolates (G1F, G3F, G4, G6, K6, K7, K17E, N1, N5, W3, W211, M60a) have been previously described (20). Tissue culture virus stocks were produced by propagation in MEFs or M210B4 cells and titers determined by standard plaque assay as previously described (32). Virulent salivary gland viral (SGV) stocks were prepared by infecting 3-wk-old female BALB/c mice i.p. with 10^4 PFU tissue culture virus and then preparing homogenates of salivary glands at 17 d postinfection. Secondary SGV stocks were prepared by infecting 3-wk-old female BALB/c mice with 10^4 PFU primary SGV stock. Viral DNA was produced from infected MEFs as previously described (33).

Construction of m157 gene-swap MCMVs and analysis of in vivo replication of MCMV

To produce m157 gene swap viruses, we used a "bacterial artificial chromosome (BAC) recombineering" approach essentially as previously described (34). The K181 BAC pARK25 DNA (35) kindly provided by A. Redwood (University of Western Australia) was transformed into the SW102 Escherichia coli strain that lacks the galK gene to give an SW102-pARK25 BAC strain. To disrupt the m157 gene in the pARK25 BAC, the galK gene sequence was amplified from the pgalK plasmid by PCR using primers containing 5' 50mer specific sequences that flank the K181 m157 gene (homology arms that facilitate homologous recombination) and downstream sequences that are specific for the galK gene (see Supplemental Table III). SW102-pARK25 BAC E. coli cells were transfected with the linear m157-flanked galK targeting PCR product and plated onto minimal media containing galactose to select for galK recombinants containing the galK gene inserted in place of the K181 m157 gene (galK-pARK25 BAC). To generate substitution mutants in which the K181 m157 gene has been replaced with the G1F m157 sequences (pARK25m157^G1F), PCR products were amplified from purified G1F viral DNA using primers containing the same flanking K181 homology arms used above and downstream specific sequences that amplify the G1F m157 sequences. The galK cassette in the galK-pARK25 BAC was replaced by transforming SW102-galK^+ pARK25 with the G1F m157 targeting PCR products and growing the bacteria on minimal medium with glycerol, chloramphenicol, and 2-deoxy-galactose. This negative selection results in loss of the galK gene through homologous recombination and substitution with the G1F m157 genes. The pARK25m157^G1F-m157^mutant and pARK25m157^G1F-m157^C2-mutant viruses were produced from the SW102-galK^+ pARK25 BAC in the same manner as the pARK25m157^G1F mutant, but using C2 m157 mutant MCMV (19) or K181 MCMV viral DNA as template for the PCR amplification. All BAC recombinants produced by this approach were streaked to give single colonies and were sequenced to verify that the m157 sequences were inserted correctly and no sequence errors were present within the gene. The vARK25-m157^G1F, vARK25-m157^G1F-m157^mutant, and vARK25-m157^C2-mutant, -m157^G1F-m157^C2-mutant, and -m157^C2-mutant viruses were recovered by transfection of MEFs with the BAC DNA preparations using FuGene 6 reagent (Roche Diagnostics). Excision of the BAC backbone was verified by loss of GFP fluorescence of virus-infected cells and confirmed by PCR across the region of BAC insertion using the following primers: forward 5'-TTGGAGCGATACGTTGACAATG-3' and reverse 5'-TCCTGATGCTTCTAATCTCACCG-3'. Viral DNA preparations were made from infected M210B4 cells or MEFs as previously described (33) and RFLP analysis performed using EcoRI and HindIII, to verify that no gross mutations had occurred elsewhere in the genome. Expression of m157 from the vARK25-m157^G1F and vARK25-m157^C2-mutant viruses was confirmed by infecting M210B4 cells in a reporter assay (data not shown). The recombinant viruses (vARK25-m157^G1F, vARK25-m157^G1F-m157^mutant, and vARK25-m157^C2-mutant) were used to freshly infect MEFs, and viral titers in the target organs (spleen, liver, lungs, and salivary glands) were compared by standard plaque assay as described (32).

Results

m157 variants display a range of specificities for Ly49H^B6 and Ly49H^29

The interaction of the laboratory Smith MCMV strain m157 glycoprotein with Ly49H^B6 was one of the first descriptions of the direct recognition of a viral protein by NK cells. However, m157 displays remarkable variation between MCMV isolates (19) (Fig. 1A). To determine whether this variation causes functional differences in binding to NK cell receptors, soluble m157–Fc fusion proteins were constructed for 14 MCMV wild isolates with variant m157 sequences and tested by incubating the Ly49H^B6-expressing BWZ-HD12 or chimeric BWZ-HI^29 reporter cells with plate-bound m157–Fc (Fig. 1B). Results were consistent with those using infected M210B4 cells (data not shown). Smith/K181, G1F, and N5 m157–Fc proteins activated both BWZ-HD12 and BWZ-HI^29 reporter cells. K17E and N1 m157–Fc proteins weakly activated chimeric BWZ-HI^29 cells, but not BWZ-HD12 cells. Although cells infected with the M16A MCMV isolate could activate BWZ-HI^29 cells to a small extent (data not shown), the M16A m157–Fc failed to do so (Fig. 1B).

To confirm these interactions, the panel of m157–Fc fusion proteins was tested by flow cytometry for the ability to bind cells expressing Ly49H^B6 (BWZ-HD12), Ly49D^B6 (BWZ-DD12), Ly49I^B6, and Ly49I^29 (Fig. 1C). Ly49D^B6– and Ly49I^B6–expressing cells acted as negative controls. Consistent with our reporter assay results (Fig. 1B), Smith, G1F, and N5 m157–Fc proteins bound to BWZ-HD12 cells. This interaction is specific for Ly49H^B6, as untransfected BWZ36 control cells showed no
Variant m157–Fc fusion proteins show a range of binding patterns to NK cells from inbred mice

The Ly49 receptor family displays remarkable diversity between mouse strains, and we hypothesized that variant m157 molecules would show a range of binding patterns to NK cells from different strains. We tested the panel of m157–Fc fusion proteins for their ability to bind NK cells from five mouse strains (BALB/c, B6, NZB, SJL/J, and 129 × 1/SvJ) that are representative of five major NK gene complex haplotypes in inbred mice (38, 39). Only laboratory strains and variant MCMV m157–Fc proteins in sequence cluster 1 (19) (Fig. 1A) could bind, and these exhibited distinct patterns of interactions with NK cells from different mouse strains (Fig. 2A). All binding observed was to cells in the NK cell (CD3−, DX5+) population, with no significant binding to CD3+ or DX5− cells above that of the secondary reagents alone. The Smith, G1F, and N5 m157–Fc proteins interacted with B6 NK cells (Fig. 2A, top row), whereas only two m157–Fc proteins, from the G1F and W8211 isolates, bound to BALB/c NK cells (Fig. 2A, second row). In addition, the Smith, G1F, K17E, and N5 m157–Fc proteins each bound strongly to NZB NK cells (Fig. 2A, third row), whereas binding to SJL/J NK cells was weak (Smith, G1F, K17E, and N5 showed highest levels) or absent (Fig. 2A, fourth row). Weak binding of Smith, G1F, K17E, and N5 m157-constructs to 129 × 1/SvJ NK cells was also observed (Fig. 2A, fifth row); it has previously been reported that m157–Fc from Smith binds to Ly49I129 (5), and our own data with the chimeric BWZ-HI129 reporter cells support this conclusion (Fig. 1C). It should be noted that in the study by Arase et al. (5), the intensity of binding of Smith m157–Ig to 129/J NK cells was also significantly less than that for B6 NK cells. These data show that m157 proteins can bind to NK cells from a range of inbred mouse strains and that individual m157 variants differ in their binding specificities.

Defining the BALB/c and B6 NK cell receptors bound by G1F and W8211 m157–Fc proteins

Because BALB/c mice lack Ly49H, the fact that G1F and W8211 m157–Fc proteins bound to BALB/c NK cells (Fig. 2A) suggested that they may bind to another receptor in this strain. The majority of cells binding these m157–Fc proteins were also positive for SE6 (anti-Ly49C/I) (Fig. 2B), indicating that, as BALB/c lack Ly49I, Ly49C(BALB/c) may be the receptor for m157 in these mice. In contrast, only ~50% of B6 cells that bind m157G1F-Fc were positive for SE6. SE6+B6 NK cells also showed weak binding to m157W8211–Fc. Overall, these data are consistent with m157G1F binding to both Ly49B6 and Ly49H6, and W8211 weakly binding only Ly49C86, and with both G1F and W8211 m157 proteins binding to Ly49C(BALB/c). The B6 SE6− cells that do not bind G1F or W8211 m157–Fc proteins (Fig. 2B, lower right quadrant) are likely expressing Ly49I and not Ly49C.

To confirm the identity of the BALB/c Ly49 receptors binding to m157 G1F–Fc and m157 W8211–Fc, cells were transduced with BALB/c Ly49 genes cloned with primer sets based on sequences available in the GenBank database (Supplemental Table II) along with the DAP12 adapter, which is required for optimal surface expression of activating Ly49 receptors (40, 41), and tested by flow cytometry for binding to m157G1F–Fc and m157W8211–Fc. Clones were obtained for Ly49B, Ly49C, Ly49E, Ly49G2, and Ly49L. Notably, we also obtained clones that lacked transmembrane domains such as for a Ly49E(BALB/c) transcript (data not shown). Both the G1F and W8211 m157–Fc proteins were found to bind Ly49C(BALB/c) (Fig. 3A), but not Ly49B, Ly49E, Ly49G2, or Ly49L (data not shown). Thus, we have identified Ly49C(BALB/c) as another inhibitory receptor capable of interacting with variant m157 proteins.
To determine whether variant m157s could bind Ly49 receptors other than Ly49H B6 in B6 mice, Ly49A B6, Ly49C B6, Ly49G1 B6, Ly49G2B6, and Ly49I B6 transfected CHO cells were incubated with a panel of variant m157–Fc proteins and analyzed by flow cytometry. Both m157 G1F–Fc and m157 W8211–Fc showed binding to Ly49C B6 (Fig. 3B). None of the m157–Fc proteins bound to Ly49IB6 (Fig. 3B) or to the other Ly49 B6 receptors tested (data not shown). Expression of Ly49I B6 was confirmed by the 5E6 mAb (Fig. 3B). The binding of m157 G1F–Fc to B6 NK cells is at least in part due to Ly49H, as m157 G1F–Fc binds to BWZ-HD12 reporter cells (Fig. 1C). The relatively weak binding by m157 W8211–Fc to Ly49C B6-transfected cells is consistent with very low levels of binding detected on 5E6 + B6 cells (Fig. 2B). m157 W8211–Fc does not bind to Ly49H B6 (Fig. 1C) nor activate BWZ-HD12 reporter cells (Fig. 1B), and W8211-infected cells do not activate BWZ-HD12 reporter cells (data not shown). Thus, we have identified FIGURE 2. Flow cytometric analysis of binding of variant m157–Fc proteins to splenocytes from five inbred mouse strains. A. Splenocytes were stained with m157–Fc chimeric proteins from the Smith laboratory strain or m157-variant wild MCMV isolates, followed by biotinylated goat anti-human IgG (Fc) and streptavidin–allophycocyanin, and also stained with FITC-conjugated DX5 mAb and with PerCP5.5-conjugated anti-CD3 mAb. NK cells were defined as being positive for the DX5 Ab (a pan–anti-NK cell reagent that binds to NK cells in all tested mouse strains) and negative for CD3 expression. Numbers indicate the percentage of CD3−, DX5+ cells that bind the various m157–Fc proteins. Plots show live, CD3− cells. B. Dual staining with PE-conjugated 5E6 to identify Ly49C/I-expressing cells binding m157–Fc proteins. Plots are gated on CD3−, DX5+ live cells. Numbers represent the percentage of cells in each quadrant. FIGURE 3. Flow cytometric analysis of binding of variant m157–Fc proteins to individual Ly49 receptors. A and C, BALB/c (A) and NZB (C) Ly49 receptor constructs were cloned into the pMX-s-IRES-GFP vector. BWZ-DAP12 cells transduced with individual constructs were analyzed for binding to m157–Fc proteins in conjunction with biotinylated anti-human IgG (Fc) mAb and streptavidin–allophycocyanin or anti-Ly49 mAbs 5E6 (anti-Ly49C/I) and 3D10 (anti-Ly49H) followed by Cy5-conjugated anti-mouse IgG by flow cytometry. Filled histograms, vector alone; solid lines, transduced cells (gated based on GFP fluorescence). All plots show gated GFP+ live cells based on exclusion of 7-AAD. No binding was detected to Ly49B-, Ly49E-, Ly49G2-, or Ly49L-transduced cells (not shown). B, CHO cells expressing Ly49B6 receptors or untransfected cells were analyzed for binding to m157–Fc proteins in conjunction with biotinylated anti-human IgG (Fc) mAb and streptavidin–allophycocyanin or anti-Ly49 mAbs 5E6 (anti-Ly49C/I) and 3D10 (anti-Ly49H) (data not shown) followed by Cy5-conjugated anti-mouse IgG by flow cytometry. Filled histograms, untransfected control CHO cells; solid lines, transduced cells. All plots show gated live cells based on exclusion of 7-AAD. No binding was detected to Ly49A B6, Ly49G1 B6, or Ly49G2 B6-expressing CHO cells (not shown).
Ly49C<sup>B6</sup> as another NK cell receptor for m157 from at least two MCMV isolates, G1F and W8211.

**Identification of Ly49 receptors in the NZB mouse strain that bind variant m157–Fc proteins**

To identify cognate Ly49 receptors from the NZB strain that were binding to the variant m157–Fc molecules, Ly49 genes were cloned using sequences available in the GenBank database (Supplemental Table II), and recombinant retroviral constructs were generated to transduce BWZ-DAP12 cells. NZB Ly49 molecules obtained included full-length clones of Ly49E, Ly49H, and Ly49C (Supplemental Fig. 1) and splice variants lacking the transmembrane domain for Ly49E, H, P, and W (not shown).

Cells transduced with full-length Ly49 receptor clones were screened for binding to the m157–Fc proteins from MCMV strains Smith, G1F, N5, and K17E, which had been found to bind NZB NK cells. Ly49C<sup>NZB</sup> showed binding to all four m157–Fc proteins. The different fluorescence intensities obtained with these molecules (Fig. 3C) is consistent with the trend seen with NZB splenocytes (Fig. 2A). The G1F and K17E m157–Fc proteins bound weakly to Ly49H<sup>NZB</sup>-transduced cells (Fig. 3C). Ly49<sup>NZB</sup> was also detected with the 3D10 mAb, consistent with the results of others (42). Thus, some variant m157 proteins are capable of binding Ly49H<sup>NZB</sup>.

**Coexpression of the inhibitory Ly49C receptor decreases BWZ-HD12 reporter cell activation by G1F m157**

Because m157G1F–Fc binds both the activating Ly49H<sup>B6</sup> and inhibitory Ly49C<sup>B6</sup> receptors, we questioned whether the inhibitory receptor could compete for binding of m157 ligands and reduce the subsequent activation of Ly49H<sup>B6</sup>-expressing reporter cells. First, we tested the binding profiles of m157<sup>Smith</sup>–Fc and m157G1F–Fc to Ly49<sup>B6</sup> and compared the binding of m157G1F–Fc to Ly49H<sup>B6</sup> and Ly49<sup>C</sup><sup>B6</sup>. We titrated the fusion proteins and analyzed the binding intensity to Ly49-transduced cells by flow cytometry from concentrations achieving saturation down to concentrations resulting in background intensity. m157<sup>Smith</sup>–Fc and m157G1F–Fc displayed similar binding patterns to Ly49<sup>B6</sup>-transduced cells (Fig. 4A). Consistent with data presented in Fig. 3B, we found that m157G1F bound to Ly49B6-expressing CHO cells, whereas m157<sup>Smith</sup> did not (Fig. 4A). The binding curves of m157G1F–Fc to Ly49B6- and Ly49C<sup>B6</sup>-expressing cells were similar, suggesting that the two Ly49 receptors have similar affinities and could compete for binding to m157G1F.

Next, we used a reporter assay to determine if simultaneous binding to Ly49B6 and Ly49C<sup>B6</sup> altered Ly49<sup>B6</sup>-dependent activation. Ly49H<sup>B6</sup>-expressing BWZ-HD12 reporter cells were transduced with the pMX-s-ires-Ly49C<sup>B6</sup> construct to generate BWZ-HD12<sup>C</sup> cells. Individual clones expressing different levels of Ly49<sup>B6</sup> and Ly49C<sup>B6</sup> were isolated by limit dilution. BWZ-HD12<sup>C</sup> clones expressing comparable levels of Ly49H to BWZ-HD12 cells were selected. Reporter cells BWZ-DAP12, BWZ-HD12, and BWZ-HD12<sup>C</sup> were co-incubated with parental BA/F3 cells or with BA/F3 expressing either m157<sup>Smith</sup> or m157G1F. Whereas BA/F3 cells did not significantly activate the reporter cells, m157<sup>Smith</sup>-expressing BA/F3 cells activated both BWZ-HD12 and BWZ-HD12<sup>C</sup> cells (Fig. 4B). Incubation of m157G1F- or m157<sup>Smith</sup>-expressing BA/F3 cells induced a similar activation in BWZ-HD12, whereas coexpression of Ly49C<sup>B6</sup> in BWZ-HD12C prevented the reporter cell activation by m157G1F–Fc (Fig. 4B). We tested several BWZ-HD12<sup>C</sup> clones and found that the inhibition of Ly49C<sup>B6</sup>-dependent activation was more profound when Ly49<sup>C6</sup> was expressed at a high density (data not shown). Blocking the Ly49C<sup>B6</sup>–m157G1F interaction with the 4LO3311 Ab restored BWZ-HD12C reporter cell activation (Fig. 4B). These data show that simultaneous binding of m157G1F to Ly49B6 and Ly49C<sup>B6</sup> impaired Ly49H<sup>B6</sup>-dependent reporter cell activation, suggesting that Ly49C<sup>B6</sup> can regulate Ly49H<sup>B6</sup> function.

**Binding of Ly49C by m157 does not augment MCMV infection in vivo**

The binding of m157G1F to both Ly49H<sup>B6</sup> and Ly49C<sup>B6</sup> in the same mouse strain provides an ideal opportunity to test the hypothesis that m157 binding to the inhibitory receptor will attenuate NK cell activation and result in increased viral replication. However, analysis of the interactions of m157G1F proteins with NK cells receptors is hampered by the poor replication of this viral isolate in vivo (20). To address this, BAC recombineering (34) was used to replace the m157 gene in the K181 laboratory MCMV strain with that of G1F or as a control with that of the C2 m157 mutant (19), which contains an 8-bp deletion resulting in a premature stop and no functional m157 protein. The m157K<sup>B1</sup> sequence was reintroduced using the same technique to create a revertant control virus. All viruses showed essentially identical growth kinetics when grown on M210B4 cells (data not shown). M210B4 cells infected with the recombinant BAC-derived viruses K181 WT, K181-m157G1F, and K181-m157C2-revertant were capable of activating BWZ-HD12 and BWZ-HD12<sup>C</sup> cells in a reporter assay in a similar manner to the K181 and G1F viruses (data not shown). This indicates that the m157G1F protein was functionally expressed in the chimeric virus. As expected, the K181-m157C2-revertant recombinant virus showed no activation of these cells (data not shown).

The viruses K181 WT, K181-m157G1F, K181-m157C2-revertant, and K181-m157K<sup>B1</sup>-revertant were used to infect adult B6 mice and viral titers in target organs (spleen, liver, lung, and salivary gland) determined by standard plaque assay at days 2, 4, 6, 10, 18, and 25.

**FIGURE 4.** Activation and binding of BWZ-Ly49 reporter cells by variant m157 proteins. A, Binding median fluorescence intensity of m157<sup>Smith</sup> (open symbols) and m157G1F (filled symbols) to BWZ cells (left panel) transfected with DAP12 only (BWZ-DAP12, circles) or DAP12 and Ly49B6 (BWZ-HD12, triangles) and to CHO cells (right panel) untransfected (circles) or transfected with Ly49B6 (triangles). These graphs illustrate one representative experiment of three performed. B, BWZ.36 reporter cells expressing DAP12 (BWZ-DAP12), DAP12 and Ly49H<sup>B6</sup> (BWZ-HD12), or DAP12, Ly49H<sup>B6</sup>, and Ly49B6 (BWZ-HD12C) were incubated with parental BA/F3 cells (open bars) or with BA/F3 expressing m157<sup>Smith</sup> (black filled bars) or m157G1F (gray filled bars) in a standard reporter assay. 4LO3311 Ly49C-blocking Ab was added on reporter cells prior to BA/F3 m157G1F (gray striped bars). Bars represent data are representative of one experiment of five performed.
postinfection (p.i.). Infection with K181-m157G1F resulted in viral replication equivalent to that observed with K181 WT (Fig. 5), suggesting that in a situation where m157 binds to both Ly49H and Ly49C, the control of MCMV by Ly49H is not significantly enhanced replication in the spleen, lungs, and salivary glands consistent with reduced NK cell control due to disruption of m157 (Fig. 5).

**Effect of m157 binding to Ly49H<sup>B6</sup> and Ly49C<sup>B6</sup> on NK cell activation**

The K181-m157G1F chimeric virus was tested for its ability to functionally inhibit or activate B6 NK cell subsets in vivo. B6 mice were infected with 5 × 10<sup>3</sup> PFU of each of the recombinant viruses, and NK cells freshly isolated from the spleen and liver at day 2 and day 4 p.i. were analyzed by flow cytometry. We detected no significant differences in the percentages of Ly49H- and/or Ly49C-expressing NK cell subsets (Fig. 6a), in the expression of CD69 or KLRG1 (data not shown) in any of these populations, or in IFN-γ production (Fig. 6b) between B6 mice infected with K181 WT or K181-m157G1F virus, consistent with the unaltered m157G1F MCMV viral replication in vivo.

NK cells possess on their surface an array of inhibitory and activating receptors, and within the Ly49H<sup>+</sup> NK cells only a proportion coexpress Ly49C. Ly49C-H<sup>+</sup> NK cells would be expected to proceed unchecked by binding to m157G1F-expressing cells. To determine more closely the intrinsic ability of distinct NK cell subsets to be activated by m157G1F-expressing cells, we analyzed the Ly49H/Ly49C NK cell subpopulations. We expanded splenic NK cells from BALB.B6-CT8 congenic mice (which display Ly49C<sup>+</sup> receptors) in IL-2-supplemented medium and sorted four subsets according to Ly49H and Ly49C expression. Cytotoxicity against syngeneic BA/F3-m157<sup>+</sup> cells elicited by the four subsets and the control unsorted NK cells was tested in a standard [<sup>51</sup>Cr] release assay. Nontransfected parental BA/F3 cells were only weakly killed by all subsets (Fig. 6c). BA/F3-m157<sup>K181</sup> or BA/F3-m157G1F cells triggered a similar killing by the two Ly49H-expressing NK cell subsets and by unsorted NK cells (Fig. 6c). NK cell-mediated killing toward BA/F3-m157G1F was triggered to a similar extent in the different Ly49H<sup>+</sup> subsets regardless of Ly49C coexpression (Fig. 6c). Ly49H<sup>+</sup> NK cells did not elicit cytotoxicity above background. These data suggest that Ly49C<sup>B6</sup> is not able to prevent Ly49H<sup>B6</sup>-mediated LAK activation upon exposure to m157G1F-expressing cells.

**Discussion**

The functional relevance of m157 in MCMV pathogenesis is unclear because it has been shown to bind to the Ly49H activation receptor in B6 mice resulting in enhanced viral clearance but also to the Ly49I inhibitory receptor in 129/J mice (5) suggesting a role in inhibition of NK cell responses. We investigated the interactions of variant m157 proteins from a range of wild mouse-derived MCMV isolates with NK cells and Ly49 receptors from inbred mouse strains and show that, as well as providing a target for B6 NK cell activation through Ly49H<sup>B6</sup>, variant m157 proteins are capable of binding to inhibitory Ly49 receptors, including Ly49C in NIZB, BALB/c, and B6 mice. The majority of m157 proteins tested did not bind to Ly49H<sup>B6</sup>-expressing BWZ-HD12 reporter cells. This extends the notion raised by our previous studies, which showed that Ly49I-like resistance to MCMV mediated by the interaction with m157 on infected cells is uncommon in both inbred (43) and wild mouse populations (44). Unlike the low frequency of Ly49H<sup>B6</sup>-binding m157 molecules, a greater proportion of m157 variants bound to Ly49I and activated Ly49I<sup>B6</sup> chimeric reporter cells. In addition, a number of these m157-Fc proteins, like m157G1F, are capable of binding Ly49C inhibitory receptors (from B6, BALB/c, and NIZB mice), which show significant homology to the Ly49H activating receptor (45). This further suggested an immune inhibitory role for m157 in preventing NK cell activation. The levels of binding of m157G1F-Fc to Ly49H<sup>B6</sup> and Ly49C<sup>B6</sup>-transduced cells were similar. However, the precise binding affinities of purified m157 molecules to these receptors remain to be determined by surface plasmon resonance analysis. When reporter cells coexpressed Ly49H<sup>B6</sup> and Ly49C<sup>B6</sup>, exposure to m157G1F-expressing targets limited Ly49H-dependent activation, and this regulatory effect was correlated with Ly49C expression levels, showing that these two receptors can both engage m157G1F and that in this context Ly49C<sup>B6</sup> has an inhibitory effect toward Ly49H<sup>B6</sup>.

Notably, we found that in vitro killing of m157G1F-expressing targets by LAKs that express both Ly49H<sup>B6</sup> and Ly49C<sup>B6</sup> was comparable with the killing exerted by LAKs expressing only Ly49H<sup>B6</sup>. In addition, the elimination of m157G1F- and m157K181-expressing targets was similar. Infection of B6 mice with the K181-m157G1F chimeric virus, which encodes the m157G1F protein capable of binding both Ly49<sup>B6</sup> and Ly49H<sup>B6</sup>, gave similar in vivo titers to the K181 WT virus, in which m157<sup>K181</sup> binds Ly49H<sup>B6</sup> only. We could not detect any significant functional consequence of the m157G1F-Ly49C<sup>B6</sup> interaction on NK cell activation at early time points p.i. Similarly, in 129/J mice, where m157<sup>Smith</sup> binds to the inhibitory receptor Ly49I but activating receptors that recognize m157 are not expressed (5), injection of an m157-deleted MCMV<sup>Smith</sup> mutant, which prevents Ly49I-mediated NK cell inhibition, does not significantly improve the control of viral replication (46). In our settings, although the m157-Fc binds well to inhibitory receptors and is easily detected in vitro, the interaction of native m157 on the surface of infected cells with Ly49C<sup>B6</sup> may be of insufficient affinity to affect virus growth in vivo.
One possible explanation for the discrepancies between in vivo and in vitro data is that the inhibitory signal provided by m157G1F binding to Ly49C may not be of sufficient strength to override the strong Ly49H B6 activation signal. Regunathan et al. (47) showed in the context of NKG2D-dependent NK cell activation that upon binding to cognate MHC class I molecules, Ly49H engagement could prevent activation of NK cells by target cells with a low NKG2D ligand density, whereas targets expressing a high ligand density were eliminated. Because Ly49H, like NKG2D, signals through the adapters DAP12 and DAP10 (48–50), it is possible that Ly49C regulation of these two receptors occurs through the same mechanism. The definition of the mechanisms operating is currently under investigation in our laboratory. It is also possible that in another host context (that of another inbred strain or wild mouse populations) where inhibitory receptors bind to variant m157 proteins may indeed be functionally relevant to viral pathogenesis. Another consideration is that, because the expression of these two proteins may indeed be functionally relevant to viral pathogenesis. In a recent publication, Lanier’s group (51) showed that “unlicensed” Ly49H+, Ly49C+ NK cells dominate the response to MCMV over Ly49H+, Ly49C+ NK cells. These results reveal that the NK cell responsiveness to MCMV infection in B6 mice does not depend only on Ly49H expression and therefore can be put in perspective with ours. To test the cytotoxic activity in different NK cell subsets in Fig. 6C, we used sorted NK cells that were initially isolated from H-2d mice and tested them against BA/F3 syngeneic targets. In the H-2d background, Ly49A and Ly49G2 are involved in NK cell recognition of self MHC class I molecules, whereas Ly49C has no endogenous ligand. Therefore, unlike in Lanier’s study, we did not compare “licensed” and “unlicensed” NK cells subsets, but we rather addressed the ability of Ly49C to display inhibitory activity on NK cell functions upon binding to m157.

The experiments illustrated in Figs. 5 and 6A, 6B have been performed in B6 mice and are thus more comparable with the experimental settings used by Lanier’s group (51). Like with MHC class I molecules, Ly49C engagement with m157 could also have overridden the “licensing” effect and impaired the responsiveness of the NK cell subset coexpressing Ly49H and Ly49C. They show that the Ly49H+,C/I+ NK cell subset contracts in wild-type B6 mouse spleen 4 d p.i. with MCMV, whereas it expands in MHC class I-deficient K\(^{d}\),D\(^{b}\) knockout mice. We found a similar reduction of the Ly49H+, Ly49C+ NK cell subset in the spleen and liver in B6 mice at this stage of the infection course. However, these decreases occurred in the context of both K181 WT and K181-m157G1F viruses. Lanier and co-workers showed that Ly49C/I+ NK cell degranulation toward MHC class I-deficient RMA-S-m157 targets and production of IFN-\(\gamma\) were slightly increased, whereas exposure to MHC class I-expressing RMA-m157 cells triggered weaker NK cell functions. They concluded that MHC class I interactions with Ly49C receptor override the responsiveness advantage gained by licensing. In our experimental
settings, we could not detect statistically significant differences in IFN-γ–producing NK cell fractions after infection with K181 WT and K181-m157G1F viruses ex vivo in B6 mice. As Lanier’s group reports (51), m157 induction occurs more quickly than MHC class I downregulation in MCMV-infected cells, also early engagement of Ly49C by H-2Dk MHC class I molecules may play a role in limiting Ly49H-dependent NK cell responsiveness in both K181 WT- and K181-m157G1F–infected mice. This possible explanation is strengthened by a recent publication from Joniec’s group (52) that demonstrates the role of MHC class I molecule in the regulation of the anti-MCMV response by NK cell subsets expressing specific inhibitory Ly49 receptors.

A significant conclusion from this study relates to the concept of “resistance” to MCMV infection, which depends on the genetic context of both host and virus. Recent studies by Belanger et al. (15) have shown that NOD mice possess a Ly49H activation receptor, but sequence differences from Ly49Hb result in the failure to control MCMV infection. The evolutionary pressures driving the emergence of activating receptors from inhibitory Ly49 homologues may be due to interactions with more conserved molecules from other pathogens or to MHC class I molecules altered by virus infection, such as is the case for the recognition of H-2Dk by the Ly49P activating receptor from MA/MY mice (53–55), where the recognition of H-2Dk is dependent on the expression of the MCMV-encoded m04 molecule (56). B6 mice are generally termed “resistant” (Cmv1), but this is true only in the context of m157Smith–Ly49H interactions and a limited number of other m157 variants. Indeed, many MCMV wild-derived isolates can replicate to high titers in this mouse strain (19). Even a low level of genetic diversity between MCMV isolates can lead to phenotypic differences in their interactions with inbred mouse strains (57). Ly49H25D was found to bind, albeit weakly, to m157 from G1F and K17E isolates, but not Smith, consistent with these mice previously being characterized as “susceptible” to Smith MCMV (42). The weak binding of Ly49HNh2 to m157G1F that we observed in vitro is consistent with our preliminary results from experiments in which NZB mice infected with MCMV K181-m157G1F did not display any resistance phenotype as would have been expected in mouse strains exhibiting a Ly49H receptor that strongly binds to m157 (A.J. Corbett and A.A. Scalzo, unpublished observations). The concept of resistance is thus receptor context dependent because it relies on the presence of both activating NK cell host receptors and cognate viral proteins capable of binding these receptors with sufficient affinity. Like in mice, selective pressure exerted by the NK cell-mediated immune response led human cytomegalovirus (HCMV) to develop escape strategies against the human immune system. Carbone’s group compared various laboratory and clinical strains of HCMV-derived UL18 proteins and demonstrated that similar to MCMV m157, the HCMV UL18 sequence is variable (58). Compared with the laboratory virus sequence, mutations found in UL18 produced by clinical virus strains were clustered in the α3 domain, which is critical for binding to the LIR-1 inhibitory NK cell receptor. Laboratory strain UL18 binds to LIR-1 with a much greater affinity than the endogenous classical and nonclassical MHC class I ligands, whereas mutations in the different clinical-derived UL18 affect its binding to LIR-1 and therefore its ability to regulate NK cell activation during the course of HCMV infection.

The role of natural selection in determining the outcome of m157 mutations and deciding the fate of m157 variant MCMV strains could also be viewed from a different angle. A virus that cannot be recognized and controlled by the host immune system will replicate rapidly, resulting in organ failure and higher mortality rates. In hosts infected with these variants, there will be more limited capacity for the virus to persist in the salivary glands and to spread through the host population. Therefore, selection pressure could favor MCMV variants that are controlled to some extent by the immune system. Andrews et al. recently demonstrated that potent anti-MCMV–mediated NK cell responses negatively regulate the duration and effectiveness of adaptive immune response by limiting exposure of T cells to infected dendritic cells, leading to sustained viral titers (59). The role of m157 in immune evasion through binding to inhibitory Ly49 receptors thus remains uncertain. The finding that most variant m157 molecules do not bind to NK cells or Ly49 raises the notion that variant m157 molecules have other functions, including perhaps binding non-Ly49 receptors, which may have important consequences for viral pathogenesis.

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