Murine cytomegalovirus m157 mutation and variation leads to immune evasion of natural killer cells

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Effective natural killer (NK) cell recognition of murine cytomegalovirus (MCMV)-infected cells depends on binding of the Ly49H NK cell activation receptor to the m157 viral glycoprotein. Here we addressed the immunological consequences of variation in m157 sequence and function. We found that most strains of MCMV possess forms of m157 that evade Ly49H-dependent NK cell activation. Importantly, repeated passage of MCMV through resistant Ly49H$^+$ mice resulted in the rapid emergence of m157 mutants that elude Ly49H-dependent NK cell responses. These data provide the first molecular evidence that NK cells can exert sufficient immunological pressure on a DNA virus, such that it undergoes rapid and specific mutation in an NK cell ligand enabling it to evade efficient NK cell surveillance.

Host–pathogen interactions are dynamic processes in which the host pits its innate and adaptive immune responses against the pathogen to limit disease severity. However, several pathogens have evolved a range of strategies to limit the efficacy of the host response, to persist and be transmitted to other hosts. Natural killer (NK) cells are an important frontline defense against a number of pathogens (1), in particular viruses belonging to the herpesvirus family (2). NK cell functions are tightly regulated by the activities of both inhibitory and activating cell surface receptors (3). Inhibitory NK cell receptors, specific for MHC class I, enable discrimination of normal healthy cells from cells that are potentially pathological, such as tumor or virus-infected cells, because the latter often express reduced levels of MHC class I. NK cell inhibitory molecules include the Ly49 receptors in rodents, the killer cell Ig-like receptors (KIR) molecules in humans, and heterodimers of CD94 and NKG2A, which are found in both species (3–5). The inhibitory signals delivered by these receptors after ligand engagement are mediated by cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (6). The activation of NK cells required to recognise tumor and virus-infected cells is mediated by engagement of activating receptors, which include activating forms of KIRs in humans (e.g., KIR2DS), Ly49H and Ly49D in mice, and NKGD, which is found in both species (3, 7–9). Signaling by activating receptors is mediated via adaptor molecules, such as DAP10 and DAP12 (5, 7).

Analysis of the NK cell response to the murine herpesvirus murine cytomegalovirus (MCMV) has provided important insights as to how NK cell receptors can specifically recognize virus-infected cells (10–13). Infection of inbred mouse strains originally identified strains with differing resistance to MCMV infection because of variation in *Cmv1*, a host resistance locus that regulates the efficacy of NK cell responsiveness (14, 15). Strains such as C57BL/6 (B6) are resistant (*Cmv1*^{r}), whereas strains such as BALB/c and DBA/2 are susceptible (*Cmv1*^{s}) to MCMV infection. Detailed genetic analyses revealed that the *Cmv1* locus maps to the distal region of mouse chromosome 6 in the NK cell gene complex (NKC) (16–18). The identity of *Cmv1* has been resolved, and the activating NK cell receptor Ly49H has been shown to account for the *Cmv1* resistance effect (19–21). Data supporting this conclusion include selective absence of Ly49H expression and *Ly49h* deletion in an MCMV-susceptible recombinant inbred mouse (BXD-8) with the C57BL/6 (resistant) haplotype (19, 21), blockade of Ly49H function with an anti-Ly49H monoclonal antibody abrogates resistance in wild-type C57BL/6 mice (19, 20), and gene transfer of *Ly49h* (on a bacterial artificial chromosome) confers resistance to otherwise susceptible mice (22).

The ligand recognized by Ly49H has also been defined (23, 24). Reporter cell lines expressing the Ly49H molecule, and its associated signaling partner DAP12, together with LacZ or GFP cassettes carrying a nuclear factor of activated T cell (NFAT) response element, which is triggered upon DAP12 activation, provided a sensitive method for identifying the ligand that could bind Ly49H (23, 24). When MCMV mutants with deletions in genes encoding potential candidate ligands (23) or cells transfected to encode MCMV class I-like proteins (24) were used, the m157 viral glycoprotein was identified as the Ly49H counterstructure. As well as binding Ly49H, m157 could engage the Ly49I inhibitory receptor in the 129/J mouse strain, suggesting that m157 may have multiple roles in the response to MCMV infection (23). However, the significance of binding an inhibitory receptor is not yet known because 129/J mice do not express the Ly49H activation receptor, and only 10% of 129/J NK cells express Ly49I. Furthermore, Ly49H$^+$ C57BL/6 mice do not express a Ly49I allele that binds m157.

The finding that an NK cell activation receptor could positively engage a viral ligand expressed on infected cells to elicit a strong NK cell response raised key questions about the dynamics of this interaction and why the m157 gene may be retained within the MCMV genome. First, is the NK cell response activated by Ly49H interaction with m157 a sufficiently strong selective pressure to drive the emergence of NK cell "escape mutants" through mutation of the m157 gene? Second, is the m157 sequence expressed by prototypic laboratory strains of MCMV representative of m157 sequences of MCMV strains present in wild mice, and hence is Ly49H engagement of m157 a universal feature of the interaction of NK cells with MCMV? In this study, we have shown that m157 sequences that do not trigger NK cells via Ly49H predominate among wild strains of MCMV and, importantly, that immune pressure from Ly49H$^+$ NK cells can

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Abbreviations: NK, natural killer; NKC, NK cell gene complex; β-gal, β-galactosidase; MCMV, murine cytomegalovirus; MEF, mouse embryo fibroblast; p.i., postinfection; SGV, salivary gland virus; pfu, plaque-forming unit.

Data deposition: The m157 sequences of the MCMV strains reported in this paper have been deposited in the GenBank database (accession nos. AY228652–AY228681).

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rapidly select for mutations in m157 that either result in a lack of expression of m157, or in the emergence of m157 mutants that do not activate NK cells via Ly49H engagement.

Methods

Animals. Female inbred C57BL/6 (Ly49H$^+$/*Cmv1^r*) and BALB/c (Ly49H$^-$/*Cmv1^s*) mice at 8 weeks of age were purchased from the Animal Resources Centre (Perth, WA, Australia). NKC congenic strains BALB.B6-*Cmv1^r* (NK1.1$^+$, Ly49H$^+$/*Cmv1^r*), BALB.B6-CT3, and BALB.B6-CT12 (both Ly49H$^+$/*Cmv1^r*) (25, 26) and BALB.B6-CT6 (Ly49H$^-$/*Cmv1^s*) (26) were bred in the Animal Services Facility at the University of Western Australia. All animal experiments were performed in pathogen-free facilities, 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. Primary mouse embryo fibroblasts (MEFs) were grown in MEM (Invitrogen, Sydney) supplemented with 10% FCS (Invitrogen). The NIH-3T12 and X63 cell lines were grown in RPM1 medium 1640 containing 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 40 μg/ml gentamicin, and 50 μM 2-mercaptoethanol (R10 medium). The BWZ-HD12 and BWZ-DD12 reporter cells (24) were maintained in R10 medium containing 200 μg/ml hygromycin (Sigma). BaF3 cells were passaged in R10 medium supplemented with 10% X63-conditioned medium (R10-X63). BaF3-m157 cells were passaged in R10-X63 containing 1 μM puromycin (Sigma). Chinese hamster ovary (CHO)-K hamster cells were grown in alpha-MEM supplemented with ribonucleosides and deoxyribonucleosides (Invitrogen), containing 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 40 μg/ml gentamicin, and 50 μM 2-mercaptoethanol.

Viruses. MCMV strains used in this study were the K181-Perth (abbreviated K181) laboratory strain and the G1B, GIF, G2, G3B, G3E, G3F, G4, G5, G6, K4, K6, K7, K10, K17A, K17B, K17E, K29, N1, N5, W2, W3, W5, W6, W7, W8, W9, W8211, W9077, and WE6 strains, all of which were isolated from wild mice trapped in Australia or Kerguelen Island in the Indian Ocean (27). Virus stocks were propagated by *in vitro* infection of MEFs and quantified by standard plaque assay on MEFs or NIH-3T12 cells (14). Virulent salivary gland viral (SGV) stocks were prepared by infecting 3-week-old female BALB/c mice with 1×10^4 plaque-forming units (pfu) of tissue culture passaged virus diluted in PBS-0.5% FCS by the i.p. route, and then preparing homogenates of salivary glands at 17 days postinfection (p.i.). Secondary SGV stocks were prepared by infecting 3-week-old female BALB/c mice with 1×10^3 pfu of the primary SGV stock.

Serial Passage of MCMV Through Ly49H$^+$/*Cmv1^r* Mice and Purification of m157 Escape Mutants. To select for m157 escape mutants, K181-Perth MCMV was sequentially passaged through 3-week-old female Ly49H$^+$/*Cmv1^r* BALB.B6-CT3 mice by infecting groups of three to four mice with 1×10^3 pfu of SGV virus administered i.p. at each sequential passage. For each passage, salivary glands were homogenized at day 17 p.i. At passage 6, spleens were removed from 2 Ly49H$^+$/*Cmv1^r* BALB.B6-CT3 at 4 days p.i. and homogenates were prepared from each of these spleens. These homogenates were serially diluted 4-fold across 96-well trays and added to MEF cultures in 96-well trays. Wells showing viral cytopathic effect derived from a single plaque were harvested for preparation of virus stocks, and this process was repeated to generate purified clonal viral stocks.

Analysis of *in Vivo* Replication of MCMV. Mice were injected i.p. with 1×10^4 pfu of MCMV and were killed at 4 days p.i., and their organs were removed for standard plaque assay as described (14).

Sequence Analysis of m157 and Other MCMV Genes. The m157 ORF was PCR amplified by using purified viral DNA and the oligonucleotide primers m157-for1 (GAGGTGGCGTGTGAAACGCAG) and m157-rev1 (GTCAGTGAGATCGTGACC) based on the published sequence of the Smith MCMV strain (28). Products from duplicate PCRs were pooled and sequenced by using the BigDye Teminator V3 sequencing kit (Applied Biosystems, Foster City, CA) with appropriate external and internal primers. Sequences were assembled with SEQED software (Applied Biosystems) and aligned with ECLUSTALW. The m144 and m152 ORFs were similarly sequenced and analyzed by using appropriate primers designed from the published MCMV sequence.

Generation of BaF3-m157/Flag Transfectants. To express the m157 protein in BaF3 cells, m157 was cloned into the pEF-PGKpuroPA vector (29), which had been reengineered to contain an human CMV (HCMV)-leader peptide/Flag-tag cassette upstream of the m157 insertion site to generate the pEF-5CMVLFlag plasmid. BaF3 cells were transfected with 5 μg of plasmid DNA and FuGene 6 transfection reagent (Roche Diagnostics) and transfectants selected by growth with 1 μM puromycin (for further details, see supporting information, which is published on the PNAS web site).

Reporter Assay for Measuring β-Galactosidase (β-gal) Activity. β-gal activity of BWZ-HD12 or BWZ-DD12 cells induced after incubation with BaF3 cells transfected with m157 or with MCMV-infected MEFs was performed as described (24) with minor modifications (see supporting information).

Statistical Analysis. For statistical analysis, the two-tailed Mann–Whitney test was performed by using the INSTAT statistical software package (GraphPad, San Diego). All data are shown as mean $\pm$ SEM.

Results

Passage of MCMV Through *Cmv1^r*/Ly49H$^+$ Mice Generates m157 Mutants. Can exposure of MCMV to a strong NK cell response elicited via m157/Ly49H interaction provide sufficient selective pressure to lead to the emergence of m157 sequence variants that cannot activate NK cells via Ly49H? To address this key question, the K181 strain of MCMV, which is routinely maintained by repeated sequential passage through salivary glands of *Cmv1^s* BALB/c mice, was sequentially passaged six times through salivary glands of resistant *Cmv1^r*/Ly49H$^+$ BALB.B6-CT3 NKC congenic mice (26). At the sixth passage, BALB.B6-CT3 mice were injected with 1×10^4 pfu of fifth passage salivary gland homogenate, and spleen homogenates prepared at 4 days p.i. To assess whether m157 mutants had emerged after passage through the Ly49H$^+$ mice, five clonal virus stocks were purified by sequential limit dilution and then designated C1, C2, C4, C5, and C6. PCR amplification of m157 was performed from DNA isolated from each clonal stock (see supporting information). Two clones, C1 and C5, have m157 amplicons that are $\approx$100 bp smaller than that of the K181 laboratory strain. The PCR products were then sequenced. All five isolates exhibit mutations in the m157 gene (Table 1). Clones C1 and C5 share the same 104-bp deletion, suggesting that they probably arose from the same mutation event at an early viral passage. Both of these clones have frame-shifts in the m157 coding sequence leading to a premature stop codon. Clones C2, C4, and C6 all have unique mutations, with clones C2 and C4 having mutations that gener-

Table 1. Mutations present in clonally purified m157 immune escape variants of MCMV

Clone designation	Type of mutation	Genomic position*	Effect on m157
C1	104-bp deletion	216,397–216,500	Frame shift and premature stop
C2	8-bp deletion	216,762–216,769	Frame shift and premature stop
C4	8-bp insertion	216,495	Frame shift and premature stop
C5	104-bp deletion	216,397–216,500	Frame shift and premature stop
C6	9-bp deletion	216,033–216,041	Deletion of residues 282–TKR–284

*Nucleotide positions are given relative to those in the prototypic Smith MCMV strain sequence (28).

ate premature stop codons. Interestingly, clone C6 has a 9-bp deletion resulting in a 3-aa deletion in the m157 protein sequence (residues 282-TKR-284). To exclude the possibility of mutations having occurred elsewhere in the MCMV genome as a result of passage through *Cmv1*^{r} mice, DNA purified from each of the five clones was subjected to restriction fragment length polymorphism (RFLP) analysis. No differences in RFLP patterns were observed between the five mutant clones and the parental K181 virus (data not shown). Furthermore, sequence analysis of the MCMV m144 and m152 ORFs, both of which function as NK cell immune subterfuge molecules (30, 31), revealed no sequence variation after serial passage (data not shown). These data indicate that the mutations in m157 arose through strong NK cell selective pressure imposed by activities mediated via the Ly49H receptor.

m157 Escape Mutants Do Not Activate Ly49H. The m157 mutant viruses generated through exposure to NK cell pressure either lack m157 expression or express altered forms of the molecule. It is predicted that cells infected with these mutant viruses will not activate cells expressing Ly49H. To test this hypothesis, BWZ-HD12 reporter cells expressing the Ly49H protein (24) were used to assess the activating potential of MEFs infected with either wild-type K181, or K181 m157 "escape mutants." Unlike K181-infected MEFs, which specifically triggered activation of the Ly49H$^+$ BWZ-HD12 reporter cells, but not of the control Ly49D$^+$ BWZ-DD12 cells, none of the five m157 "escape mutants" triggered activation of the BWZ-HD12 cells, indicating that the m157 gene has been functionally disrupted (Fig. 1*a*).

The *in vivo* consequences of the m157 mutations on the ability of MCMV to replicate in Ly49H$^+$/*Cmv1*^{r} mice were assessed. All five m157 mutants replicated to high titers in the spleens of Ly49H$^+$ BALB.B6-*Cmv1*^{r} mice, with titers comparable to those seen for the replication of wild-type K181 MCMV in susceptible BALB/c mice ($P > 0.05$) (Fig. 1*b*). These data confirm that the *Cmv1*^{r} phenotype of resistance to MCMV, observed in Ly49H$^+$ mouse strains, is critically dependent on the presence of a functional m157 protein on the surface of MCMV-infected cells.

Rapid Emergence of m157 Mutants Resulting from NK Cell Pressure. Viruses isolated after six passages through Ly49H$^+$ mice were 100% m157 mutants (five clones), suggesting that at the late stages of sequential viral passage m157 mutants have totally replaced the wild-type virus. To investigate the kinetics of emergence of m157 mutants, viral stocks were prepared from each of the six sequential passages and used to infect resistant B6 mice. Infection of B6 (Ly49H$^+$/*Cmv1*^{r}) and BALB.B6-CT6 (Ly49H$^-$/*Cmv1*^{s}) mice with wild-type K181 gave low and high titers, respectively, in the spleen at day 4 p.i. (Fig. 2*a*). Infection of B6 mice by the serial passage viral stocks resulted in progressively higher viral titers, with the passage 6 stock replicating in resistant mice to levels equivalent to those observed in susceptible mice infected with wild-type virus (Fig. 2*a*). These data suggest that after repeated passage in resistant mice, the virus population consists of a greater proportion of m157 mutants that are capable of effective replication in Ly49H$^+$ mice.

The temporal emergence of m157 mutants was further explored by PCR analysis of DNA purified from viral stocks prepared at each serial passage. The timing of appearance of the 104-bp mutant of m157 was monitored as an indicator of when mutation in m157 was occurring. The 104-bp m157 truncation mutant was clearly present by the third passage through Ly49H$^+$ resistant mice (Fig. 2*b*). Collectively, the viral replication and PCR data indicate that MCMV can rapidly mutate when subjected to immunological pressure from Ly49H-activated NK cells, and thus evade NK cell mediated immune responses.

Natural Variants of m157. The data presented above indicate that MCMV can rapidly evolve in response to selective pressure from the host and can consequently modify the outcome of the host–pathogen interaction. Because the Smith and K181 MCMV

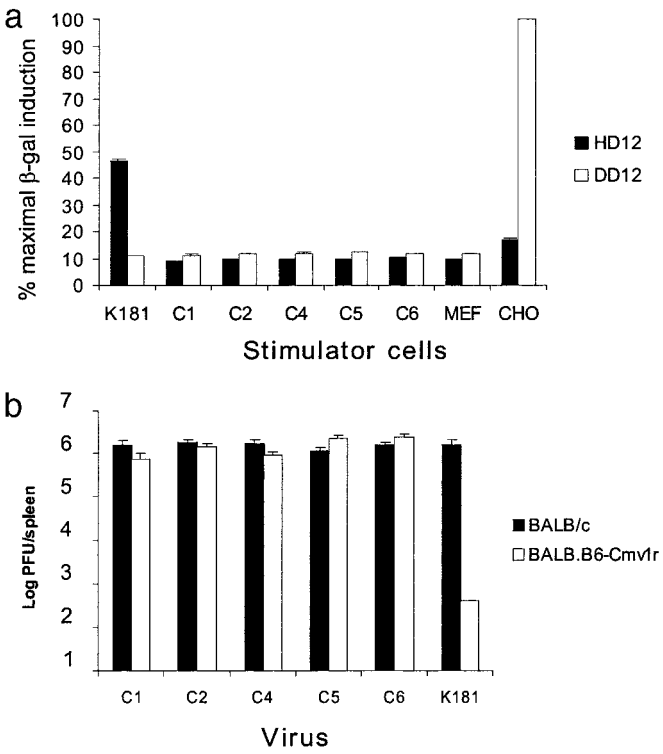

Fig. 1. Mutations in m157 result in escape from Ly49H-mediated surveillance. (*a*) Clonal viral stocks derived from passage through *Cmv1*^{r}/Ly49H$^+$ mice were tested for their ability to interact with Ly49H by infecting MEF cultures with each clonal stock (C1, C2, C4, C5, or C6) or parental K181 virus, and then adding BWZ-HD12 or BWZ-DD12 reporter cells. β-gal activity was then quantified by the addition of chlorophenol red β-D-galactoside (CPRG) substrate. The percent maximal β-gal induction was quantified relative to BWZ-HD12 or BWZ-DD12 reporter cells activated with PMA/ionomycin. (*b*) BALB/c (*Cmv1*^{s}/Ly49H$^-$) or BALB.B6-*Cmv1*^{r} (*Cmv1*^{r}/Ly49H$^+$) congenic mice were infected with 10^4 pfu of salivary gland stocks of the C1, C2, C4, C5, or C6 clonal viruses or parental K181. At 4 days p.i., spleens were harvested and homogenized, and viral titers were determined by standard plaque assay.

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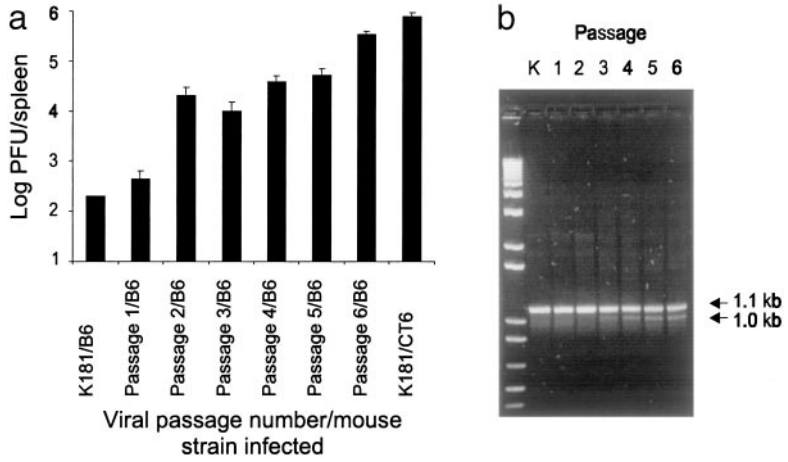

Fig. 2. MCMV m157 escape mutants rapidly emerge after passage in Ly49H$^+$ mice. (*a*) Salivary gland homogenate viral stocks prepared at each of the six sequential passages were used as inocula to infect *Cmv1*^{r}/Ly49H$^+$ B6 mice. Groups of four mice were infected i.p. with 10^4 pfu of each sequential SGV homogenate. B6 and BALB.B6-CT6 mice were also infected with 10^4 pfu of wild-type K181 virus. At 4 days p.i., spleens were collected and homogenized and MCMV titers were quantified by plaque assay. (*b*) The emergence of the 104-bp mutation in m157 was assessed by performing m157 PCR amplifications with DNA purified from MEFs infected with the sequential passage SGV stocks. The PCR products were resolved on a 2% agarose gel.

laboratory strains share identical amino acid sequences (23), it was possible that sequence variation in m157 might be rare, and that NK cell recognition of MCMV-infected cells by the Ly49H activation receptor may be a characteristic feature of this virus. However, in view of the data presented above, it may be that most MCMV strains seek to elude NK cell surveillance by expressing variant sequences of m157 that cannot be detected by Ly49H.

To test whether MCMV m157 is highly conserved or variable, the m157 gene from 29 MCMV strains isolated from wild mice was sequenced. Alignment of m157 amino acid sequences from these viral strains indicates a high degree of variation within the m157 protein sequence (see supporting information). Two of the 29 MCMV strains (K4 and K17A) had sequences identical to the prototype sequence of Smith and K181 laboratory strains. All other strains exhibited variation from the prototypic MCMV sequence. Six strains (G1F, N5, N1, K17E, W5, and W8211) belong to a cluster that exhibited the lowest degree of variation (80.4–87.9% identity). In contrast, m157 sequences from the remaining 21 strains, including K17B, G4, G5, G3E, G6, and K6, belong to a group of viruses with related sequences, which were considerably different from that of Smith m157 (63.9–68.2% identity). These data indicate that, in wild mice, most MCMV strains possess m157 variants dissimilar to Smith and K181 m157.

Viruses with Variant m157 Sequences Replicate to High Levels in Spleens of Ly49H$^+$ Mice. To explore whether m157 sequence variation affects the activation of Ly49H$^+$ NK cells *in vivo*, the pathogenesis of a subset of MCMV strains possessing m157 variant sequences (N1, G4, G5, and K6), together with K4, a strain that shares the same prototype m157 sequence as K181 and Smith, were analyzed in resistant BALB.B6-CT12 (*Cmv1*^{r}/Ly49H$^+$) and susceptible BALB.B6-CT6 (*Cmv1*^{s}/Ly49H$^-$) congenic mice. All MCMV strains replicated to high levels in the spleens of the susceptible Ly49H$^-$ strain BALB.B6-CT6 (Fig. 3). In the resistant BALB.B6-CT12 strain, which showed low levels of replication for K181 and K4 MCMV strains, the m157 variant strains (N1, G4, G5, and G6) all exhibited high viral titers in the spleen, comparable to those observed in the susceptible BALB.B6-CT6 strain.

m157 Sequence Variants Are Not Recognized by Ly49H. The failure of Ly49H$^+$ mice to effectively control the replication of MCMV strains with variant m157 sequences suggests that Ly49H may not recognize these m157 variant glycoproteins. To test this hypothesis, MEFs infected with either K181 or MCMV m157 variants were assessed for their ability to activate BWZ-HD12 reporter cells. BWZ-HD12 cells co-cultured with K181-, K17A-, or G1F-infected cells, but not G4-, G5-, K6-, or N1-infected cells, resulted in significant stimulation ($P < 0.05$) of the reporter cells (Fig. 4*a*). An additional seven viral strains (W2, W3, W8211, G3B, G2, K17B, and K17E) did not activate BWZ-HD12 cells (data not shown). The finding that G1F-infected cells could trigger activation of the Ly49H reporter cells is of interest because the m157 sequence of this viral strain shows a high degree of homology with the m157 sequence from the prototype K181 and Smith strains.

To extend the data obtained from virus-infected cells, BaF3 cells were transfected with plasmid constructs expressing m157 variants N-terminally tagged with a "Flag" epitope. Flag-tagged constructs were prepared for m157 sequences from the isolates K6, K17A, K17E, N1, G1F, and G5. Reporter assays were then performed. Only BaF3 cells transfected with K17A or G1F m157

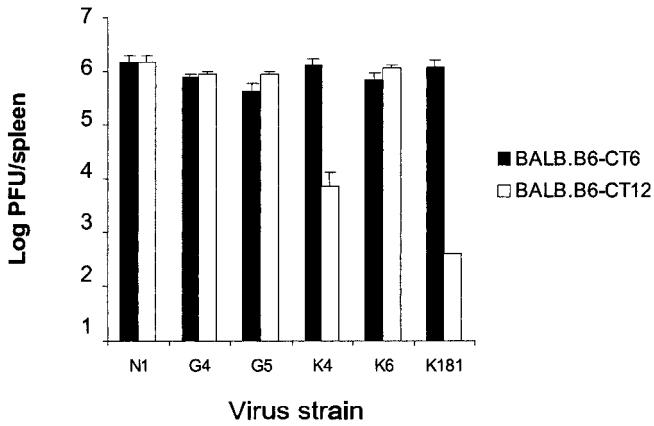

Fig. 3. MCMV m157 variants replicate to high titers in *Cmv1*^{r}/Ly49H$^+$ mice. MCMV-susceptible BALB.B6-CT6 (*Cmv1*^{s}/Ly49H$^-$) and MCMV-resistant BALB.B6-CT12 (*Cmv1*^{r}/Ly49H$^+$) NKC congenic mice were infected by the i.p. route with 1×10^4 pfu of the MCMV strains listed, and spleens were harvested at 4 days p.i. for quantification of virus replication by standard plaque assay.

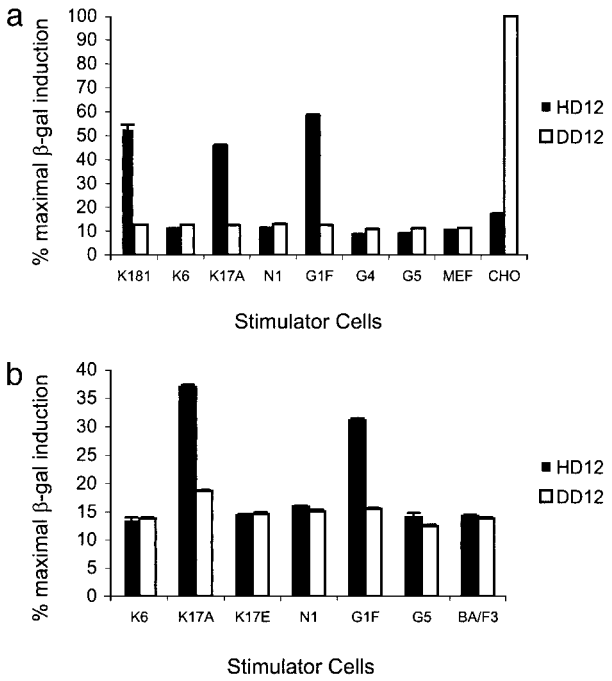

Fig. 4. Ability of MCMV m157 variants to activate via interaction with Ly49H. (*a*) Confluent MEFs in 96-well trays were infected with different strains of MCMV at a moi of 5 pfu per cell and then incubated for 24 h before BWZ-HD12 or BWZ-DD12 reporter cells were added. Controls included wells containing uninfected MEFs or Chinese hamster ovary (CHO)-K cells. After a further 24-h incubation, β-gal production was quantified by addition of chlorophenol red β-D-galactoside (CPRG) substrate. (*b*) BaF3 cells transfected with N-terminally Flag-tagged m157 constructs for K6, K17A, K17E, N1, G1F, or G5, or untransfected BaF3 cells were added to BWZ-HD12 or BWZ-DD12 reporter cells and incubated for 24 h before assaying for β-gal induction. The percentage of maximal β-gal induction was quantified relative to BWZ-HD12 or BWZ-DD12 reporter cells activated with PMA/ionomycin.

constructs significantly activated reporter cells ($P < 0.05$), relative to untransfected BaF3 cells (Fig. 4*b*). Expression of m157 molecules on the surface of BaF3 cells was confirmed for each of the constructs by flow cytometry (data not shown). BaF3 cells transfected with a flag-tagged N5 strain m157 molecule also stimulated activation of the BWZ-HD12, but not the BWZ-DD12 reporter cells (data not shown). N5 m157, like G1F m157, possesses a high degree of identity to m157 of the prototype laboratory strains (see supporting information).

Discussion

Efficient activation of NK cells is critical to limiting virus replication and severity of disease caused by a number of viruses. Interaction of the Ly49H NK cell activation receptor with the MCMV m157 glycoprotein elicits a potent NK cell response (23, 24). The m157 glycoprotein is a member of the m145 family of MCMV glycoproteins, many of which have immune evasion properties (m144 and m152) (30, 31). Thus, the observation that m157 could trigger a potent NK cell response via Ly49H was enigmatic. Here, we investigated the functional dynamics of m157/Ly49H interactions. We found that most strains of MCMV encode m157 variants unable to activate NK cells via Ly49H. Importantly, when MCMV strains possessing Ly49H-activating forms of m157 are exposed to immunological selective pressure, by serial passage through Ly49H$^+$ mice, they can rapidly evolve to generate m157 escape mutants that do not activate Ly49H.

The analysis of m157 sequences in MCMV strains derived from wild mice indicated that only a few strains (K4 and K17A) carry m157 sequences identical to that of laboratory MCMV strains. The other strains analyzed belong to 2 sequence groups that share common sequence elements, but differ in their overall level of sequence identity (see supporting information). Only two of these strains, G1F and N5 (86.7% and 84.6% identity to Smith m157, respectively), were capable of activating Ly49H$^+$ reporter cells. These data suggest that in wild populations of mice most circulating MCMV strains (≈86%) encode m157 proteins that are unable to activate NK cells via Ly49H. The fact that m157 sequences from MCMV strains derived from wild mice fall into two groups suggests that the m157 protein may have evolved different functions. Smith m157 can bind Ly49I from the 129/J mouse strain, as well as Ly49H from B6 mice (23). It is thus possible that m157 sequences from wild-derived MCMV strains bind to Ly49I and/or other Ly49 proteins. The high degree of variability in m157 contrasts with the high level of conservation observed in MCMV structural proteins, such as gM (M100) (32), but is similar to variation observed in gB (M55), which, because of significant immune pressure from neutralizing antibodies, is highly variable (33). Given the high degree of sequence variability in m157, it will be of interest to know whether other MCMV immunomodulatory proteins also exhibit such variability, which may indicate that MCMV continually evolves to acquire additional functions.

Although the majority of MCMV strains from wild mice cannot activate NK cells via Ly49H, it is possible that these strains induce NK cell responses, albeit less efficiently, by interactions with other activating receptors, such as NKG2D, which recognizes the class I-like ligands H60 and Rae1 (34), or the recently described murine ULBP-like transcript 1 (MULT1) protein (35). Because MCMV can interfere with NK cell responses via m152-mediated down-regulation of NKGD ligands (31, 36), it will be important to determine whether all MCMV strains can mediate this effect, or whether some viral strains trigger more effective NK cell responses in *Cmv1*^{s}/Ly49H$^-$ mouse strains.

The rapid emergence of m157 escape mutants after two to three passages through *Cmv1*^{r}/Ly49H$^+$ mice indicates that exposure of MCMV to NK cell surveillance imposes an extremely strong pressure for selection of viral isolates with mutations in the m157 gene. By contrast, over many years of MCMV passage in *Cmv1*^{s} mice, we have not observed mutations in m157 as shown in resistant *Cmv1*^{r} mice. Because the replication cycle of MCMV is ≈24 h, the virus could undergo ≈17 rounds of replication and Ly49H$^+$ NK cell selective pressure up to the time of harvest of the infected salivary glands. Long-term exposure of human CMV to antiviral drugs results in the emergence of resistant isolates in patients over periods of several months (37). The viral gene products targeted by anti–viral drugs are essential for virus replication, and the majority of mutations result in loss of amino acids in these genes. In contrast, the observed m157 out-of-frame mutations, as well as in-frame deletions, did not compromise virus survival *in vitro* or *in vivo*. Notwithstanding this, in Ly49H$^+$ mouse strains m157 mutations confer a growth advantage, as demonstrated by the higher viral titers recovered after infection with m157 escape mutants relative to wild-type K181. The m157 protein of the C6 escape mutant, which does not activate Ly49H, has a 3-aa deletion (282-TKR-284) in the C terminus of the m157 protein. This observation suggests that these residues must be critical for interaction with Ly49H and for delivery of activation signals to NK cells. Although the protein sequence of B6 Ly49H and 129/J Ly49I are very similar (38), it is uncertain whether the 282-TKR-284 deletion in the C6 virus would also result in reduced binding of m157 to 129/J Ly49I.

The emergence of "NK cell-escape" variants has been reported for Pichinde virus (PV), a member of the arenavirus family (39). Two mutants were generated by serial *in vivo* passage through C3H/HeSnJ mice that were pretreated with poly(IC),

an IFN inducer leading to increased NK cell activation. Injection of BALB/c severe combined immunodeficient (SCID) mice resulted in a persistent viral infection, and led to the emergence of a NK cell resistant virus isolate. All three mutant viruses exhibited enhanced viral replication and reduced susceptibility to NK cell control relative to the parental NK cell sensitive virus. However, unlike the present study, the molecular basis responsible for the enhanced NK cell resistance exhibited by the mutant PV viruses was not defined. It is conceivable that, as for MCMV m157, a PV protein, recognized by an activation receptor, mutated resulting in reduced host NK cell recognition.

It has been speculated that selective pressure from viruses, such as MCMV, resulted in evolution of the Ly49H activating receptor from the highly sequence related inhibitory Ly49I receptor (10, 23). Although the present study does not provide any direct evidence for or against this hypothesis, it does shed important insights into the dynamics of interactions between MCMV and host NK cells. Most MCMV strains that occur naturally in wild mice have m157 proteins that do not activate NK cells via Ly49H. It is possible that m157 in these viruses has evolved so that it does not activate NK cells. However, because Ly49H is uncommon in laboratory (9) and wild mice (A.A.S. C.A.F., and M. G. Brown, unpublished data), the selective pressure required for the emergence of m157 variants unable to bind Ly49H may not exist in nature, and m157 variants might have evolved to mediate other functions, such as binding to inhibitory NK cell receptors.

Our results show that m157, the specific viral ligand for the Ly49H activation receptor, exists in most MCMV strains in forms incapable of activating NK cells via Ly49H. Importantly, when MCMV strains with Ly49H-activating forms of m157 are placed under selective pressure from Ly49H$^+$ NK cells, m157 can very rapidly mutate to evade NK cell recognition. This study thus provides molecular evidence that CMV can very rapidly respond to innate immune pressures mediated by NK cells that threatens its survival. Future studies will determine whether m157 variants that are not recognized by Ly49H can bind inhibitory NK cell receptors to mediate attenuation of host NK cell responses.

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