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Materials and Methods

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Direct Recognition of Cytomegalovirus by Activating and Inhibitory NK Cell Receptors

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Natural killer (NK) cells express inhibitory receptors for major histocompatibility complex (MHC) class I antigens, preventing attack against healthy cells. Mouse cytomegalovirus (MCMV) encodes an MHC-like protein (m157) that binds to an inhibitory NK cell receptor in certain MCMV-susceptible mice. In MCMV-resistant mice, this viral protein engages a related activating receptor (Ly49H) and confers host protection. These activating and inhibitory receptors are highly homologous, suggesting the possibility that one evolved from the other in response to selective pressure imposed by the pathogen.

Natural killer (NK) cells mediate innate immunity against viruses, bacteria, parasites, and tumors by using an array of cell surface receptors that regulate their response (*1*). In rodents, the *Ly49* family of genes encodes both activating and inhibitory NK cell receptors (*2*). The inhibitory Ly49 receptors recognize major histocompatibility complex (MHC) class I and suppress NK cell attack against healthy cells but permit a response against cells that have lost class I expression (*3*). The function of the activating Ly49 receptors has remained elusive. However, recent reports have implicated the activating Ly49H receptor that is expressed on NK cells from mouse cytomegalovirus (MCMV)–resistant mice [strain C57BL/6 (B6)] in immune protection against MCMV infection (*4–6*).

We considered several possibilities to explain Ly49H-mediated protection against MCMV. Because other Ly49 receptors recognize mouse H-2 class I proteins, Ly49H might recognize self H-2 presenting a viral peptide. Alternatively, Ly49H may recognize a host MHC class I protein that is induced after viral infection of cells, in a manner similar to the NKG2D receptor, which recognizes the stress-induced class I–like molecule MIC in human CMV (HCMV)–infected cells (*7*). Finally, Ly49H could directly bind to an MCMV-encoded protein.

In order to test these possibilities, we transfected a mouse T cell hybridoma carrying a NFAT–green fluorescent protein (GFP) reporter construct with Ly49H and the DAP12 signaling adapter protein (*8*). Cross-linking with monoclonal antibody (mAb) to Ly49H induced GFP expression, confirming that the receptor was functional (*9*). When these Ly49H reporter cells were cocultured for 18 hours with mouse NIH-3T3 cells infected with MCMV Smith strain or with the K181 strain (*9*), they turned green, indicating the presence of a Ly49H ligand (Fig. 1A). Ly49H reporter cells cultured with uninfected NIH-3T3 cells or parental T hybridoma cells

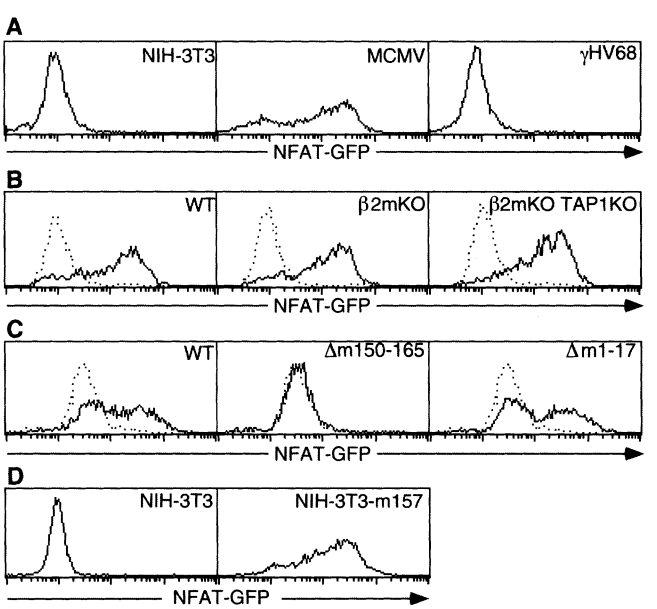

Fig. 1. Activation of Ly49H reporter cells by MCMV-infected cells. (**A**) Ly49H reporter cells were cocultured with MCMV or γ-herpesvirus 68–infected NIH-3T3 cells for 18 hours, and GFP expression was analyzed by flow cytometry with a FACSCaliber flow cytometer (Becton Dickinson, San Jose, CA). (**B**) β2 microglobulin–deficient, β2 microglobulin– and TAP-deficient, or wild-type mouse SV40-transformed embryonic fibroblasts were infected with MCMV and cocultured with Ly49H reporter cells for 2 days. GFP expression was analyzed by flow cytometry. Histograms of Ly49H reporter cells cocultured with uninfected cells (dotted lines) are superimposed over histograms of MCMV-infected cells (solid lines). KO, knockout. (**C**) NIH-3T3 cells were infected with wild-type MCMV or ΔMS 94.5 (m150-m165 deletion mutant) or with ΔMS 94.7 (m1-m17 deletion mutant) MCMV. Thereafter, Ly49H reporter cells were cocultured with the infected (solid lines) or uninfected (dotted lines) cells, and GFP expression was analyzed. (**D**) Ly49H reporter cells were cocultured with parental NIH-3T3 cells or NIH-3T3 cells transduced with m157, and GFP expression was analyzed.

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not expressing Ly49H and DAP12 failed to respond (*9*). Ly49H recognition was MCMV-specific, as shown by the fact that reporter cells did not respond to NIH-3T3 cells infected with the mouse γ-herpes virus 68 (Fig. 1A).

Expression of conventional MHC class I on the cell surface requires association with β2-microglobulin and peptides, and most Ly49 receptors bind only to the fully assembled class I antigen. In contrast, as shown in Fig. 1B, Ly49H reporter cells were able to recognize MCMV-infected fibroblasts established from mice deficient in β2-microglobulin, fibroblasts that lacked β2-microglobulin and TAP-1 (the peptide transporter required for mature class I assembly), and fibroblasts lacking *H-2K* and *H-2D* genes (*9*). These findings are in accordance with studies indicating that NK cells in wild-type and β2-microglobulin–deficient mice respond equivalently to MCMV infection (*10*). Thus, it appeared unlikely that Ly49H recognized a viral peptide presented by MHC class I or a conventional mouse MHC class I antigen.

MCMV encodes a viral glycoprotein, m144, with homology to MHC class I (*11*); however, NIH-3T3 cells transfected with m144 did not activate the Ly49H reporter cells (*9*). Next, in order to identify the viral gene responsible for activation of the Ly49H reporter cells, we analyzed a panel of MCMV deletion mutants. The ΔMS94.5 deletion virus lacks the genes between m150 and m165 (*12*), the RV7 deletion virus lacks genes m137 through m141 (*13*), and the ΔMS94.7 deletion virus lacks genes between m1 and m17 (*14*). Both the ΔMS94.7 virus and the RV7 virus (*9*) activated the Ly49H reporter cells, but ΔMS94.5 did not (Fig. 1C). This mapped the responsible gene to the region containing m150 through m165. An m152-deficient MCMV activated the Ly49H reporter cells, excluding this gene from consideration (*9*). By assaying the Ly49H reporter cells against NIH-3T3 cells transfected individually with each of the other genes in the implicated region, m157 was identified as the ligand (Fig. 1D) (*8*). Reporter cells were activated equivalently when cocultured with either m157-transfected NIH-3T3 cells or with the m157-transfected cells infected with the ΔMS94.5 deletion mutant virus (*9*), indicating that m157 does not require other viral factors to activate Ly49H.

m157 is predicted to encode a type I glycoprotein that attaches to the cell membrane by means of a glycophosphatidylinositol anchor. By introducing a FLAG epitope tag onto the NH_2-terminus of m157, we confirmed that it was expressed on the cell surface of transfectants and was cleaved by treatment with phosphatidylinositol–phospholipase C (*9*). An analysis of the primary structure of m157 failed to demonstrate homology to any known viral or host protein other than r157, a rat CMV homolog of m157 (*15*). However, when evaluated with the 3D-PSSM program, which recognizes protein folds using one-dimensional (1D) and 3D sequence profiles coupled with secondary structure and solvation potential (*16*), m157 was identified as a MHC-like protein containing α1, α2, and α3 domains. The analysis predicted significant similarity to the non-classical MHC class I proteins H-2M3, MICA, and CD1d. Thus, like other Ly49 receptors, the viral ligand of Ly49H appears to be a MHC-like protein, although confirmation of this awaits a full structural analysis of m157.

m157 was confirmed as a ligand for Ly49H by the demonstration that a soluble, recombinant, m157–immunoglobulin (Ig) fusion protein (*8*) stained the Ly49H reporter cells but not the parental T cell hybridoma (*9*). m157-Ig stained NK cells isolated from B6 mice, and a similar proportion of NK cells reacted with an Ly49H-specific mAb (Fig. 2A). In addition, binding of m157-Ig to NK cells was blocked completely by a mAb reactive with Ly49H (Fig. 2B), confirming the specificity of the interaction. Ly49H is associated with the DAP12 signaling adapter protein (*17*), and expression of Ly49H is substantially reduced on NK cells in DAP12-deficient mice (*18*). Accordingly, m157-Ig showed diminished binding to NK cells from $DAP12^{-/-}$ NK cells (Fig. 2A). Furthermore,

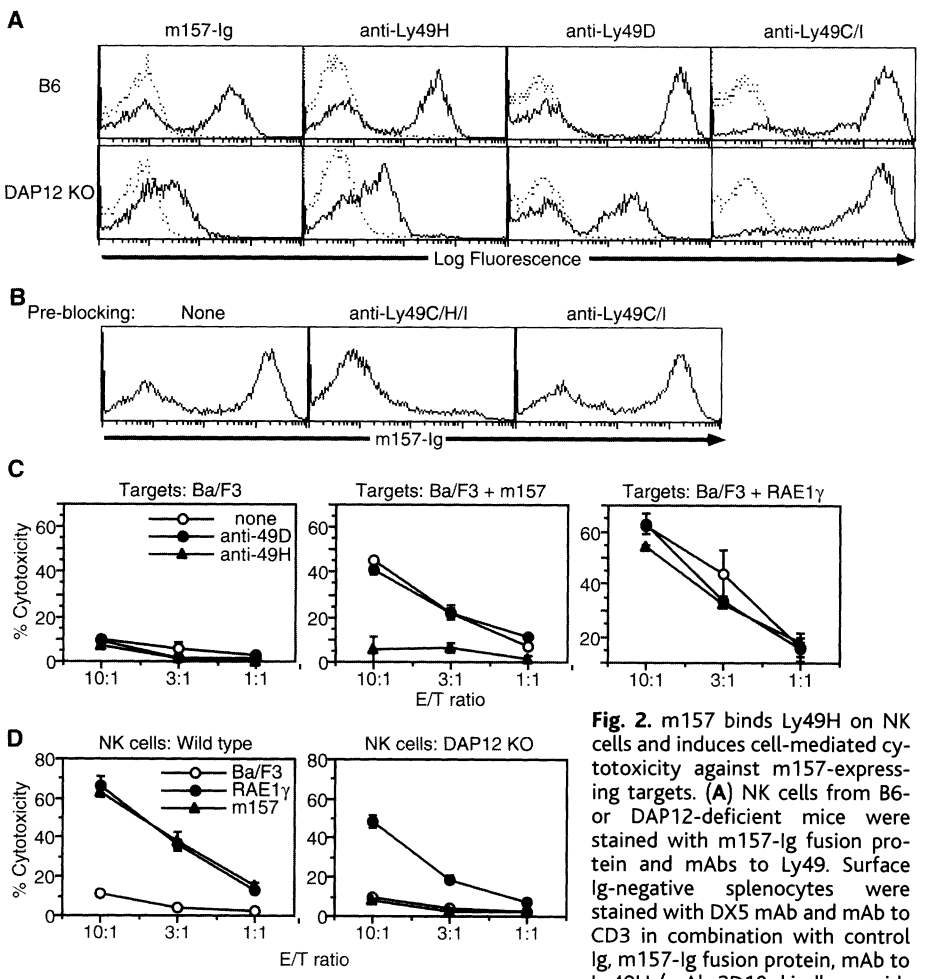

Fig. 2. m157 binds Ly49H on NK cells and induces cell-mediated cytotoxicity against m157-expressing targets. (**A**) NK cells from B6- or DAP12-deficient mice were stained with m157-Ig fusion protein and mAbs to Ly49. Surface Ig-negative splenocytes were stained with DX5 mAb and mAb to CD3 in combination with control Ig, m157-Ig fusion protein, mAb to Ly49H (mAb 3D10, kindly provided by W. Yokoyama), mAb to Ly49D (4E5, BD PharMingen, San Diego, CA), or mAb to Ly49C/I (5E6, BD PharMingen). Data are displayed gating on $CD3^-$, $DX5^+$ NK cells. Histograms of control Ig-stained cells (dotted lines) are superimposed over histograms of cells stained with m157-Ig or anti-Ly49 (solid lines), as indicated. (**B**) Blocking of m157-Ig staining by mAb to Ly49H. NK cells were preincubated with mAb to Ly49C/H/I/U (1F8, kindly provided by M. Bennett) or mAb to Ly49C/I (5E6), then stained with biotinylated m157-Ig, followed by phycoerythrin-conjugated streptavidin. (**C**) Purified NK cells from B6 mice were expanded with IL-2 for 7 days, and cytotoxicity against Ba/F3, m157-transfected Ba/F3, or RAE1γ-transfected Ba/F3 cells was analyzed in the presence of mAb to Ly49C/H/I/U (10 μg/ml) (1F8, solid triangles), mAb to Ly49D (4E5, solid circles), or in the absence of mAbs (open circles). (**D**) Cytotoxicity of IL-2–expanded NK cells from B6- and DAP12-deficient mice (backcrossed to B6 for eight generations) against parental Ba/F3 (open circles), m157-transfected Ba/F3 (solid triangles), or RAE1γ-transfected Ba/F3 cells (solid circles).

NK cells from wild-type B6 mice efficiently killed mouse Ba/F3 pro–B cells transfected with m157 and NIH-3T3 cells transfected with m157 (*9*), and this was blocked in the presence of mAb to Ly49H (Fig. 2C). DAP12-deficient NK cells failed to kill m157-bearing targets but were able to kill other NK-sensitive cells (Fig. 2D) (*8*). Collectively, these findings demonstrate that m157 is an MHC-like MCMV-encoded ligand for the Ly49H receptor that activates NK cells in MCMV-resistant B6 mice.

The ability of NK cells to attack cells expressing m157 raises the question of why this viral gene evolved and how it has been able to persist. This prompted us to investigate whether m157 is recognized by NK cells in MCMV-susceptible versus MCMV -resistant mouse strains. Although B6 mice have the *Cmv1r* gene (recently identified as *Ly49H*), which confers NK cell–mediated resistance to MCMV (*19*), many other strains are relatively susceptible to this virus (*20*, *21*). A recent study by Lee *et al.* (*22*) suggested that there are two independent origins for MCMV susceptibility in mice that map to the *Ly49* complex region. MCMV-susceptible strains of mice were grouped on the basis of relatedness, with mice similar to BALB/c forming one cluster and mice related to 129/J forming another subset. The Ly49 receptor family is polygenic and polymorphic, demonstrating considerable variation in different mouse strains (*23*). We analyzed NK cells from BALB/c and 129/J mice because they were identified as different in their haplotypes for the Ly49 region (*22*) and because the *Ly49* genes in these strains are well characterized and these mice are known to lack Ly49H (*4*–*6*, *24*). m157-Ig did not bind to NK cells from BALB/c mice but did react with a subset of NK cells from 129/J mice (Fig. 3A). In contrast to NK cells from B6 mice, interleukin-2 (IL-2)–activated NK cells from MCMV-susceptible 129/J and BALB/c mice were unable to kill m157-transfected Ba/F3 cells but were able to kill Ba/F3 cells expressing a ligand for the activating NKG2D receptor (Fig. 3B).

Although susceptibility to MCMV could be explained by the lack of an activating receptor for m157 in BALB/c mice, we considered the possibility that MCMV susceptibility in certain mouse strains might be caused by m157 binding to an inhibitory Ly49 receptor. 129/J mice lack Ly49H but express two highly related molecules: an inhibitory Ly49I receptor and an activating Ly49U receptor (*24*). m157-Ig bound to 293T cell transfectants expressing the inhibitory Ly49I allele from 129/J but did not react with transfectants expressing the activating Ly49U receptor from 129/J or the inhibitory Ly49I or Ly49C alleles from B6 (Fig. 4) (*8*). These results are consistent with the lack of a receptor for m157 on BALB/c NK cells and the presence of the inhibitory Ly49I receptor for m157 on only a subset of 129/J NK cells. Whether m157 inhibits the function of 129/J NK cells expressing Ly49I remains to be confirmed by expression of m157 in an appropriate NK-sensitive target. However, the immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain of Ly49I predicts that it will suppress NK activation (*24*). Although the inhibitory Ly49I receptor able to bind m157 was found only on a minor subset of NK cells in 129/J mice, it remains to be determined whether it is more broadly distributed in outbred mice or in other strains of inbred mice.

Viral immunity in MCMV-resistant mice is in part conferred by secretion of IFN-γ by NK cells (*25*, *26*). Therefore, we analyzed the ability of MCMV or the ΔMS94.5 deletion virus (lacking m150-m165) to induce production of IFN-γ by NK cells in vitro. IL-2–

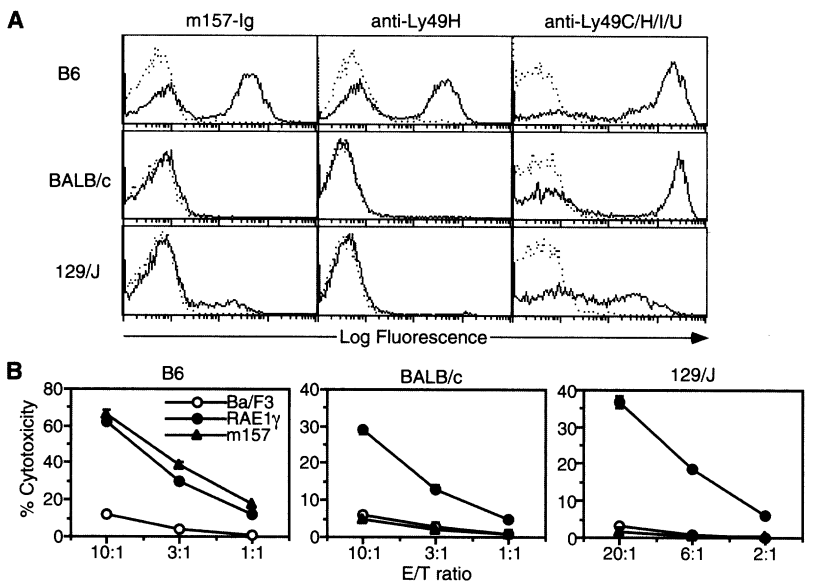

Fig. 3. Binding of m157-Ig to NK cells from MCMV-susceptible mice and NK cell–mediated cytotoxicity against m157-bearing targets. (**A**) Surface Ig-negative splenocytes from B6, BALB/c, and 129/J mice were stained with DX5 mAb and mAb to CD3 in combination with control Ig, m157-Ig fusion protein, mAb to Ly49H (mAb 3D10), or mAb to Ly49C/H/I/U (mAb 1F8). Data are displayed gating on $CD3^-$, $DX5^+$ NK cells. Histograms of control Ig-stained cells (dotted lines) are superimposed over histograms of cells stained with m157-Ig or mAb to Ly49 (solid lines), as indicated. (**B**) Purified NK cells from B6, BALB/c, and 129/J mice were expanded with IL-2 for 7 days, and cytotoxicity against parental Ba/F3 (open circles), m157-transfected Ba/F3 (solid triangles), or RAE1γ-transfected Ba/F3 cells (solid circles) was analyzed.

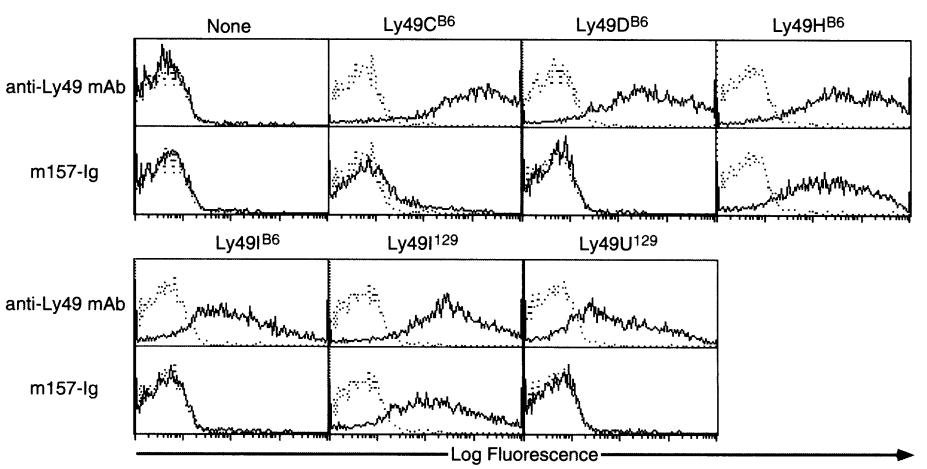

Fig. 4. Binding of m157-Ig to both inhibitory and activating Ly49. cDNAs for Ly49C^{B6}, Ly49D^{B6}, Ly49H^{B6}, Ly49I^{B6}, Ly49I^{129}, and Ly49U^{129} were transfected into 293T cells carrying FLAG-tagged DAP12. After 2 days, transfected cells were stained with control Ig, 1F8 mAb to Ly49C/H/I/U, 4E5 mAb to Ly49D, or m157-Ig, as indicated. Cells were analyzed by flow cytometry with a small desktop Guava Personal Cytometer with Guava ViaCount and Guava Express software (Burlingame, CA).

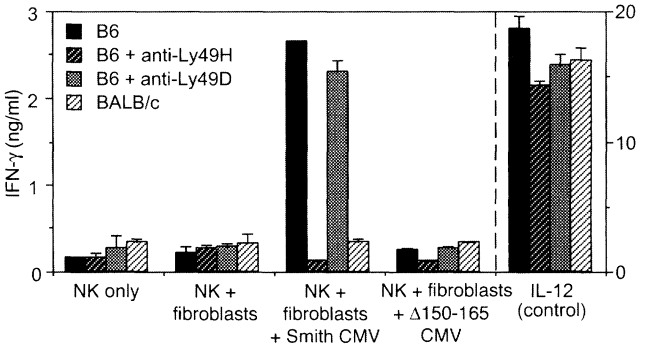

Fig. 5. IFN-γ production by NK cells stimulated with MCMV-infected cells. SV40-transferred embryonic fibroblasts were infected with wild-type MCMV or ΔMS94.5 deletion virus. Infected cells were fixed with 4% paraformaldehyde 36 hours after infection and were cocultured with IL-2–expanded B6 NK cells for 18 hours in the presence or absence (black bar) of 1F8 mAb to Ly49C/H/I/U (10 μg/ml) (dark hatched bar) or 4E5 mAb to Ly49D (gray bar). BALB/c NK cells were also cocultured with virus-infected cells in the absence of mAbs (light hatched bar). As a positive control, NK cells were stimulated with IL-12 (1 ng/ml). Concentrations of IFN-γ were measured by enzyme-linked immunosorbent assay.

activated NK cells from B6 and BALB/c mice were cocultured with SV40-transformed embryonic fibroblasts infected with virus (*8*). B6 NK cells, but not BALB/c NK cells, produced significant amounts of IFN-γ in response to stimulation with wild-type MCMV-infected fibroblasts; NK cells from these two strains were not activated by the ΔMS94.5 virus (Fig. 5). Cytokine production by B6 NK cells stimulated with MCMV-infected fibroblasts was completely blocked by a mAb against Ly49H but was not affected by a mAb to Ly49D used as a control. Because Ly49H exclusively recognized m157, we conclude that the response of B6 NK cells to MCMV-infected fibroblasts in vitro is dominated by the activating Ly49H receptor. These results support and suggest a mechanism to explain the prior observations that treatment of CMV-resistant C57BL/6 mice in vivo with mAb reactive against Ly49H resulted in higher viral titers and increased mortality (*4*, *5*).

Based on these findings, we propose a speculative model for the pathogen-driven evolution of activating NK cell receptors. Double-stranded DNA viruses, such as cytomegalovirus and poxviruses, have large genomes and encode proteins that share homology with chemokines, chemokine receptors, tumor necrosis factor–related proteins, and MHC antigens, presumably to permit viral propagation and persistence in the host (*27*). We propose that the MHC class I–related m157 antigen may have evolved to provide a selective advantage for the virus by engaging an inhibitory NK cell receptor, thereby dampening the host immune response. It is intriguing to note that the Ly49I alleles from B6 and 129/J mice both bind selectively to H-2K^d, whereas the Ly49I receptor in MCMV-resistant B6 mice fails to bind m157. Also notable, the activating Ly49H receptor does not recognize any known H-2 ligand but demonstrates high affinity binding to m157. The origin of m157 and whether it was selected by interaction with an inhibitory receptor similar to Ly49I cannot be traced, because if such selection did occur, it most certainly predates the generation of inbred strains of mice. Nonetheless, the observation that m157 binds to both an inhibitory and a highly homologous activating Ly49 receptor suggests the possibility that one evolved from the other as a consequence of selection by the pathogen.

Although functional *Ly49* genes are not found in humans, the KIR family of NK cell receptors probably mediates the same immune functions. Like Ly49, the *KIR* genes show remarkable genetic diversity within the population (*28*) and encode both inhibitory and activating receptors that are expressed on subsets of NK cells (*29*). Although HLA class I ligands have been identified for most of the inhibitory KIRs, the activating KIRs appear not to bind HLA class I or do so with low affinity (*30*). A comparison of *KIR*s in humans and chimpanzees revealed that these genes are evolving rapidly, possibly faster than the evolution of the MHC in these species (*31*). Based on this study, Parham and colleagues predicted that selection by pathogens might provide the force driving diversification of these receptors (*31*). Our present observations provide evidence supporting this idea. The paradox of having highly related activating and inhibitory receptors is not restricted to NK cells but applies to several families of immune receptors (*32*). This suggests a general strategy whereby the inhibitory receptors may evolve to prevent autoimmunity while the activating receptors are busy combating pathogens. This scenario predicts that other activating NK cell receptors will also recognize pathogen-encoded ligands. Although completely speculative, the possibility is worth consideration.

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