Tolerance of NK cells encountering their viral ligand during development

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During development, T and B cells encountering their cognate ligands via antigen-specific receptors are deleted or rendered anergic. Like T and B cells, natural killer (NK) cells express certain receptors, such as Ly49H, associated with immunoreceptor tyrosine-based activation motif–bearing adaptor proteins that transmit activating signals through Syk family kinases. Ly49H binds with high affinity to a mouse cytomegalovirus (MCMV)–encoded glycoprotein, m157, but does not recognize self-antigens. For comparison with the behavior of immature T and B cells exposed to foreign antigens, we addressed the fate of Ly49H$^+$ NK cells that encountered their viral ligand during development by retroviral transduction of bone marrow stem cells with m157. In chimeric mice expressing m157, we observed a reduction in Ly49H$^+$ NK cells in multiple tissues and less Ly49H on the cell surface. NK cells exposed to m157 during development appeared less mature, produced less interferon γ when stimulated through Ly49H, and were unable to kill m157-bearing target cells. After MCMV infection, these NK cells were severely impaired in their ability to proliferate. Thus, if immature NK cells encounter ligands for their activating receptors, regulatory mechanisms exist to keep these cells in an unresponsive state.

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Abbreviations used: ITAM, immunoreceptor tyrosine-based activation motif; MCMV, mouse CMV; MFI, mean fluorescence intensity.

Terms such as positive selection and deletion are most commonly used to describe events occurring during thymocyte development. Thymocytes that have not correctly rearranged their TCRs undergo a process referred to as death by neglect and do not survive (1). Although NK cells are not generally thought to undergo such selective processes, recent evidence suggests that developing NK cells may be "licensed" or "armed" in the bone marrow to acquire a fully functional status (2–6). Similar to positive selection of immature T cells that have correctly rearranged a functional TCR able to interact with self-peptide–MHC, immature NK cells expressing inhibitory receptors may be given the proper signals via interactions with their autologous MHC class I molecules to complete their functional maturation (2, 3). In the absence of these interactions, NK cells may either fail to complete their maturation or, alternatively, may be chronically stimulated, with each case resulting in NK cells that are hyporesponsive (2, 3). Therefore, selective pressures apparently are placed on immature NK cells expressing inhibitory receptors to ensure self-tolerance.

NK cells expressing activating receptors might also undergo a selection process that would eliminate or inactivate developing NK cells bearing an activating receptor recognizing a high affinity ligand expressed in the host's bone marrow in a manner similar to negative selection in thymocytes. NK cells bearing the DAP12-associated activating Ly49H receptor interact with the mouse CMV (MCMV)–encoded protein m157 on infected cells (7, 8), thus protecting mice containing this NK cell subset against MCMV infection (9). In a specific pathogen-free mouse colony, immature NK cells will not encounter this viral ligand during development in the bone marrow. This provides a model system to determine how immature Ly49H$^+$ NK cells behave when they encounter a ligand for an activating receptor during development. Would immature Ly49H$^+$ NK cells be deleted in a manner analogous to thymocytes that have rearranged a self-reactive TCR? Would these NK cells mature and become chronically activated in the periphery?

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Would these NK cells be desensitized to the ligand and become hyporesponsive when challenged with infection during adult life?

Studies to determine how T and B cells respond when exposed to high affinity foreign antigen during development have typically used TCR or BCR transgenic mice against antigens such as ovalbumin or hen egg lysozyme (10, 11). Because the Ly49H receptor has a high affinity for the viral glycoprotein m157 but does not recognize any self-antigen (12), this provides an analogous model system to evaluate the fate of NK cells receiving strong immunoreceptor tyrosine-based activation motif (ITAM)–mediated signaling during development. To address this question, we generated mice that express m157 in the bone marrow and examined the development of the Ly49H$^+$ NK cell subset. To our surprise, we observed that Ly49H$^+$ NK cells were found in the periphery of m157-expressing mice; however, these NK cells were present at lower numbers and were severely defective in their ability to mediate cytotoxicity and proliferate in response to MCMV infection.

RESULTS

Expression of m157 in cell lines and chimeric mice

The goal of this study was to determine the effects of early and chronic stimulation of the Ly49H receptor with its viral ligand, m157, on the development and function of NK cells. We cloned m157 into the MigR1 retroviral vector and assessed expression of m157 by transient transfection of 293T cells and co-culturing with an Ly49H-NFAT-GFP reporter cell line. m157-transfected 293T cells activated >70% of reporter cells, as measured by GFP expression (Fig. 1 A). Using an antibody specific for m157 (13), we demonstrated expression of this viral protein on the surface of packaging cells transfected with the Mig-m157 vector, on hematopoietic stem cells infected with the Mig-m157 retrovirus, and on bone marrow cells from lethally irradiated B6 mice reconstituted with m157 retrovirus–infected hematopoietic stem cells 3 mo after transplantation (Fig. 1 B). In contrast, m157 was not detected on cells from the Mig vector control group (Fig. 1 B).

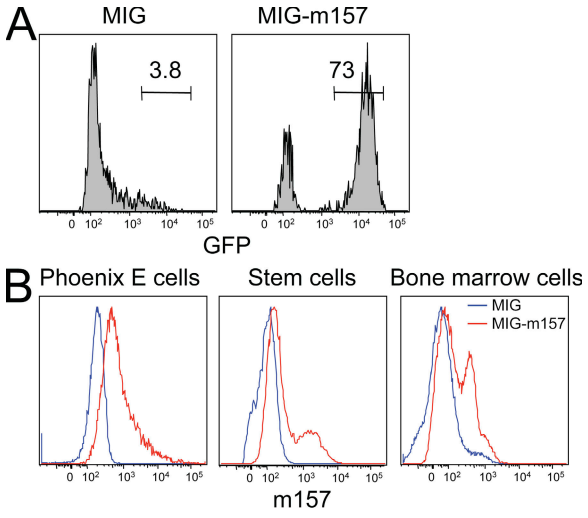

Figure 1. Expression of MCMV-encoded m157 protein in cell lines and stem cell chimeric mice. (A) 293T cells transfected with Mig control vector or Mig-m157 were co-cultured with the Ly49H-NFAT-GFP reporter cell line. Percentages of reporter cells expressing GFP are shown. (B) m157 expression in transfected Phoenix E cells, retrovirus-infected bone marrow cells, and bone marrow cells from hematopoietic stem cell–reconstituted irradiation chimeric mice was measured by using an antibody against m157. Cells expressing m157 in the cells infected with Mig control vector and the Mig-m157 construct are shown. The efficiency of transduction with the control Mig and the Mig-m157 vectors, as determined by GFP expression, was comparable (not depicted).

Ly49H$^+$ NK cell numbers and Ly49H expression are reduced in m157-expressing mice

NK cells in peripheral blood from the chimeric mice expressing m157 or the control vector were analyzed. Because stem cells derived from congenic CD45.1 mice were injected into irradiated CD45.2 recipient mice, donor cells were tracked by staining with an antibody against CD45.1 (Fig. 2 A). Peripheral blood revealed a >50% reduction in the percentage of Ly49H$^+$ NK cells from m157-expressing mice compared with Mig control mice, whereas the numbers of NK cells bearing another DAP12-associated activating receptor, Ly49D, were unchanged (Fig. 2 A). Ly49D and Ly49H are coexpressed on a subset of double-positive cells (14); interestingly, the percentage of these double-positive NK cells was diminished, replaced by an increased frequency of single-positive Ly49D$^+$ NK cells (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072448/DC1). Furthermore, surface Ly49H expression, as measured by the mean fluorescence intensity (MFI) of cells stained with anti-Ly49H mAb, was diminished on NK cells from Mig-m157 mice compared with Mig control mice (Fig. 2 B). As a control, the amount of Ly49D on NK cells was similar between the two groups (Fig. 2 B), even on the Ly49D$^+$, Ly49H$^+$ double-positive NK cells, suggesting that the lower expression of Ly49H observed in Mig-m157 mice is specific to the expression of the Ly49H ligand. Similarly, NKG2D was expressed at similar levels in the two groups (Fig. 2 B). NK cell subsets expressing inhibitory receptors also showed no differences between m157-expressing and control mice (Fig. S2). In addition, expression of inhibitory Ly49 receptors on the Ly49H$^+$ NK cell subset from m157-expressing mice showed no differences compared with control mice (unpublished data). Reduced Ly49H$^+$ NK cell numbers and Ly49H expression were observed in the peripheral blood of m157-expressing mice at multiple time points after stem cell reconstitution (Fig. 2 C).

3 mo after reconstitution, m157-expressing and control Mig mice were killed, and the NK cells from multiple organs were analyzed. The overall number of NK cells in all organs showed no significant differences between the two groups (unpublished data). Similar to peripheral blood, Ly49H$^+$ NK cell numbers and Ly49H surface expression were reduced in the spleen, liver, and bone marrow of m157-expressing compared with control mice (Fig. 3 A). Ly49D$^+$ NK cell numbers

and Ly49D expression remained unchanged between the two groups (unpublished data). NKG2D was also unaffected on NK cells in all tissues examined from m157-expressing or control mice (Fig. 3 A). Although the percentages of Ly49H$^+$ NK cells were diminished by >60% in the spleen and liver of m157-expressing mice, only a small decrease was observed in the bone marrow (Fig. 3 B). It should be noted that although the amount of Ly49H on the surface of the NK cells in the m157-expressing mice was lower than in control mice, the Ly49H$^+$ and Ly49H$^-$ subsets were still clearly resolved in the m157-expressing animals. Therefore, the decrease in the percentage of Ly49H$^+$ NK cells in the m157-expressing mice is not simply caused by an inability to discriminate between the Ly49H positive and negative subsets.

Ly49H$^+$ NK cells from m157-expressing mice are phenotypically less mature

NK1.1 is expressed early on immature NK cells, before expression of the Ly49 receptors (15–17). No differences were observed in the amounts of NK1.1 expressed on Ly49H$^+$ NK

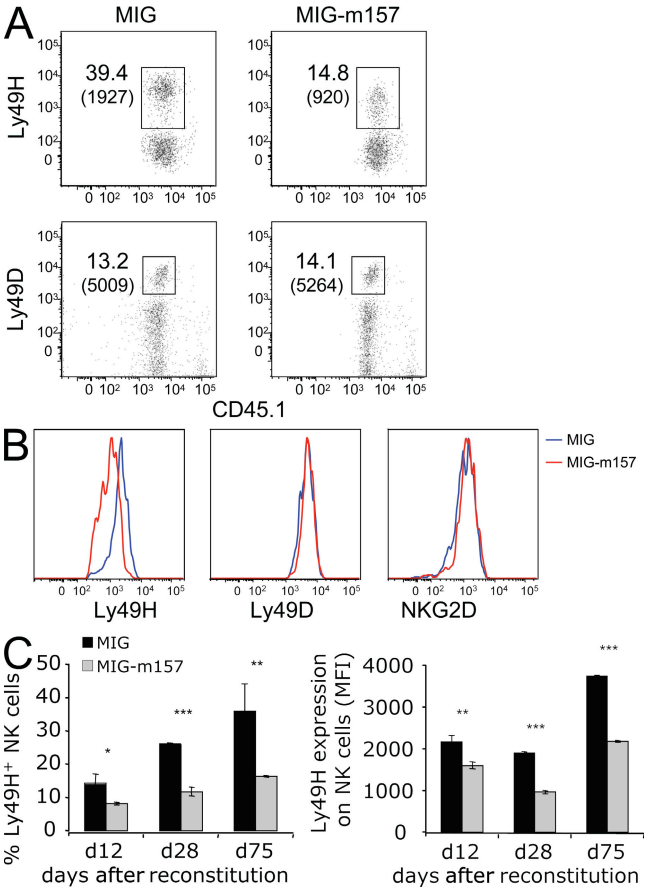

Figure 2. Reduced Ly49H$^+$ NK cell numbers and Ly49H expression in the peripheral blood of m157-expressing chimeric mice. (A) 28 d after reconstitution of irradiated recipient mice with Mig control or Mig-m157–transduced stem cells (CD45.1$^+$), peripheral blood was analyzed for Ly49H$^+$ and Ly49D$^+$ NK cells in chimeric mice. Plots are gated on CD3$^-$, NK1.1$^+$ cells, and percentages of Ly49H$^+$ (top) and Ly49D$^+$ (bottom) NK cells (CD45.1$^+$) are shown. MFIs for boxed regions are shown in parentheses. (B) Overlay of histogram plots comparing cell surface amounts of Ly49H, Ly49D, and NKG2D on NK cells that express the activating receptor from Mig control and Mig-m157 chimeric mice. (C) Graphs show the percentages of Ly49H$^+$ cells (left) and the MFI for Ly49H expression (right) at 12, 28, and 75 d after reconstitution of irradiated recipient mice. Data are presented as the mean ± SEM of three to five mice at each time point. Statistical differences in the percentage of Ly49H$^+$ NK cells and the MFI of Ly49H expression between control and m157-expressing mice are indicated (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$). Data are representative of three independent experiments.

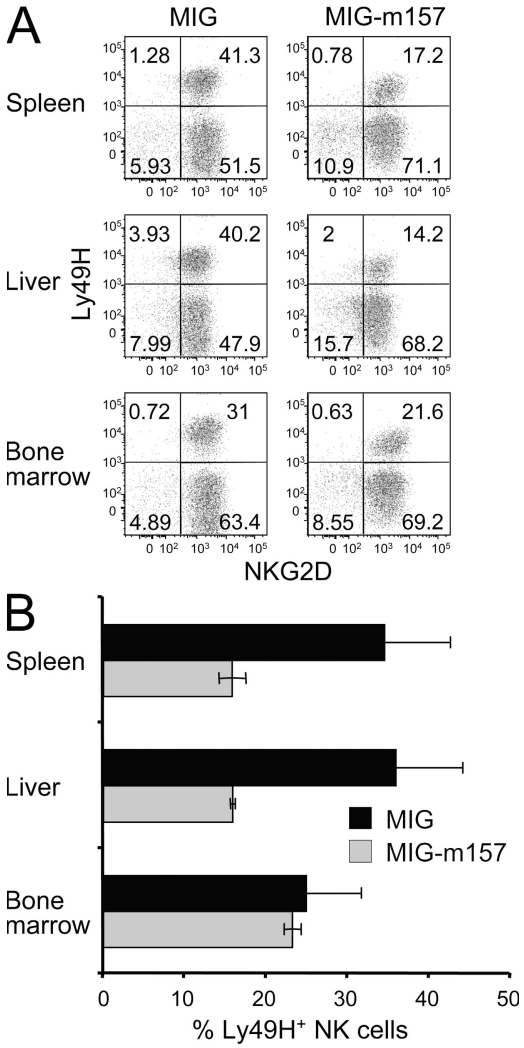

Figure 3. Reduced Ly49H$^+$ NK cell numbers in the spleen, liver, and bone marrow of m157-expressing mice. 3 mo after hematopoietic stem cell reconstitution of irradiated recipient mice with either Mig control or Mig-m157–transduced stem cells, mice were killed and NK cells from the spleen, liver, and bone marrow were analyzed. (A) Plots show the percentages of CD3$^-$, NK1.1$^+$ cells from the indicated organs that express Ly49H and NKG2D. (B) Bar graph shows the percentages of Ly49H$^+$ NK cells in tissues examined, presented as the mean ± SEM of three to five mice. Differences in percentages of Ly49H$^+$ NK cells between control and m157-expressing mice were statistically significant in the spleen and liver ($P < 0.01$). Data are representative of three independent experiments.

cells in the spleen, liver, and bone marrow from m157-expressing mice and control mice (Fig. 4 A). Other receptors expressed before the Ly49 receptors, such as CD122 (15–17), were unaltered on Ly49H$^+$ NK cells from m157-expressing compared with control Mig mice (unpublished data). Expression of CD117 (c-Kit), a developmental marker that is turned on after Ly49H expression but then down-regulated as NK cells mature (15–17), remained higher on Ly49H$^+$ NK cells from m157-expressing compared with control mice, with the sharpest contrast seen in the bone marrow (Fig. 4 A). Other markers expressed at high levels late during NK cell maturation, such as CD43 and CD11b (Mac-1) (16–18), were present at lower amounts on Ly49H$^+$ NK cells from m157-expressing mice compared with controls (Fig. 4 B). In m157-expressing mice, Ly49H$^-$ NK cells did not appear phenotypically immature compared with controls, indicating a preferential effect on the development of the Ly49H$^+$ NK cells (unpublished data). Collectively, these results suggest that NK cells encountering m157 during development do not properly mature.

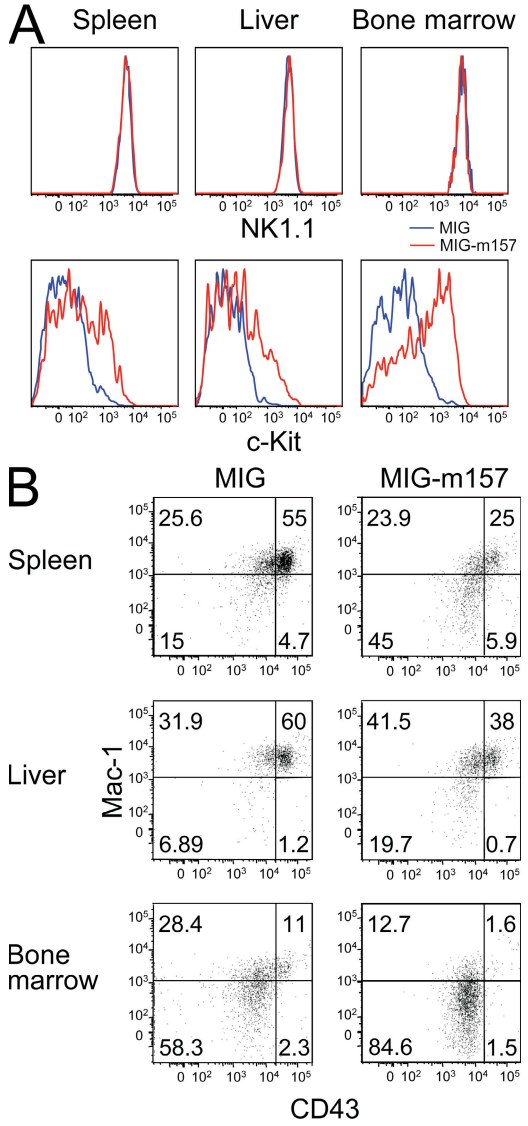

Figure 4. Ly49H$^+$ NK cells in the spleen, liver, and bone marrow of m157-expressing mice are phenotypically less mature. (A) Histograms show the expression of the developmental markers NK1.1 (top) and c-Kit (bottom) on Ly49H$^+$ NK cells in the spleen, liver, and bone marrow of Mig control or Mig-m157 chimeric mice. (B) Plots show expression of the maturation markers CD43 and CD11b (Mac-1) on Ly49H$^+$ NK cells in the spleen, liver, and bone marrow of Mig control or Mig-m157 chimeric mice. Quadrant markers were arbitrarily set to allow visual comparison of the most mature NK cell populations that coexpressed high amounts of both CD43 and Mac-1 in the different tissues. Percentages are shown for each quadrant in the dot plots. Data are representative of three independent experiments, with three to five mice per group.

Ly49H$^+$ NK cells from m157-expressing mice produce less IFN-γ and exhibit diminished cytotoxicity ex vivo

Because of the altered phenotype of the Ly49H$^+$ NK cells from m157-expressing mice, we tested the functionality of these cells ex vivo. Splenocytes were incubated with plate-bound antibodies against NK1.1, Ly49D, or Ly49H for 4 h in the presence of Brefeldin A, and intracellular IFN-γ production was measured. Although anti-NK1.1 and anti-Ly49D stimulation gave comparable numbers of IFN-γ–producing NK cells between m157-expressing and control mice, anti-Ly49H stimulation resulted in robust IFN-γ production only by NK cells from Mig control mice and not NK cells from m157-expressing mice (Fig. 5). However, this selective hyporesponsiveness to anti-Ly49H mAb stimulation was partially rescued if the NK cells from m157-expressing mice were cultured in the presence of high concentrations of IL-2 (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072448/DC1).

We tested the ability of Ly49H$^+$ NK cells from 28-d bone marrow–reconstituted control and m157-expressing mice to kill m157-bearing target cells. Splenocytes from both groups of mice were enriched for NK cells by using magnetic bead cell sorting. Comparable numbers of Ly49H$^+$ NK cells (as determined by flow cytometry) were coincubated with m157-transduced Ba/F3 targets or untransduced Ba/F3. Although Ly49H$^+$ NK cells from Mig control mice were able to efficiently lyse m157-bearing target cells, Ly49H$^+$ NK cells from m157-expressing mice showed a severely diminished cytotoxic response (Fig. 6 A). At 3 mo after reconstitution, Ly49H$^+$ NK cells from m157-expressing mice continued to show a decreased ability to kill BaF3-m157 target cells compared with Mig control mice (Fig. 6 B). Overall, NK cell–mediated cytotoxicity in m157-expressing mice remained intact because lysis of Ba/F3 targets expressing Rae-1ε (an NKG2D ligand) or YAC-1 targets was comparable to control mice (Fig. 6, C and D). Together with the cytokine data, these experiments demonstrate that NK cells that develop in the presence of m157 show severely diminished effector functions when stimulated through the Ly49H receptor.

Reduced expansion of activated Ly49H$^+$ NK cells from m157-expressing mice

Because the Ly49H$^+$ NK cells from m157-expressing mice were less responsive in vitro when stimulated with anti-Ly49H

or co-cultured with m157-bearing target cells, we tested the in vivo functionality of these cells. Splenic NK cells from irradiation bone marrow chimeric mice expressing m157 or vector control were purified and adoptively transferred into DAP12-deficient recipient mice (Fig. 7 A). We have previously shown that in DAP12-deficient mice, Ly49H is expressed at much lower levels than in wild-type mice (19). We then measured the activation and proliferation of the transferred wild-type Ly49H$^+$ NK cells from m157-expressing or control mice after MCMV infection (Fig. 7 A). Donor Ly49H$^+$ NK cell numbers were normalized before injection so that the same percentage of Ly49H$^+$ cells was present in the NK cell population of the recipient mice before infection (Fig. 7 B). The donor Ly49H$^+$ NK cells from m157-expressing mice remained Ly49H^{lo} even after adoptive transfer into recipient mice (Fig. 7 B). NK cells from both control and m157-expressing mice displayed the typical early activation profile attributable to the massive inflammatory response induced immediately after viral infection (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20072448/DC1); however, the transferred NK cells (CD45.1$^+$) analyzed 36 h after MCMV infection showed very little expansion in the spleen at this early time point (not depicted). Interestingly, 7 d after infection, the Ly49H$^+$ NK cells from m157-expressing mice were unable to expand as well as their counterpart Ly49H$^+$ NK cells from control Mig mice (Fig. 7 C). The endogenous Ly49H^{dim} population (CD45.1$^-$) found in DAP12-deficient recipients demonstrate much less expansion in response to MCMV and remain at <50% of the entire NK cell pool (Fig. 7 C and not depicted). After infection, Ly49H$^+$ NK cells from both m157-expressing and control mice expressed high amounts of KLRG1 on the cell surface (Fig. 7 C), signifying activation of these proliferating cells (20). The adoptively transferred Ly49H$^+$ NK cells from control Mig mice expanded 5–10-fold (a statistically significant amount; $P = 0.004$) more than the Ly49H$^+$ NK cells from m157-expressing mice in response to MCMV infection (Fig. 7 D). These experiments demonstrate that Ly49H$^+$ NK cells exposed to their cognate viral ligand during development are defective in their ability to "sense" m157 during MCMV infection and respond in a rapid manner.

In these studies, we examined the in vivo response of the NK cells from control Mig or Mig-m157 mice to MCMV by adoptive transfer, rather than by simply challenging the chimeric mice with MCMV, for several reasons. The adoptive transfer experiments allowed us to match the number of transferred control and m157-exposed NK cells in the

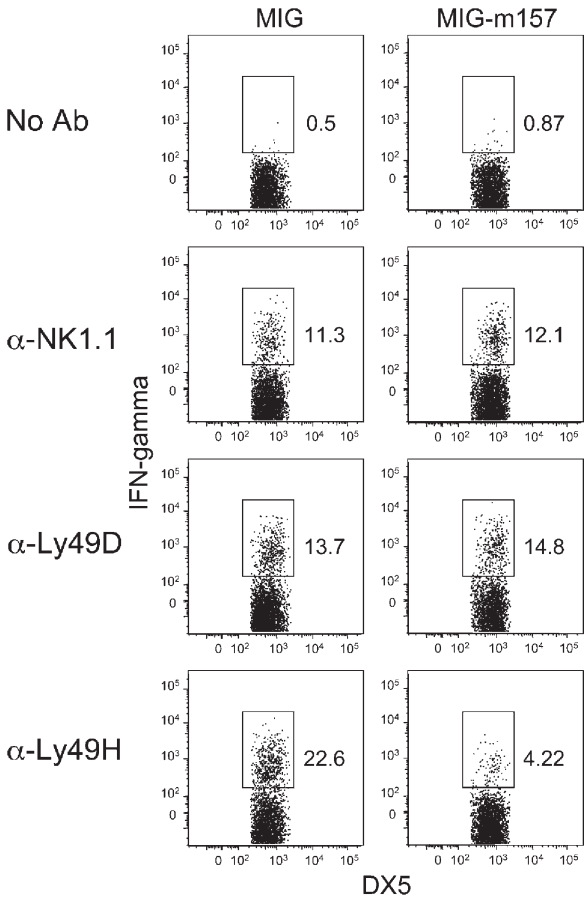

Figure 5. NK cells from m157-expressing mice produce less IFN-γ when stimulated with anti-Ly49H. Freshly isolated splenocytes from either Mig control or m157-expressing mice were stimulated with plate-bound antibodies against the NK cell surface markers NK1.1, Ly49D, Ly49H, or control (no antibody). Plots show the percentages of CD3$^-$, CD11b$^+$, DX5$^+$ cells that produce IFN-γ. Data are representative of three independent experiments.

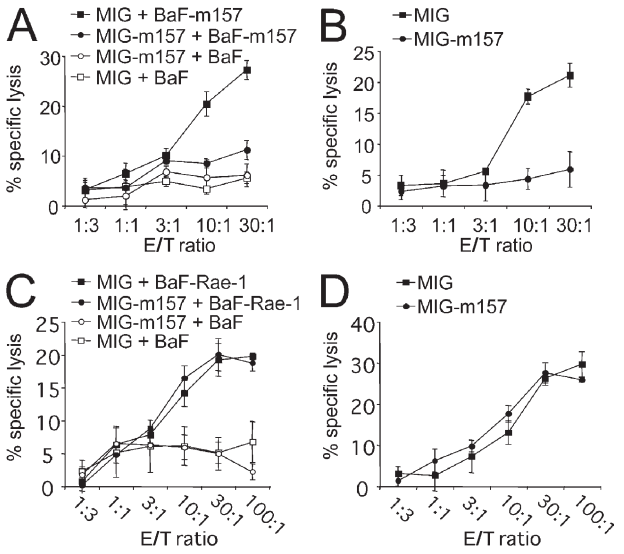

Figure 6. Ly49H$^+$ NK cells from m157-expressing mice exhibit diminished cytotoxicity against m157-bearing targets. NK cells from Mig control or Mig-m157 chimeric mice at 28 d (A) or 3 mo (B) after reconstitution were MACS purified. Equivalent numbers of Ly49H$^+$ NK cells, as determined by flow cytometry, were incubated with Ba/F3 or m157-transfected Ba/F3. (C) Total NK cells from Mig or Mig-m157 mice were incubated with Ba/F3 or Rae-1–transfected Ba/F3 target cells. (D) Total NK cells from Mig or Mig-m157 mice were incubated with YAC-1 target cells. Data are representative of three independent experiments.

recipient mice. Moreover, if we had simply infected the Mig-m157 chimeric mice with MCMV, the host hematopoietic cells in the mice would express m157 so that the Ly49H$^+$ NK cells would be unable to distinguish between MCMV-infected and normal host cells expressing the transduced m157.

Inhibition of Ly49H$^+$ NK cell maturation in trans by cells expressing m157

To determine whether the immature phenotype and hyporesponsiveness of Ly49H$^+$ NK cells from m157-expressing mice could be caused by expression of m157 on an adjacent cell during development, we made 1:1 Mig/Mig-m157 mixed bone marrow chimeric mice and compared NK cell populations with m157-expressing and control Mig mice. To track individual cell populations in 1:1 mixed chimeras, Mig-infected control bone marrow was derived from B6 (CD45.2$^+$) mice, and m157-expressing bone marrow was derived from congenic CD45.1 mice. Peripheral blood on day 28 after reconstitution revealed a >50% reduction in the percentage of Ly49H$^+$ NK cells from 1:1 mixed chimeras compared with Mig control mice, with percentages similar to Ly49H$^+$ NK cell numbers from m157-expressing mice (Fig. 8 A). Similar to m157-expressing mice, surface Ly49H expression was diminished on all NK cells from 1:1 mixed chimeric mice compared with Mig vector control mice (Fig. 8 A). In contrast, no differences in Ly49D$^+$ NK cell numbers or Ly49D surface expression were observed between the three groups (Fig. 8 A). When gating on Mig-control (CD45.2$^+$) or Mig-m157 (CD45.1$^+$) cells within NK cell populations from 1:1 mixed chimeras, similarly diminished Ly49H expression was observed at days 12 and 28 after reconstitution compared with NK cells from control Mig mice (Fig. 8 B). These findings suggest that the regulatory effects of m157 on the Ly49H$^+$ NK cell population can occur in trans and that expression of m157 does not have to be intrinsic to the NK cell being rendered less responsive.

DISCUSSION

The innate immune system is evolutionarily older than the adaptive immune system. Although NK cells are considered members of the innate immune system, they bear many striking phenotypic and functional similarities to T cells (21–23). NK cells might constitute a lineage of cells that provides an evolutionary link between the innate immune system and the antigen-specific receptor-bearing cells of adaptive immunity, possessing certain attributes of both. If NK cells are indeed a bridge between innate and adaptive immunity, then hallmarks of the adaptive immune system may have evolved first in this cell type. In fact, the paradigm is beginning to shift, given

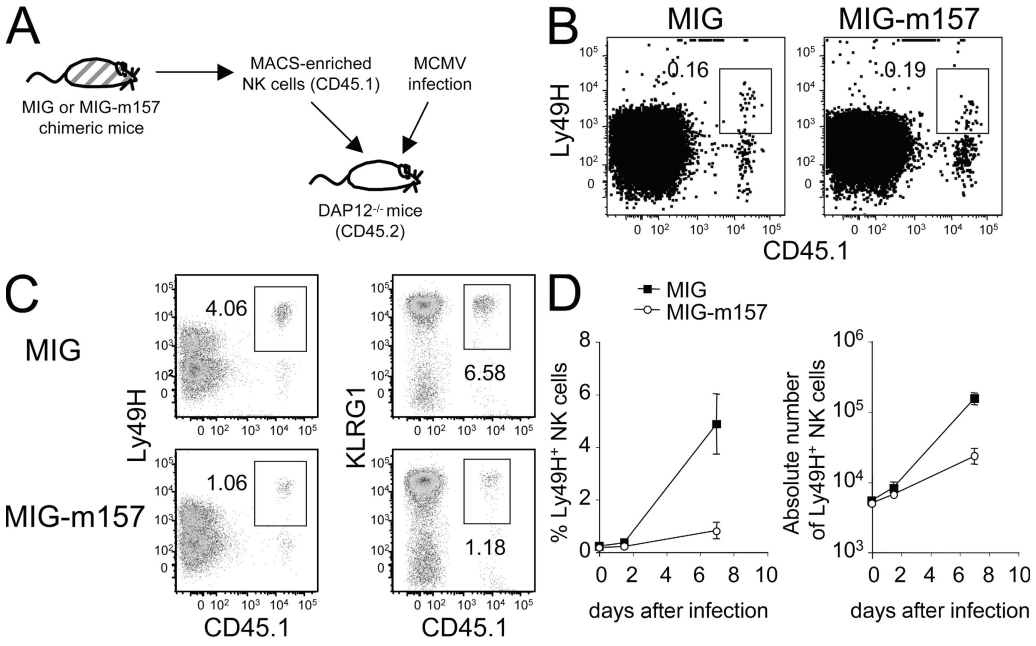

Figure 7. Reduced expansion of Ly49H$^+$ NK cells from m157-expressing mice. (A) Schematic diagram of experiment. NK cells from Mig control or m157-expressing mice were MACS purified, and equivalent numbers of Ly49H$^+$ NK cells were adoptively transferred to DAP12-deficient recipient mice. Recipient mice were infected with MCMV 1 d later. Splenic NK cells were analyzed 36 h and 7 d after infection. (B) Plots show transferred NK cells (CD45.1$^+$) in the spleen of recipient DAP12-deficient (CD45.2$^+$) mice before infection. Percentages of Ly49H$^+$ cells within the total NK cell population are found in the gated region of each plot. (C) Plots show transferred NK cells (CD45.1$^+$) in recipient DAP12-deficient mice 7 d after MCMV infection. Percentages of Ly49H$^+$ cells within the total NK cell population are gated in each plot. Activation marker KLRG1 was measured on total transferred NK cell populations. (D) Expansion of the transferred Ly49H$^+$ NK cells is shown in the graphs. The percentages of transferred Ly49H$^+$ cells in the entire NK cell population (left) and the absolute number of transferred Ly49H$^+$ NK cells (right) are plotted for 0, 1.5, and 7 d after infection. Data are presented as the mean ± SEM of two or three mice at each time point. Differences in the percentage and absolute number of Ly49H$^+$ NK cells between control and m157-expressing mice were statistically significant at day 7 after infection ($P = 0.004$). Data are representative of three independent experiments.

recent data documenting selective pressures on developing NK cells (2, 3), clonal expansion of NK cell subsets (24–26), and possibly even immune memory in NK cells (27).

Although T cell development is relatively well understood, little is known about how NK cells are regulated during their development (21–23). Recently, two groups have proposed comparable, yet distinct, models for mechanisms that regulate the development of NK cells bearing inhibitory receptors for self–MHC class I (2, 3). Although the precise mechanism for self-tolerance is yet to be determined, it is clear that immature NK cells undergo selective events in the bone marrow that determine their fate in the periphery. The question of whether developing NK cells bearing activating receptors experience similar selective pressures was unaddressed. Therefore, we sought to determine whether self-tolerance occurs in developing NK cells bearing ITAM-signaling activating receptors in a manner similar to developing T or B cells.

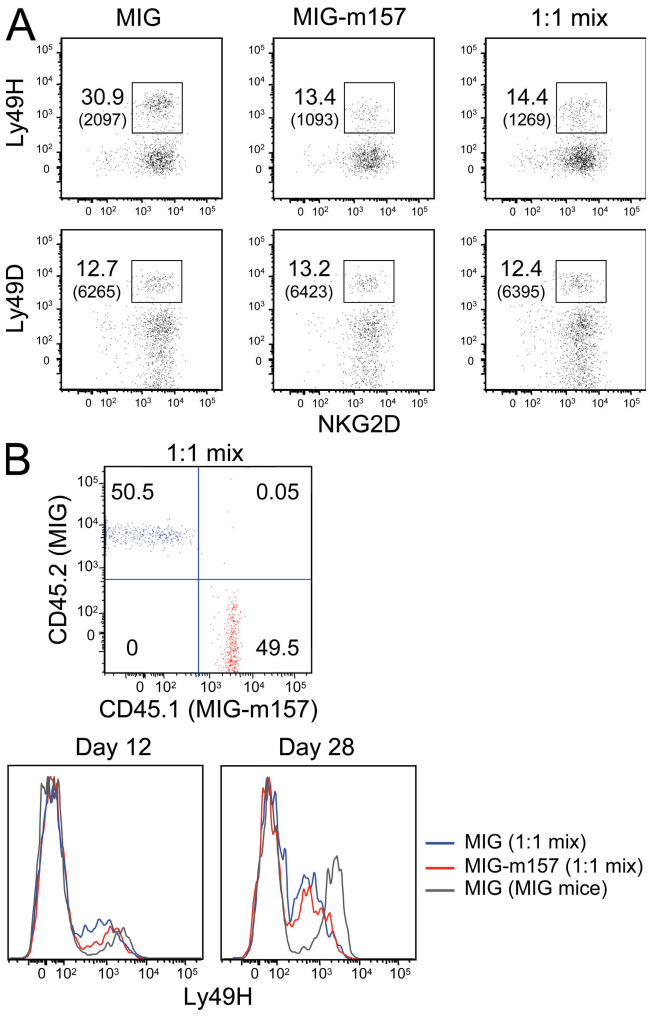

Figure 8. Expression of m157 results in trans down-regulation of Ly49H expression and Ly49H$^+$ NK cell numbers. (A) 28 d after hematopoietic stem cell reconstitution of irradiated recipient mice with Mig control, Mig-m157, or a 1:1 mixture of Mig control/Mig-m157 stem cells, peripheral blood was analyzed for Ly49H$^+$ and Ly49D$^+$ NK cells in mixed chimeric mice. Plots are gated on CD3$^-$, NK1.1$^+$ cells, and percentages of NKG2D$^+$ Ly49H$^+$ (top) and Ly49D$^+$ (bottom) NK cells are shown. MFIs for boxed regions are shown in parentheses. (B) Overlay histogram plots examine the amounts of Ly49H on the cell surface of NK cells derived from Mig control (CD45.2$^+$) and Mig-m157 (CD45.1$^+$) stem cell populations within the 1:1 mixed chimeric mice. Peripheral blood from 12 and 28 d after reconstitution are shown. Data are representative of three independent experiments, with three to five mice per group at each time point.

For comparison with previous studies using TCR or BCR transgenic mice exposed to high affinity antigens during development, we established a model system whereby the ITAM-signaling Ly49H receptor expressed on NK cells would encounter the foreign m157 glycoprotein during their development in the bone marrow. Using this model, we demonstrate that when developing Ly49H$^+$ NK cells encounter their cognate viral ligand, certain regulatory mechanisms render the cells in the periphery hyporesponsive. Rather than a complete deletion, as seen in thymocytes possessing TCRs that undergo high affinity interactions with self-peptide–MHC complexes (Fig. 9 A), Ly49H$^+$ NK cells that interact with m157 survive but populate the periphery in lower numbers and express diminished amounts of the Ly49H receptor on the cell surface. Furthermore, these Ly49H-bearing NK cells from mice that express m157 were defective in their ability produce effector cytokines and mediate killing via Ly49H-specific stimulation and to specifically expand after MCMV infection. Perhaps the term "disarming" is best applied to these activating receptor–bearing NK cells that are actively rendered hyporesponsive by bone marrow–derived cells expressing cognate ligand for Ly49H (Fig. 9 B). Just as NK cells lacking inhibitory receptors for self–MHC class I can exit the bone marrow and populate the periphery but are refractory to stimulation through their activating receptors, disarmed NK cells bearing activating receptors that have encountered their cognate ligand during development undergo a similar fate (Fig. 9 B). Interestingly, there is a small population of Ly49H$^+$ NK cells that eludes tolerance in our system, and they can produce cytokines (Fig. 5) and undergo proliferative expansion (Fig. 7) via specific Ly49H receptor ligation. Further studies are required to determine the mechanism of this escape and establish whether or not these NK cells behave similarly to self-reactive T cells that have evaded central tolerance mechanisms and are either rendered anergic or mediate autoimmunity in the periphery.

We have used m157 as a model system to evaluate the consequences of early exposure of NK cells during their development to a high affinity non–self-ligand for an ITAM-bearing receptor. Although MCMV persists in the host after resolution of the primary infection, MCMV is preferentially localized in the salivary gland. Therefore, it is unlikely that NK cells, which develop predominantly in the bone marrow, would encounter cells expressing m157 during their early development. Our present studies do not address the consequences of prolonged exposure of mature NK cells, as compared with developing NK cells, to m157. In adult mice infected with MCMV, there is expansion of mature Ly49H$^+$ NK cells, followed by a contraction phase in which most of these NK

cells presumably die by apoptosis (24–26). Whether the responsiveness of the remaining MCMV-experienced NK cells is affected has not yet been addressed.

Concurrent with our studies using retroviruses to express m157 in mice, Tripathy et al. generated m157-expressing transgenic mice (see Tripathy et al. [28] on p. 1829 of this issue). In accordance with our findings, these investigators also observed a diminished number and functional impairment of Ly49H$^+$ NK cells in these m157-transgenic mice. In our adoptive transfer studies, we demonstrated a profound defect in the proliferation of Ly49H$^+$ NK cells from m157-expressing mice after recipient mice were infected with MCMV. This severe impairment in the expansion of these m157-exposed Ly49H$^+$ NK cells would likely result in inefficient clearance of MCMV in these hosts because the Ly49H$^+$ NK cell subset is known to be responsible for the NK cell–mediated protection of B6 mice (9, 25, 29). Indeed, Tripathy et al. (28) have observed that m157-transgenic mice are more susceptible to MCMV infection than wild-type mice, with the lack of resistance attributed in part to the diminished cytotoxic function of Ly49H$^+$ NK cells in the transgenic mice. Although there were some differences observed in these two experimental models (e.g., apparently a more profound impairment of non-Ly49H receptors in the m157-transgenic mice), this might be accounted for by the amount of m157 expressed by the strong β-actin promoter in the transgenic mice compared with the lower amounts of m157 produced by the retroviral vector. Nonetheless, the major conclusions from both of these complementary studies are in agreement.

Previous studies have examined the consequences of transgenic expression of ligands for certain other NK cell receptors. In transgenic B6 mice constitutively expressing Rae-1, NKG2D (a receptor for Rae-1) was down-regulated on developing and mature NK cells (30, 31). These transgenic mice were unable to reject Rae-1–bearing tumors that are rejected by syngeneic, nontransgenic mice and were unable to reject allogeneic bone marrow grafts expressing Rae-1. Our current study fundamentally differs from these experiments involving NKG2D ligand expression in several ways. Rae-1 is a self-protein, whereas m157 is foreign. Moreover, although Rae-1 is not expressed in most healthy adult tissues, we have detected constitutive expression of Rae-1 in the liver, a site where NK cells are known to traffic (32). In addition, the signaling pathways used by Ly49H and NKG2D are distinct.

Previous studies have also investigated the DAP12-associated Ly49D receptor in mice bearing H-2D^d, a ligand of Ly49D. In contrast to our findings with m157 and Ly49H, Ly49D$^+$ NK cells developing in a host expressing H-2D^d were not diminished in frequency or number (33). Moreover, in mixed bone marrow chimeras in which B6 Ly49D$^+$

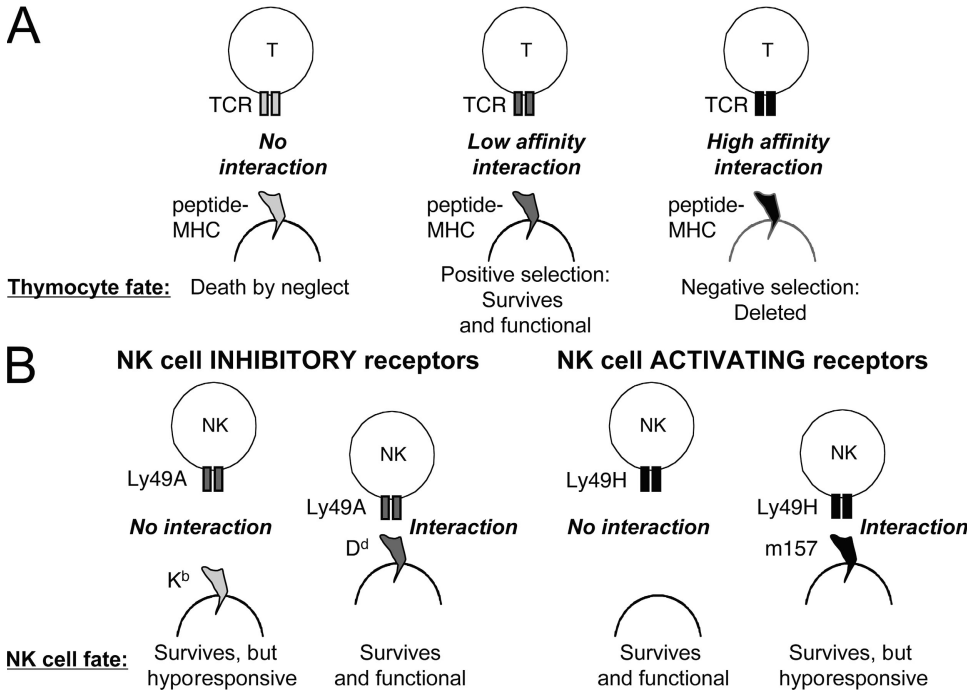

Figure 9. Regulatory events during T and NK cell development. (A) Possible events and outcomes during T cell development in the thymus. Thymocytes unable to interact with peptide-MHC do not survive and undergo death by neglect. Thymocytes interacting with peptide-MHC at low affinity are positively selected and survive to populate the periphery. Thymocytes interacting with peptide-MHC at high affinity will die by negative selection. (B) Possible events and outcomes during NK cell development in the bone marrow. Immature NK cells expressing inhibitory receptors that cannot interact with cognate MHC will survive but are hyporesponsive. Immature NK cells expressing inhibitory receptors are able to interact with cognate MHC and populate the periphery as functional effector cells. Immature NK cells expressing activating receptors do not interact with cognate ligand and will survive to become functional effector cells. Immature NK cells expressing activating receptors encounter cognate ligand and survive but are hyporesponsive.

NK cells were exposed to H-2D^d cells, there was no down-modulation of the Ly49D receptor on these cells (34). Studies of the functional responses of these Ly49D$^+$ NK cells developing in H-2D^d–bearing hosts are complicated by the fact that a majority of Ly49D$^+$ NK cells coexpress inhibitory Ly49 receptors, such as Ly49A, Ly49C, Ly49I, and Ly49G2, that also recognize H-2D^d as a ligand. Moreover, although functional studies indicate that H-2D^d is a ligand for Ly49D (35, 36), there has as yet been no evidence that Ly49D can directly bind to H-2D^d (37). Indeed, we have failed to observe activation of NFAT-GFP reporter cells expressing Ly49D and DAP12 co-cultured with H-2D^d–bearing cells (unpublished data). These results suggest that Ly49D is likely a very low affinity receptor for H-2D^d, much less than the affinity of the inhibitory Ly49 receptors binding to H-2D^d. In contrast, there is no evidence that Ly49H binds to any known H-2 ligand, but it does bind with high affinity to m157 (12). Therefore, in establishing a model system to explore the consequences of engaging an ITAM-signaling Ly49 receptor with a high affinity ligand on NK cell development, Ly49H and m157 provide a much simpler and better-defined receptor–ligand pair to address these questions.

As we are beginning to uncover the events that dictate survival and function in NK cell development, further studies are required to elucidate the precise molecular mechanisms and signals by which NK cells mature and become effector cells in peripheral organs. Understanding the regulatory mechanisms at work during NK cell development that safeguard against peripheral autoimmunity will have important clinical implications.

MATERIALS AND METHODS

Mice. C57BL/6 and congenic (CD45.1) mice were purchased from the National Cancer Institute. *Rag2*$^{-/-}$ × IL-2R common–γ chain$^{-/-}$ B6 mice were purchased from Taconic. B6 DAP12-deficient mice were bred at UCSF. Experiments were performed according to the UCSF Institutional Animal Care and Use Committee guidelines.

Retroviral vectors. m157 was cloned into the MigR1 vector, and virus was generated using the Phoenix ecotropic packaging cell line (38). Cells were incubated for 24–48 h, and analyzed for expression of m157 surface protein by flow cytometry and by culturing overnight with Ly49H-NFAT-GFP reporter cells, as previously described (7). An empty MigR1 vector containing only GFP was used as a control.

Bone marrow chimeric mice. Retrovirus supernatant was added to B6 bone marrow cells (38) with 4 μg/ml of polybrene in 6-well culture dishes and spun at 1,300 *g* for 2 h at 37°C. After centrifugation, cells were incubated overnight and the transduction was repeated the following day. On the fourth day, 10^6 cells were injected into lethally irradiated (1,000 rad) mice maintained on antibiotic water. Rag2$^{-/-}$ × γc$^{-/-}$ or DAP12-deficient B6 mice were used as recipients because they contain no NK cells (39, 40) or express Ly49H$^+$ NK cells that respond inefficiently to MCMV, respectively. Note that a subset of DAP12-deficient NK cells express very low levels of Ly49H on the cell surface (19).

Flow cytometry and intracellular staining. Cells were directly stained with antibodies against m157, NK1.1, DX5, CD3, Ly5.1, Ly49H, Ly49D, NKG2D, CD69, KLRG1, c-Kit, CD43, and Mac-1 (CD11b; all obtained from eBioscience or BD Biosciences). Anti-m157 and anti-Ly49H were provided by W. Yokoyama (Washington University, St. Louis, MO). Intracellular IFN-γ staining (BD Biosciences) was performed after in vitro culture in media containing Brefeldin A for 4 h at 37°C (41). Flow cytometry was performed on an LSRII and data were analyzed with CellQuest software (both from Becton Dickinson).

Ex vivo NK cell stimulation assay. 2 × 10^6 splenocytes were cultured in 24-well plates coated with the antibodies indicated in the figures at 10 μg/ml. Intracellular staining was performed after a 4-h incubation at 37°C in the presence of Brefeldin A. To avoid NK cell activation via CD16, cells were incubated with the neutralizing anti-CD16/32 mAb, 2.4G2, before performing stimulation assays. Because antibodies against NK1.1 were used in some experiments for stimulation, the percentages of IFN-γ–producing NK cells were determined by gating on CD3$^-$, CD11b$^+$, DX5$^+$ cells.

Ex vivo NK cell–mediated cytotoxicity assays. Splenocytes were enriched for NK cells by using an NK cell isolation kit (Miltenyi Biotec) followed by autoMACS magnetic bead separation. These ex vivo NK cells were used as effector cells in a 6-h ^{51}Cr release assay (42) against Ba/F3, m157-transfected Ba/F3 (7), YAC-1, and Rae-1ε–transfected Ba/F3 (43) targets.

Adoptive transfer and MCMV infections. NK cells were purified from the spleens of m157-expressing or control mice by using an NK cell isolation kit followed by autoMACS magnetic bead separation. 5 × 10^5 Ly49H$^+$ NK cells from each group were adoptively transferred into DAP12-deficient recipient mice. The following day, 5 × 10^4 PFU of a salivary gland stock of MCMV (Smith strain) was injected i.p.

Statistical analysis. Statistical differences in percentage and absolute number of Ly49H$^+$ NK cells and MFI of Ly49H expression between control and m157-expressing mice were determined by using the two-tailed unpaired Student's *t* test.

Online supplemental material. Fig. S1 shows specific Ly49H$^+$ and Ly49D$^+$ NK cell subsets in the peripheral blood and spleens of control and m157-expressing mice. Fig. S2 shows the expression of activating and inhibitory NK cell receptors in control and m157-expressing mice. Fig. S3 shows IFN-γ production by NK cells from control and m157-expressing mice in the presence of anti-Ly49H mAbs and IL-2. Fig. S4 shows the early activation phenotype of Ly49H$^+$ NK cells from control and m157-expressing mice after MCMV infection. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20072448/DC1.

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