Continuous engagement of a self-specific activation receptor induces NK cell tolerance

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Natural killer (NK) cell tolerance mechanisms are incompletely understood. One possibility is that they possess self-specific activation receptors that result in hyporesponsiveness unless modulated by self–major histocompatability complex (MHC)–specific inhibitory receptors. As putative self-specific activation receptors have not been well characterized, we studied a transgenic C57BL/6 mouse that ubiquitously expresses m157 (m157-Tg), which is the murine cytomegalovirus (MCMV)–encoded ligand for the Ly49H NK cell activation receptor. The transgenic mice were more susceptible to MCMV infection and were unable to reject m157-Tg bone marrow, suggesting defects in Ly49H$^+$ NK cells. There was a reversible hyporesponsiveness of Ly49H$^+$ NK cells that extended to Ly49H-independent stimuli. Continuous Ly49H–m157 interaction was necessary for the functional defects. Interestingly, functional defects occurred when mature wild-type NK cells were adoptively transferred to m157-Tg mice, suggesting that mature NK cells may acquire hyporesponsiveness. Importantly, NK cell tolerance caused by Ly49H–m157 interaction was similar in NK cells regardless of expression of Ly49C, an inhibitory receptor specific for a self-MHC allele in C57BL/6 mice. Thus, engagement of self-specific activation receptors in vivo induces an NK cell tolerance effect that is not affected by self-MHC–specific inhibitory receptors.

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Abbreviations used: LAK, lymphokine-activated killer; m157-Tg, m157 transgenic; MCMV, murine cytomegalovirus; poly I:C, polyinosinic:polycytidylic acid.

NK cell activation receptors play important roles in both viral infection and tumor surveillance. One such activation receptor, Ly49H, accounts for genetic resistance to murine cytomegalovirus (MCMV) (1–3). Coupled to the DAP12 signaling chain, Ly49H is expressed on a subset of NK cells in C57BL/6 mice (4–6) and recognizes the MCMV-encoded m157 molecule, a glycophosphatidylinositol-linked protein (7–12). To date, m157 is the only known ligand for the Ly49H receptor, and in C57BL/6 mice only Ly49H interacts with m157.

Activated NK cells release proinflammatory cytokines and cytolytic granules that can ultimately destroy the susceptible target cell (13). These potent effector responses must be controlled, as NK cells must recognize and distinguish the abnormal cells that they destroy from normal self-tissues that are spared (14, 15). One mechanism to prevent aberrant NK cell activation involves inhibitory receptors specific for MHC class I molecules on target cells. Engagement of these inhibitory receptors delivers negative signals, explaining the "missing self" hypothesis (16). In general, the integration of both positive and negative stimuli determines if an NK cell becomes activated during effector responses.

The MHC class I–specific inhibitory receptors also appear to have another function (17). Engagement of inhibitory Ly49 receptors by their cognate MHC class I ligands, expressed as self-MHC, allows NK cells to acquire functional competence to be triggered through their activation receptors. In this process, termed licensing, the self-MHC class I–specific inhibitory receptors appear to signal the licensing event because their cytoplasmic domains, specifically the immunoreceptor tyrosine-based inhibitory motif, are required (17). Though further understanding of its mechanism is required, most groups now agree that the engagement of inhibitory receptors by

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self-MHC class I plays an important role in acquisition of NK cell function (14, 15, 18).

At least two major models have been proposed to account for the role of the self-MHC class I–specific receptors in licensing (14, 15). One model ("arming" or "stimulatory" model) suggests that the inhibitory receptor directly signals the licensing event. Another model ("disarming" or "inhibitory" model) suggests that, when unimpeded, a self-specific activation receptor results in a "hyporesponsive" state, akin to peripheral T cell anergy. The self-MHC class I–specific inhibitory receptor is postulated to modulate the function of the activation receptor and thereby allows licensing to occur. Current data do not allow conclusive discrimination between these models (14, 15). To more fully understand the potential contribution of self-specific NK cell activation receptors to NK cell tolerance and to begin testing the disarming (inhibitory receptor) model for licensing, we borrowed a strategy that was critical for detailed characterization of B and T cell tolerance, i.e., the generation of transgenic mice expressing ligands for the BCR and TCR, respectively. For example, a foreign antigen (hen egg lysozyme) expressed in this context is perceived by the immune system as "self," allowing further elucidation of B cell self-tolerance mechanisms (19). This transgenic strategy was required because there are few other NK cell activation receptors where the ligands are unequivocally defined and are not present in normal mice. Herein, we generated mice on the C57BL/6 background that ubiquitously expresses m157. These mice displayed a defect in NK cell function that is manifested in vivo by increased susceptibility to MCMV infection, as well as an inability to reject m157-transgenic (m157-Tg) BM cell transplants. The data indicate that the persistent engagement of self-activation receptors influences the functional status of the mature NK cell.

RESULTS

Production of m157 transgenic mice

We generated a transgenic construct for expression of m157 under the control of the human β-actin promoter (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072446/DC1). The construct was directly injected into C57BL/6-derived fertilized ova. Transgenic founders were identified by PCR genotyping, as was the cell surface expression of m157 on peripheral blood lymphocytes and BM cells. The m157-Tg mice were phenotypically normal and healthy. Their growth and development were similar to their nontransgenic (WT) littermates. As compared with WT mice, they had similar numbers of splenocytes and there were no significant differences in the percentages of B or T cells (Fig. S2 and not depicted). Thus, even though the m157-Tg mice expressed the ligand for the Ly49H activation receptor, they did not display any overt signs of autoimmunity.

m157-Tg mice are defective in MCMV control and BM rejection

Because the Ly49H receptor plays a vital role in susceptibility to MCMV infection, we assessed responses to MCMV infection in vivo. After injection of 1.5 × 10^5 PFU MCMV, <50% of the m157-Tg mice survived, with deaths beginning around the time of onset of lethality in genetically susceptible BALB/c mice (Fig. 1 A). In contrast, all of the WT C57BL/6 littermates survived. In addition, infected m157-Tg mice displayed significantly higher splenic MCMV titers at 3 d after infection compared with WT littermates (Fig. 1 B). The m157-Tg mice had splenic viral titers comparable to susceptible BALB/c mice. To determine if mature m157-Tg splenocytes were defective in controlling MCMV outside the context of an m157-Tg mouse, we performed adoptive transfer experiments in neonatal mice whereby transferred splenocytes provide NK cell–dependent resistance to MCMV infections (20). However, neonatal mice were not protected by adoptively transferred m157-Tg splenocytes, similar to PBS-treated controls, whereas WT splenocytes provided a significant increase in survival (Fig. 1 C). Collectively, these data indicate that m157-Tg mice behave similarly to BALB/c mice lacking *Ly49h*, and m157-Tg splenocytes are less efficient than their WT counterparts in controlling MCMV infection, suggesting a defect in m157-Tg Ly49H$^+$ NK cells.

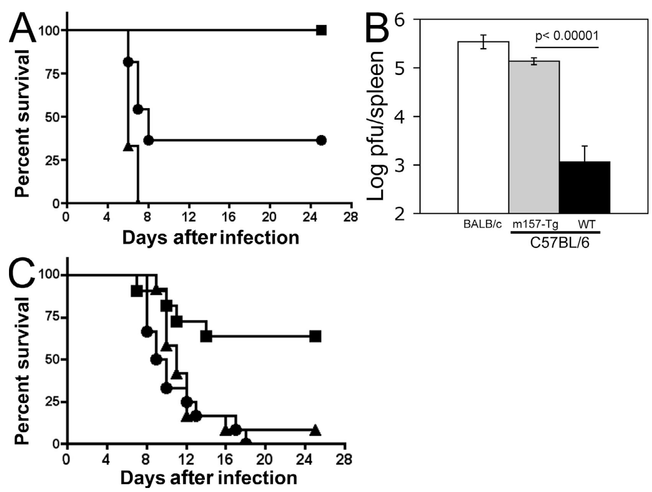

Figure 1. Increased mortality and splenic titers in m157-Tg mice infected with MCMV. (A) Survival curve after MCMV infection. BALB/c mice (▲, 3 per group), m157-Tg (●, 11 per group), or WT littermates (■, 11 per group) were injected i.p. with 1.5 × 10^5 PFU MCMV (Smith strain) and were followed for 30 d after infection. The percentage of mice surviving within each group was determined daily. (B) Splenic titers after MCMV infection. BALB/c (white, *n* = 9), m157-Tg (gray, *n* = 8), and WT littermates (black, *n* = 8) were injected with 2 × 10^4 PFU MCMV Smith strain. The spleens were harvested on day three after infection, and viral titers were determined with a standard viral plaque assay. The results are presented as Log PFU/spleen (mean ± SEM). For one mouse in the WT group with a titer below level of detection of this assay, the minimum number of detectable PFU (10^2) was assumed to determine the mean. P value determined using a two-tailed Student's *t* test. (C) WT, but not m157-Tg, splenocytes protect neonatal mice from MCMV infection. Neonatal mice were injected i.p. with PBS (▲, *n* = 12), 5 × 10^7 WT splenocytes (■, *n* = 11), or 5 × 10^7 m157-Tg splenocytes (●, *n* = 12). 24 h later, the mice were injected i.p. with 870–1,000 PFU MCMV. The percentage of mice surviving within each group was determined daily. These data are a combination of two separate experiments, each of which showed similar results.

The presence of normal hematopoiesis in the m157-Tg mice suggested that the m157-Tg BM either did not express m157 or the Ly49H^{+} NK cells were tolerized in the m157-Tg mice. The observation that m157 was detected on the surface of BM cells derived from m157-Tg mice (Fig. S1) suggests that Ly49H^{+} NK cells in m157-Tg mice were tolerized. To further evaluate this possibility, we performed BM transplantation experiments. It has been previously demonstrated that irradiation-resistant NK cells can reject MHC-deficient BM grafts (21, 22) or BM-expressing ligands for an activation receptor such as NKG2D (23). Similar to these results, we found that the engraftment of m157-Tg BM was lower than WT BM in WT mice, at every BM dose (Fig. 2 A). Although engraftment of m157-Tg BM was never as low as $\beta_2m^{-/-}$ BM, rejection by WT mice was prevented by depletion of NK cells with the anti-NK1.1 mAb or administration of the nondepleting anti-Ly49H mAb (Fig. 2 B). Therefore, these experiments demonstrate that WT Ly49H^{+} NK cells recognize m157 on m157-Tg BM cells, and that WT rejection of m157-Tg BM was NK cell dependent, and more specifically, Ly49H dependent.

More importantly, the preceding experiments also suggested that m157-Tg mice might be tolerant to m157 because of normal hematopoiesis in the m157-Tg mice. To study this further, lethally irradiated m157-Tg mice were injected with 2×10^6 BM cells from m157-Tg mice. The m157-Tg mice, unlike WT mice, failed to reject BM cells from m157-Tg donors (Fig. 2 B). Of note, the m157-Tg mice were still able to reject $\beta_2m^{-/-}$ BM (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072446/DC1). This is likely explained by a role for

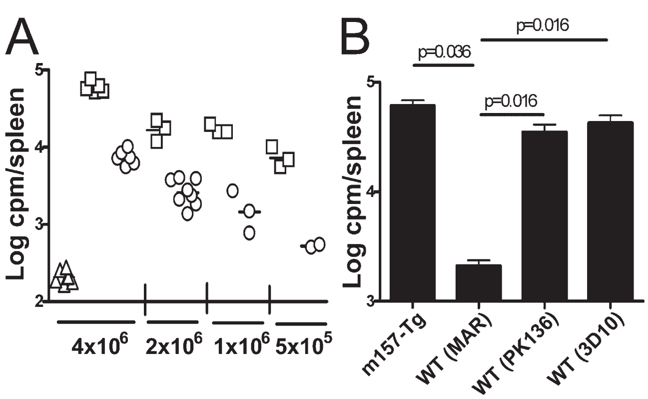

Figure 2. WT, but not m157-Tg, mice reject m157-Tg BM transplants. (A) WT C57BL/6 recipient mice were lethally irradiated, and then injected i.v. with the indicated number of donor WT (□), m157-Tg (○), or $\beta_2m^{-/-}$ (△) donor BM cells. ^{125}I-deoxyuridine uptake was measured in the spleen as an index of engraftment. Each point represents an individual mouse. The line represents the mean of the log counts per minute (cpm)/spleen of the group. (B) m157-Tg (n = 3) or WT recipient mice were lethally irradiated and then injected i.v. with 2×10^6 m157-Tg donor BM cells. WT mice were pretreated 2 d before BM transplant with 200 μg i.p. of anti-NK1.1 mAb (PK136; n = 4), anti-Ly49H mAb (3D10; n = 4), or control mouse anti-rat mAb (MAR; n = 5). Fig. 2 B represents one of two similar experiments. P values were determined using Mann-Whitney test. There was no statistical difference between m157-Tg recipients and WT recipient mice pretreated with either anti-NK1.1 mAb or anti-Ly49H mAb.

Ly49H^{-} NK cells (which do not interact with m157) that probably participate in the rejection of $\beta_2m^{-/-}$ BM. Taken together with the MCMV experiments, these data indicate a defect in the response of m157-Tg Ly49H^{+} NK cells to m157-expressing targets (either on MCMV-infected cells or on m157-Tg BM).

Down-regulation of Ly49H in vivo

There are many potential explanations for the apparent dysfunction of the Ly49H^{+} NK cells in the m157-Tg mice. To investigate these defects further, we analyzed the expression of Ly49H on the surface of peripheral blood and splenic NK cells in numerous founders. In all m157-Tg mice examined, there was a slight decrease in the measured percentage and number of Ly49H^{+} NK cells (Fig. 3 and Fig. S2). This was specific to the Ly49H receptor because the expression of other

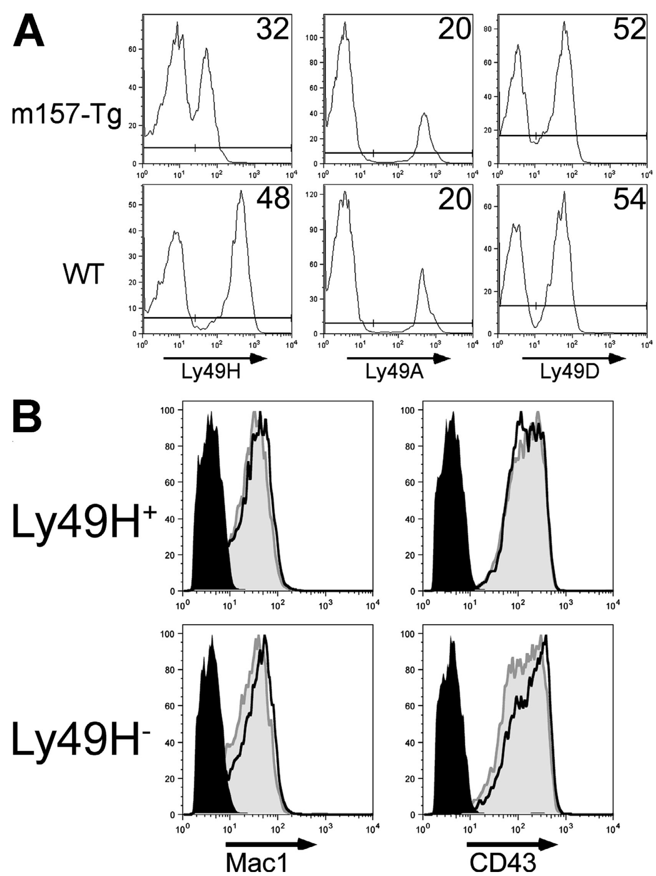

Figure 3. Decreased Ly49H expression on m157-Tg NK cells. (A) Flow cytometry of splenic NK cells from m157-Tg or WT littermates. Representative histograms demonstrating staining of NK cells from either m157-Tg or WT mice for Ly49H, Ly49A, or Ly49D, as indicated. The numbers in the histograms represent the percentage of positive-staining cells. (B) Representative histograms depicting Mac1 or CD43 staining of either Ly49H^{+} or Ly49H^{-} NK cells from m157-Tg (open histogram) and WT (shaded) mice. Negative control staining is shown in black histograms. Approximately 85–90% of cells stained positive for Mac1, and 99% were positive for CD43 in both m157-Tg and WT mice. Histograms in Fig. 3 (A and B) were gated on NK1.1^{+}, CD3^{-}, CD19^{-} cells. Histograms in Fig. 3 B were generated on Ly49H^{+} or Ly49H^{-} gates, as indicated.

Ly49 receptors (Ly49D, Ly49A, Ly49C, Ly49G, and Ly49I) and NKG2D were unchanged (Fig. 3 A and not depicted). As the total number of NK cells was only modestly changed, these data suggest that the majority of Ly49H+ NK cells were not deleted (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20072446/DC1). Comparable effects on Ly49H expression were seen in the BM (Fig. S5). A similar down-regulation of Ly49H expression was previously observed on WT Ly49H+ NK cells responding to m157-expressing targets in vitro (8–10), suggesting that most, if not all, Ly49H+ NK cells engaged m157 in the m157-Tg mice.

To determine if the apparent recognition of m157 resulted in a maturation defect in the Ly49H+ NK cell subset, we assessed various markers of development and activation. Comparable to WT NK cells, splenic Ly49H+ NK cells from the m157-Tg mice expressed both Mac1 and CD43, markers associated with mature, functional NK cells (Fig. 3 B). In addition, comparable levels of c-Kit (CD117) were expressed on Ly49H+ BM NK cells from m157-Tg and WT mice (Fig. S5). Moreover, they did not express the CD69 activation marker (not depicted). Thus, even though decreased cell surface expression of Ly49H implied engagement with m157, the NK cells appeared to be mature and did not appear to be activated, which is consistent with an "anergic" or hyporesponsive phenotype.

Functional defects of Ly49H+ NK cells from m157-Tg mice

To further explore the hyporesponsive phenotype, we studied the function of Ly49H+ NK cells from m157-Tg mice in vitro. Upon exposure to m157-expressing targets, m157-Tg Ly49H+ NK cells did not produce IFN-γ, whereas WT Ly49H+ NK cells responded (Fig. 4 A). Moreover, activated WT Ly49H+ NK cells showed decreased levels of Ly49H, as previously observed (8–10). Interestingly, Ly49H levels on m157-Tg NK cells did not change further after stimulation; their levels on freshly stimulated isolated NK cells were already similar to the levels on WT Ly49H+ NK cells activated by m157 targets.

Surprisingly, m157-Tg Ly49H+ NK cells were also defective when exposed to Ly49H-independent stimuli. Ly49H+ NK cells from the m157-Tg mice did not produce IFN-γ when stimulated by plate-bound anti-NK1.1 (Fig. 4 A). In contrast, Ly49H− NK cells from both m157-Tg and WT littermates produced similar levels of IFN-γ after anti-NK1.1 stimulation. Generally, WT Ly49H+ NK cells were somewhat better responders to anti-NK1.1 than WT Ly49H− NK cells, an observation that was not previously reported in WT mice, but may be related to the propensity of NK cells that express one NK cell activation receptor to express another simultaneously (6). To explore this further in terms of the m157-Tg mice, we compared the ratio of IFN-γ production by Ly49H+ NK cells versus Ly49H− NK cells (Fig. 4 B). When splenocytes were stimulated with cells stably transfected with m157 (RMAs-m157) or by anti-NK1.1, the ratio was significantly lower for m157-Tg NK cells than for WT cells. Moreover, similar results were seen with Chinese hamster ovary and YAC targets. Though the defect was not as severe, Ly49H+ NK cells from m157-Tg mice also produced less IFN-γ than Ly49H+ cells from WT mice upon stimulation with IL-12 and -18 (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20072446/DC1). These findings are consistent with a global defect in Ly49H+ NK cell function with these stimuli.

On the other hand, there was no difference in the ratio when splenocytes were stimulated with RMA cells (Fig. 4 B) and treatment of splenocytes with PMA and ionomycin resulted in the production of IFN-γ by the Ly49H+ NK cells in the m157-Tg mouse (Fig. 4 A), indicating that the NK cells were not "exhausted" from the apparent constant stimulation through Ly49H.

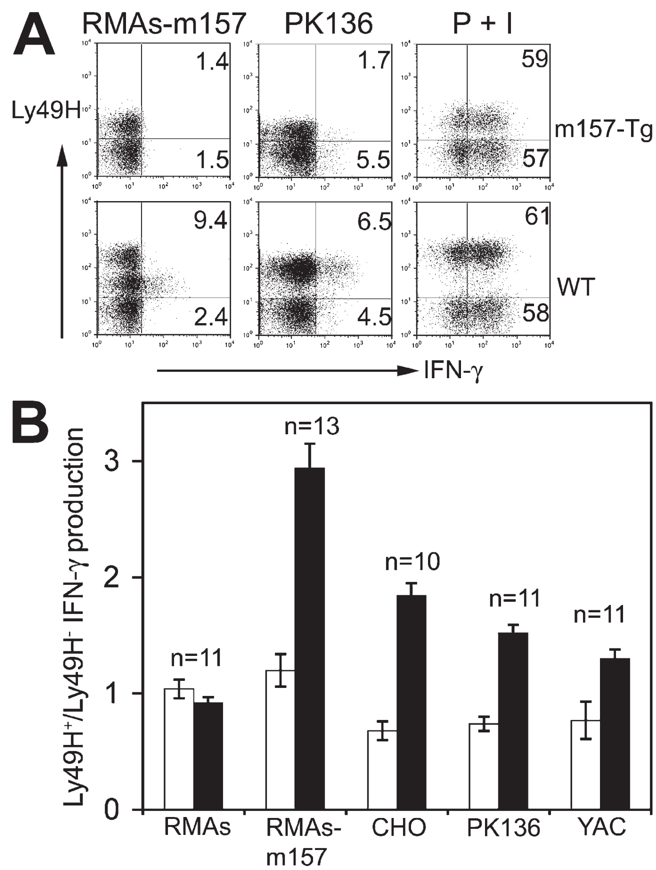

Figure 4. Decreased IFN-γ production by Ly49H+ NK cells in the m157-Tg mice. (A) Representative dot plots demonstrating IFN-γ production by freshly isolated NK cells stimulated with either RMAs-m157 cells, plate-bound anti-NK1.1 mAb (PK136), or PMA and ionomycin (P+I). The numbers represent the percentage of Ly49H+ or Ly49H− NK cells producing IFN-γ. The dot plots were gated on NK1.1+, CD3−, CD19− cells. (B) The ratio of the percentage of IFN-γ–producing Ly49H+ NK cells to the percentage of IFN-γ–producing Ly49H− NK cells in m157-Tg mice (open bars) and WT mice (shaded bars). The graph represents the mean ± the SEM. Ratios were calculated after stimulation with RMAs cells, RMAs-m157 cells, Chinese hamster ovary cells, plate-bound anti-NK1.1 (PK136), and YAC cells. The number of mice used in each group is displayed. The difference in ratio between WT and m157-Tg mice was significant ($P < 0.05$) for all stimulation groups except RMAs cells. P values determined using a two-tailed Student's *t* test.

To determine if the apparent hyporesponsiveness could be overcome by in vivo polyclonal NK cell stimulation, mice were preinjected with polyinosinic:polycytidylic acid (poly I:C), a known inducer of type I IFNs, 24 h before in vitro stimulation. Ly49H$^+$ NK cells from the m157-Tg mice, in contrast to Ly49H$^+$ NK cells from WT mice, remained unable to produce IFN-γ (regardless of Ly49H-dependent or Ly49H-independent stimulation; Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20072446/DC1). Collectively, these results indicate that Ly49H$^+$ NK cells are globally hyporesponsive when derived from mice expressing their m157 ligand as a self-protein.

Functional defect of lymphokine-activated killer (LAK) cells from m157-Tg mice

Although poly I:C treatment did not overcome the hyporesponsiveness of the m157-Tg NK cells, we further evaluated their function by analysis of LAK cells grown in vitro with high doses of IL-2. Of note, by day four in culture, Ly49H levels on the LAK cells from m157-Tg were comparable to those on LAK cells from WT mice (Fig. S4). Also, the difference in the percentage of Ly49H$^+$ NK cells in the m157-Tg versus the WT LAK populations was minimal (Fig. S4). These data also suggest that the Ly49H$^+$ NK cells were not deleted in the m157-Tg mice.

Despite the normalization of Ly49H expression and percentage of Ly49H$^+$ cells, the bulk LAK cell population from m157-Tg mice was not able to kill m157-expressing targets (Fig. 5). YAC cells (Fig. 5), as well as BaF-Rae-1ε and OP9 cells (not depicted), were killed to a similar extent by both m157-Tg and WT LAK cells, suggesting there was no defect in Ly49H-independent killing. To more precisely study this problem, we used sorted LAK cells. Sorted Ly49H$^+$ LAK cells from m157-Tg mice were unable to kill the m157-expressing targets (Fig. 5 B). This was likely not caused by blockade of the Ly49H receptor by the anti-Ly49H mAb used for sorting because we washed and incubated the cells for 2 d after sorting. In addition, sorted WT Ly49H$^+$ LAK cells were able to specifically kill the m157 transfectants. Sorted Ly49H$^+$ LAK cells from both m157-Tg and WT mice were comparable in their ability to kill other targets through Ly49H-independent mechanisms. These data indicate that high doses of IL-2 could overcome the hyporesponsiveness to Ly49H-independent stimuli, but not the hyporesponsiveness to Ly49H-dependent stimuli.

Trans interaction is important for down-regulation of Ly49H and Ly49H function

Given that the human β-actin promoter used to generate the m157-Tg mouse should result in ubiquitous expression, m157 is also expressed on NK cells themselves (unpublished data). This expression could result in the interaction of Ly49H and m157 on or in the NK cell itself (cis interaction) and give rise to down-regulation of Ly49H expression and other functional effects. To determine if this was the case, we transferred WT BM from B6.SJL-*Ptprca* (Ly5.1) mice into irradiated Ly5.2 mice that were either WT or m157-Tg. Down-regulation of Ly49H was observed on WT NK cells when WT (Ly5.1) BM was transferred into m157-Tg mice, but not when WT BM was transferred into WT mice (Fig. 6 A). The Ly49H$^+$ NK cells obtained from WT→m157-Tg BM chimeras were defective in IFN-γ production upon stimulation with plate-bound

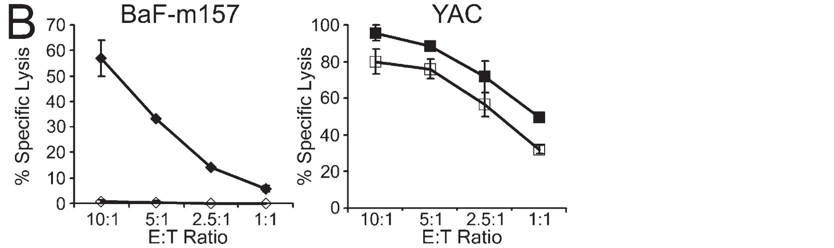

Figure 5. NK cells from m157-Tg mice kill m157-expressing targets less efficiently. (A) A standard 4-h ^{51}Cr-release assay was performed with LAK cells from m157-Tg (open symbols) or WT littermates (closed symbols). LAK cells were incubated with the indicated targets at various effector-to-target (E:T) ratios. In BaF-m157 and RMAs-m157 graphs, both m157-expressing targets (diamonds) and nonexpressing parental lines (squares) are shown. The data are presented as the mean ± the SEM for BaF and BaF-m157 targets ($n \geq 12$ mice), RMAs and RMAs-m157 targets ($n \geq 2$ mice), and YAC targets ($n \geq 14$ mice). (B) A standard 4-h ^{51}Cr-release assay was performed with Ly49H-sorted LAK cells from m157-Tg or WT mice. The sorted Ly49H$^+$ LAK cells from WT mice (closed symbol) and m157-Tg mice (open symbol) were incubated with the indicated targets at various E:T ratios. The data are presented as the mean ± the SEM for four mice in two separate experiments.

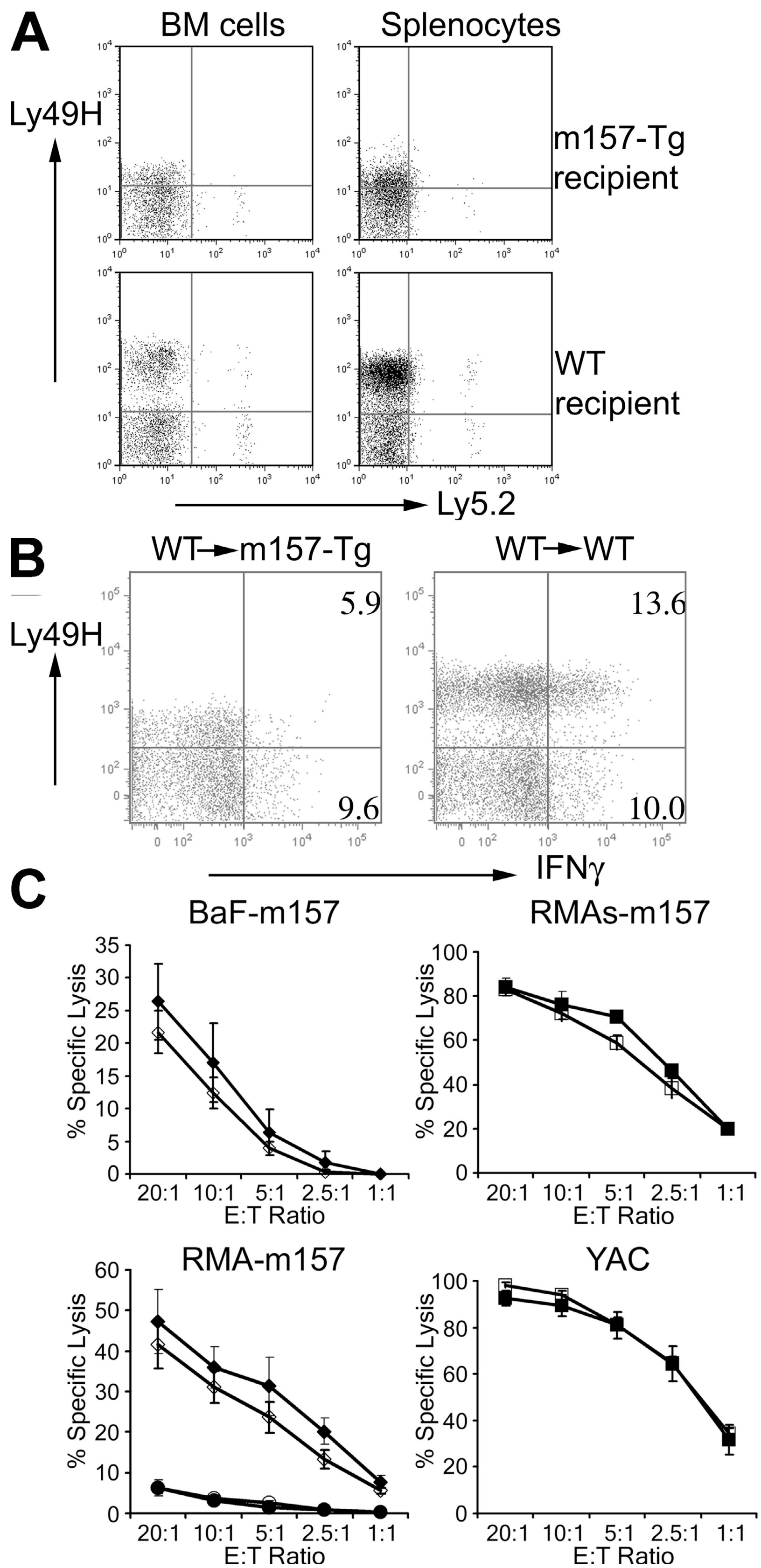

Figure 6. Trans interactions play an important role in the m157-Tg phenotypes. (A) A dot plot of Ly49H and Ly5.2 expression on BM cells and splenocytes from chimeric mice. The chimeric mice were generated by injecting WT donor BM (Ly5.1) into lethally irradiated m157-Tg or WT (Ly5.2) recipients.

anti-NK1.1 mAb as compared with Ly49H$^+$ NK cells from WT→WT BM chimeras (Fig. 6 B). In contrast, comparable IFN-γ production was seen in the Ly49H$^-$ NK cell populations in both BM chimeras. Thus, a trans interaction between m157 and Ly49H is sufficient to alter Ly49H receptor surface expression and function.

Furthermore, transfer of mature WT spleen cells (Ly5.1) into m157-Tg mice (Ly5.2) resulted in a down-modulation of Ly49H on the donor NK cells when examined 9 d after transfer (Fig. 7). Moreover, there was a defect in IFN-γ production by the Ly49H$^+$ donor NK cells upon stimulation with plate-bound anti-NK1.1. These data suggest that persistent engagement of Ly49H on mature NK cells is sufficient to recapitulate the functional defect of m157-Tg NK cells.

Continuous m157–Ly49H engagement is necessary for Ly49H dysfunction

To determine if functional defects persisted in vitro in the Ly49H$^+$ NK cells from the chimeric mice, we generated LAK cells. Unlike LAK cells from m157-Tg mice, LAK cells generated from WT→m157-Tg chimeric mice were able to kill m157-expressing targets (Fig. 6 C). This capacity was comparable to WT→WT chimeric mice. Both populations also killed YAC cells (Ly49H-independent mechanism), indicating that absence of Ly49H–m157 engagement during in vitro culture with cytokines can reverse the hyporesponsive phenotype (as compared with Fig. 5). These data demonstrate that the functional defect seen in the Ly49H$^+$ NK cells from the m157-Tg mice is not fixed, and suggests that hyporesponsiveness requires continuous engagement of the activation receptor.

Licensing does not reverse Ly49H$^+$ NK cell hyporesponsiveness in m157-Tg mice

To address if the licensing process had any effect on m157-Tg Ly49H$^+$ NK cell hyporesponsiveness, we assessed the function of cells expressing Ly49C that is specific for a self-MHC class I allele (H2K^b) in C57BL/6 mice. In WT mice, regardless of whether the freshly isolated NK cells were Ly49H$^+$ or Ly49H$^-$, anti-NK1.1 induced a higher percentage of the Ly49C$^+$ population to produce IFN-γ than the Ly49C$^-$ population (Fig. 8 A), which is consistent with previous results (17). For m157-Tg cells, the Ly49H$^-$ subset behaved similarly to the Ly49H$^-$ subset in WT mice, with a higher percentage of the Ly49C$^+$ population producing IFN-γ. However, the Ly49H$^+$ subset in the m157-Tg mice was hyporesponsive to anti-NK1.1, regardless of Ly49C expression (Fig. 8 A). Similar results were seen when WT→m157-Tg BM chimeras were analyzed, suggesting that trans interactions between Ly49H and m157 were sufficient for these observations (Fig. 8 B). Finally, chimeras made from WT→WT mice behaved like WT mice (Fig. 8 B). Collectively, these data demonstrate that licensing cannot overcome the functional hyporesponsiveness resulting from trans engagement of Ly49H with m157.

DISCUSSION

In this study, we provide evidence that the engagement of the NK cell–specific Ly49H activation receptor with its ligand, which is caused by transgenic expression of m157, results in down-regulation of Ly49H receptor expression and "hyporesponsiveness" in Ly49H$^+$ NK cell function. Similar results were observed by Sun and Lanier using retroviral-transduced expression of m157 in BM stem cells (see Sun and Lanier [24] on p. 1819 of this issue). Any differences between our results and theirs could be related to the means by which m157 was expressed. In the studies described here, the defect in Ly49H function was reversible and independent of the level of Ly49H expressed and the percentage of Ly49H$^+$ NK cells in the m157-Tg mice.

The hyporesponsiveness in Ly49H$^+$ NK cell function was of two types, with one specifically involving only the Ly49H pathway. The second extended beyond stimulation through the Ly49H receptor because we also observed global defects in signaling by other activation receptors that do not signal through Ly49H and DAP12, and use other signaling chains, as well as responses to cytokines, such as IL-12 and -18. Moreover, stimuli that bypass proximal activation signals could equally stimulate normal and hyporesponsive cells, suggesting that the signaling defect is distal to the signaling chains, but upstream of signals mimicked by PMA and ionomycin. It is possible that continuous engagement of Ly49H with m157 results in negative feedback that leads to the down-regulation of not only Ly49H but also downstream signaling molecules (e.g., protein tyrosine kinases) that are shared by multiple activation pathways. Alternatively, continuous engagement of Ly49H with m157 could result in the sequestration of downstream signaling molecules that are shared by multiple activation receptor pathways. In this manner, NK cells could be rendered hyporesponsive to multiple activation pathways. Clearly, future studies will need to be performed to identify the mechanisms of global hyporesponsiveness. Regardless of the mechanism, these data, taken together with data from Sun and Lanier and others (24–27), lend strong support to the hypothesis that an activation receptor with specificity for a self-ligand can

The dot plot was gated on NK1.1$^+$, CD3$^-$, CD19$^-$ cells. Note that almost all NK cells were of donor origin (Ly5.2$^-$). (B) IFN-γ production by chimeric NK cells after stimulation with plate-bound anti-NK1.1 mAb. Chimeric mice were generated as described in Fig. 6 A. The numbers represent the percentage of Ly49H$^+$ or Ly49H$^-$ NK cells producing IFN-γ. The dot plot was gated on NK1.1$^+$, CD3$^-$, CD19$^-$ cells. (C) A standard 4-h ^{51}Cr-release assay was performed with LAK cells from WT→m157-Tg (open symbols) or WT→WT (closed symbols) chimeric mice. LAK cells were incubated with the indicated targets at various E:T ratios. In the RMA-m157 graph, both m157-expressing targets (diamonds) and nonexpressing parental lines (circles) are shown. The data are presented as the mean ± the SEM for BaF-m157 and YAC targets (n = 6–8 mice) and for RMA-m157 and RMAs-m157 targets (n = 4–5 mice).

confer a generalized state of hyporesponsiveness to the NK cell, as previously hypothesized (15).

Interestingly, our data suggest that hyporesponsiveness can occur when mature Ly49H$^+$ NK cells are exposed to m157. Even when they developed in a ligandless environment, WT Ly49H$^+$ NK cells became hyporesponsive when transferred into the m157-Tg mice. We examined NK cells 9 d after transfer when the cells had down-regulated Ly49H expression, but were no longer making IFN-γ at baseline, suggesting that they specifically became hyporesponsive by engagement of Ly49H, and that maturation in the m157-Tg environment was not necessary to demonstrate the hyporesponsive phenotype. This surprising result requires additional examination because it also suggests that WT NK cells may show a state of hyporesponsiveness after activation receptor triggering during a normal innate immune response.

In the m157-Tg NK cells, the global functional hyporesponsiveness had in vivo consequences because MCMV control and m157-Tg BM rejection were affected. Although it is possible that responses of m157-Tg mice after MCMV infection are partially a result of the decreased Ly49H expression on NK cells, hyporesponsiveness of the Ly49H$^+$ NK cells likely also plays a major role. Our in vitro data corroborate our in vivo findings, indicating an NK cell functional impairment, as we demonstrate a defect in the Ly49H$^+$ NK cells to produce IFN-γ in response to Ly49H-mediated as well as non-Ly49H–mediated stimuli. Furthermore, LAK cells from m157-Tg mice were defective in killing m157-expressing targets, even when normalized for the number of Ly49H$^+$ NK cells.

Of note, the global effects of Ly49H engagement could be overcome by cytokines in certain situations (high levels of IL-2 in vitro), but not in others, such as poly I:C administration. The inability to reverse the global hyporesponsiveness of Ly49H$^+$ NK cells with poly I:C is relevant to MCMV infections because poly I:C induces Toll-like receptor stimulation of dendritic cells, resulting in type I IFN release and concomitant NK cell stimulation (28). A similar pathway is activated in MCMV infections and is required for NK cell control of MCMV, even when Ly49H is present (29, 30). Thus, hyporesponsiveness through an activation receptor may not be overcome by certain proinflammatory cytokines, accounting for the persistent defect in MCMV clearance in the m157-Tg mice despite host production of cytokines during infection.

Functional hyporesponsiveness appeared to require continued receptor–ligand interaction. Persistent in vivo interaction in trans was sufficient for global Ly49H$^+$ NK cell hyporesponsiveness, as revealed by our BM chimeric mouse experiments and adoptive transfer of mature WT NK cells. Interestingly, hyporesponsiveness caused by trans effects was reversed during in vitro culture with IL-2. For example, if the NK cells did not express m157, such as NK cells from WT→m157-Tg BM chimeric mice, then in vitro culture in IL-2 reversed hyporesponsiveness. On the other hand, selective hyporesponsiveness of the Ly49H pathway persisted even in the presence of high doses of IL-2 if we used m157-Tg NK cells in which receptor–ligand interactions likely continued via cis effects or trans interactions with neighboring cells. The putative cis effects may be another example by which such interactions could affect NK cell receptor function as recently described (17, 31) and reviewed (32). In either case, these persistent effects on Ly49H function did not appear to be caused by lowered levels of Ly49H expression because nearly equal levels of Ly49H were seen when WT and m157-Tg NK cells were

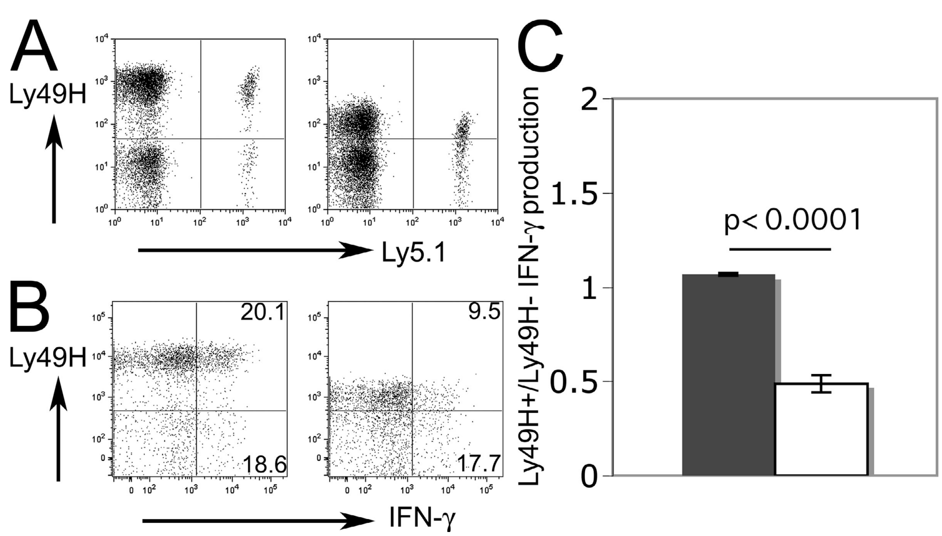

Figure 7. Mature donor WT Ly49H$^+$ NK cells display defective phenotype upon transfer into m157-Tg mice. (A) A dot plot of Ly49H and Ly5.1 expression on NK cells from adoptively transferred mice. The mice were generated by injecting WT donor splenocytes (Ly5.1) into WT (Ly5.2) or m157-Tg (Ly5.2) recipients. The dot plot was gated on NK1.1$^+$, CD3$^-$ cells. (B) IFN-γ production by donor NK cells after stimulation with plate-bound anti-NK1.1. The numbers represent the percentage of Ly49H$^+$ or Ly49H$^-$ cells producing IFN-γ. The dot plot was gated on NK1.1$^+$, CD3$^-$, CD19$^-$, Ly5.1$^+$ (donor) NK cells. (C) The ratio of the percentage of IFN-γ–producing Ly49H$^+$ NK cells to the percentage of IFN-γ–producing Ly49H$^-$ NK cells from WT (filled, *n* = 3) or m157-Tg (open, *n* = 5) mice after stimulation with plate-bound anti-NK1.1. The results are presented as the mean ± the SEM.

cultured in vitro. Collectively, these data indicate that the NK cell hyporesponsive state can be manifested by the presence of the ligand in trans and potentially in cis, and may be affected by the cytokine milieu, but persistent interactions between activation receptor and its ligand may result in permanent hyporesponsiveness of the given activation receptor pathway.

There are some similarities, but also notable differences between our current study and previous investigations of mice with transgenic expression of ligands for another NK cell activation receptor, NKG2D (26, 27). In transgenic mice constitutively expressing Rae-1ε or MICA, down-regulation of NKG2D and generalized defects in NK cell function were observed. Moreover, trans effects on NKG2D were noted

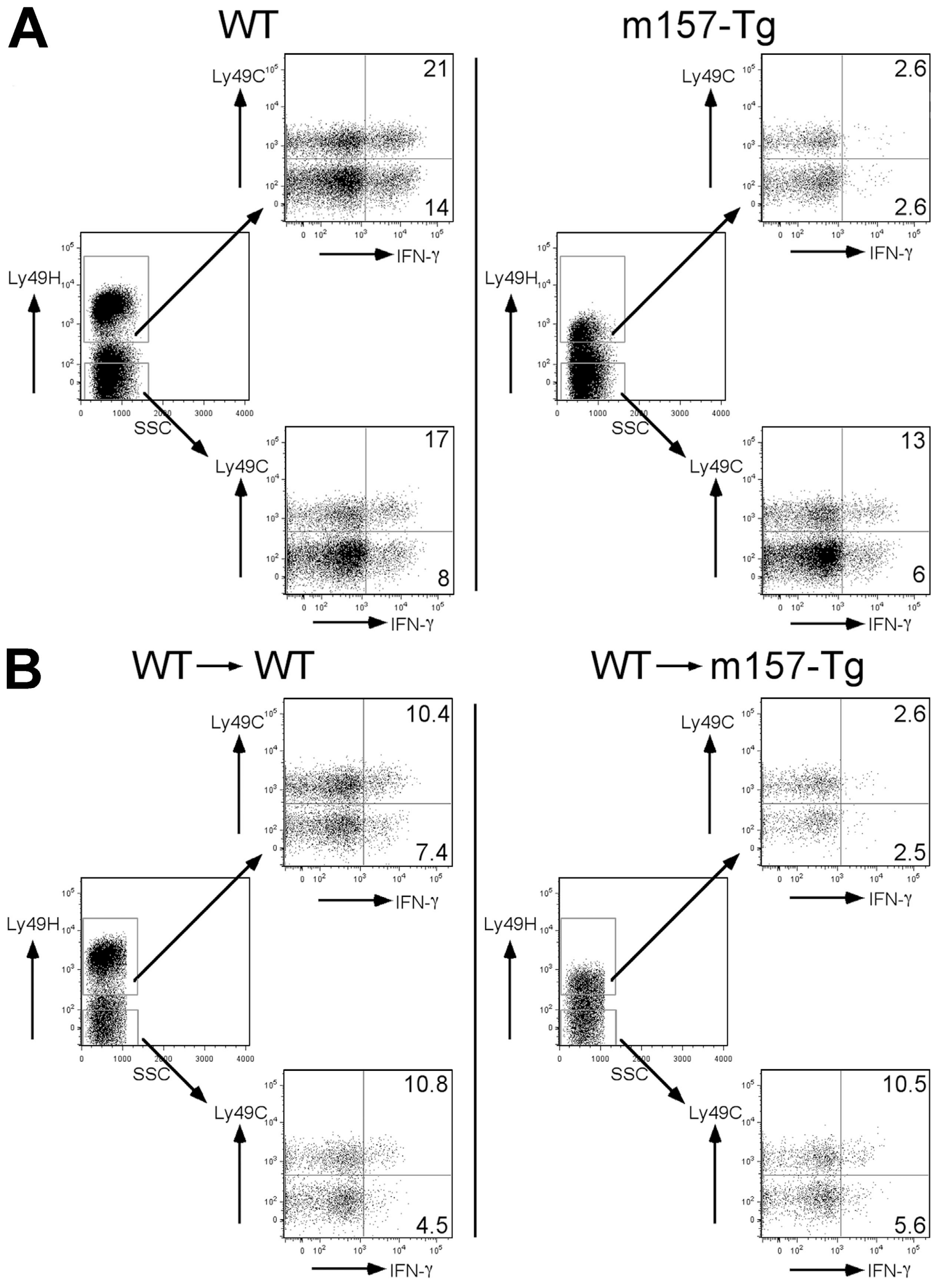

Figure 8. Licensing does not reverse Ly49H$^+$ hyporesponsiveness in m157-Tg mice. (A) IFN-γ production by NK cells from WT (left) or m157-Tg (right) after stimulation with plate-bound PK136. The first dot plot (Ly49H vs. side scatter) was gated on NK (NK1.1$^+$, CD3$^-$, CD19$^-$) cells. The boxes represent the Ly49H$^+$ and Ly49H$^-$ subsets used in further analysis for Ly49C and IFN-γ expression. (B) IFN-γ produced by NK cells from WT→WT and WT→m157-Tg chimeric mice. The analysis was identical to Fig. 8 A. The numbers represent the percentage of Ly49H$^+$ or Ly49H$^-$ NK cells producing IFN-γ.

through examination of mice with tissue-specific expression of Rae-1ε (27). These results are similar to the Ly49H down-regulation and the defects in the Ly49H$^+$ NK cells in m157-Tg mice that were observed both by us and by Sun and Lanier (24). However, in the Rae-1ε and MICA-Tg models, all NK cells were altered with potential contributions from soluble NKG2D ligands (26, 27). In contrast, in the m157-Tg mice, only the Ly49H$^+$ subset of NK cells was altered, indicating a specific effect caused by receptor engagement. In addition, there was no evidence for soluble m157 (unpublished data). Furthermore, it is possible that other immune cells expressing NKG2D may have contributed to the NKG2D functional effects noted in vivo in the Rae-1ε or MICA-Tg mice. For example, there was increased susceptibility to squamous cell cancers in the Rae-1ε-Tg mice and increased susceptibility of MICA-Tg mice to MICA-expressing tumors, but tumor resistance in these models can also be mediated by $\alpha\beta$ T cells and $\gamma\delta$ T cells that express NKG2D (26, 27). Similarly, there was increased susceptibility of the MICA-Tg mice to *Listeria* infection, but dysfunction of other cell types, such as NKG2D-expressing CD8$^+$ T cells, may contribute to the phenotype observed (26).

NKG2D and its ligands have additional layers of complexity. Mouse NKG2D is expressed on all NK cells in two different isoforms, (33–35) and two different signaling molecules (DAP12 and DAP10) (36). Also, mouse NKG2D has multiple endogenous ligands, including those that are constitutively expressed as well as being up-regulated on "stressed" cells (37–41). In addition, NKG2D ligands can be expressed as soluble forms that modulate NKG2D function (42). Finally, NKG2D itself is expressed on non-NK cell populations (40). Thus, although prior studies of NKG2D ligand-Tg mice demonstrate NKG2D-induced hyporesponsiveness, our current studies demonstrate that the in vivo functional effects of persistent activation receptor interactions, specifically on NK cell functions (because Ly49H is expressed only on NK cells), has no known endogenous ligands, and that m157 does not display any detectable binding to other cells in C57BL/6 mice.

Previous work has also demonstrated that Ly49D$^+$ NK cells appear to be defective in mice expressing H2D^d (25), a putative ligand for Ly49D (43, 44). However, physical interaction between Ly49D and H2D^d has been difficult to detect (45), suggesting that H2D^d may not be a Ly49D ligand or that other, as yet undefined, parameters affect H2D^d binding to Ly49D. In addition, H2D^d is recognized by other Ly49 inhibitory receptors (46), suggesting that its effect on Ly49D$^+$ NK cells could be caused by other receptor–ligand interactions. On the other hand, the Ly49H-m157 interaction exploited for the studies reported here does not appear to be subject to these concerns.

Our studies also provide additional insight into the issue of NK cell tolerance with respect to MHC class I because we provide a test of the "disarming" model. Herein, we demonstrated that licensing (engagement of inhibitory Ly49 receptor with self-MHC class I molecules) could not overcome the hyporesponsiveness caused by engagement of the activation receptor Ly49H with its ligand (m157) expressed as self. However, it is theoretically possible that licensing could overcome the activation receptor-induced hyporesponsive state if the interactions between the activation receptor and its ligand, or between the relevant inhibitory receptor and MHC class I, were either decreased or increased, respectively. For example, affinity differences could modulate the resultant functional competence of the NK cell, although recent biophysical studies indicate that the affinity of Ly49H for m157 approximates that of Ly49 receptors for MHC class I ligands (K_d = $\sim$1 μM) (47). Similarly, it is possible that the expression levels of the relevant receptors and ligands could affect avidity, or the simultaneous participation of several different receptors on an individual NK cell may be relevant. However, the current data suggest that hyporesponsiveness induced by a self-specific activation receptor may be difficult to overcome by licensing through a self-MHC class I–specific inhibitory receptor, i.e., the data currently do not support the disarming model.

Nevertheless, the interactions of activation receptors with their self-specific ligands do result in NK cell tolerance. Perhaps this represents another mechanism for self-tolerance, distinct from licensing by self-MHC via the "arming" model. NK cell tolerance would therefore be achieved by multiple mechanisms, somewhat analogous to central versus peripheral tolerance for T cells (48, 49), for example. Clearly, further studies are warranted because NK cell tolerance for self appears to be more complex than originally conceptualized (16).

MATERIALS AND METHODS

Mice. C56BL/6 mice were obtained from the National Cancer Institute (Bethesda, MD). B6.β_2M$^{-/-}$ mice and B6.SJL-*Ptprca* (CD45.1, Ly5.1) mice were purchased from The Jackson Laboratory. Mice were maintained under specific pathogen–free conditions and used after 8 wk of age. Animal studies were approved by the Animal Studies Committee at Washington University (St. Louis, MO).

m157 transgenic mice. A transgenic construct for the expression of m157 was generated as follows: the m157 cDNA was obtained by PCR (amplification of a 1,000-bp m157 fragment from pHSE3′m157 using primers that contained NotI restriction sites) (9, 10). The resulting m157 fragment was cloned into the pCMV plasmid (BD Biosciences) digested with NotI. A 1,400-bp fragment from pCMVm157 with XhoI and HindIII ends was cloned into the pBAP vector that had been digested with SalI and HindIII to generate pBAPm157. Microinjection of the purified ClaI-digested fragment from pBAPm157 was performed by the Mouse Genetics Core at Washington University supported by the Rheumatic Diseases Core Center. DNA obtained from tail tissue of the founders and offspring were screened by PCR using the following primer sets that generated a 250-bp fragment: m157-F (5′-TCAACTTCCGACGCAAAAGAAAT-3′) and m157-R (5′-ACCGTCGATTCGTCAGTAACG-3′). Peripheral blood lymphocytes were also stained for the expression of m157 using a biotinylated mAb against m157 (6H121) that has been previously described (10).

Virus and infection of mice. Smith strain MCMV was a generous gift of H. Virgin (Washington University). A salivary gland stock of MCMV generated in our laboratory was used in all of the in vivo experiments (50). For the survival curve, the mice were injected i.p. with 1.5 × 10^5 PFU MCMV. The percentage of mice surviving within each group was determined daily.

Moribund mice were killed per institutional guidelines. To measure splenic titers, mice were injected i.p. with 2×10^4 PFU MCMV, and titer was determined by plaque assays, as previously described (51). In brief, spleens from infected mice were obtained on day 3 after infection. They were placed in 1 ml of DME containing 10% calf serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine and frozen at –80°C until assayed. Spleens were thawed and homogenized with Dounce homogenizer on ice. Serial dilutions of the splenic lysate were then used to infect a monolayer of NIH-3T12 cells in triplicate on 6-well plates. After a 1-h incubation, the infected cells were overlaid with overlay media (DME, 5% calf serum, 1% Hepes, and 0.5% noble agar). On day three, the infected cells were again overlaid with overlay media. Titers were read on day four by two independent observers using a light microscope at 4× magnification.

Adoptive transfer into neonatal mice. Recipients were neonatal (3–4 d old) C57BL/6 mice. Before the experiments, neonatal mice from several litters were pooled and randomly redistributed to lactating mothers. Groups of 5–7 mice were given an i.p. injection of PBS (vehicle control), 5×10^7 WT splenocytes, or 5×10^7 m157-Tg splenocytes. The cells were injected in 50 μl of PBS. The next day, the mice were challenged with 870–1,000 PFU of MCMV i.p. in a total volume of 50 μl. The percentage of mice surviving in each group was determined daily.

BM graft rejection, BM chimeras, and splenocyte transfers. WT or m157-Tg mice were irradiated with 9.5 Gy. Irradiated WT or m157-Tg mice were injected via tail vein with 4×10^6, 2×10^6, 10^6, or 5×10^5 BM cells isolated from the femora and tibiae of WT, m157-Tg, or $\beta 2m^{-/-}$ mice. 5 d later, the mice were injected via the tail vein with 3 μCi ^{125}I-deoxyuridine (GE Healthcare). After 20–24 h, the mice were killed and the spleens were removed, rinsed extensively with PBS, and counted in a gamma counter (Gamma 5500; Beckman Coulter). Mice pretreated with anti-NK1.1, anti-Ly49H, or mouse anti–rat mAbs were injected i.p. 2 d before irradiation with 200 μg of the respective mAb. For BM chimera assays, BM cells were prepared from femora and tibiae of B6.SJL-*Ptprca* (Ly5.1) mice. A total of 10^7 BM cells was injected via tail vein into 9.5 Gy irradiated WT (Ly5.2) or m157-Tg (Ly5.2) mice. The spleen cells were analyzed at least 6 wk after transplantation. For the adoptive transfer of mature splenocytes, 4×10^7 splenocytes from WT (Ly5.1) mice were injected via tail vein into WT (Ly5.2) or m157-Tg (Ly5.2) mice. 90 d later, the mice were killed, spleens were removed, and splenic NK cells were assayed for cell surface expression and cytokine production.

Flow cytometry. Spleen cell suspensions were generated and stained for cell surface markers as previously described (9). The following antibodies were obtained from BD Biosciences: APC-PK136 (anti-NK1.1), PerCP-Cy5.5-145-2C11 (anti-CD3) and PerCP-Cy5.5-1D3 (anti-CD19), and FITC-M1/70 (anti-CD11b) and FITC-S7 (anti-CD43). Biotinylated-3D10 (anti-Ly49H) (6), FITC-4E4 (anti-Ly49D) (52), 4LO3311 (anti-Ly49C) (53), and FITC-JR9 (anti-Ly49A) (6) were purified from hybridomas and conjugated to FITC using FITC-celite (Calbiochem) or biotin using EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Fisher Scientific) according to manufacturer's protocol. Digoxigenin labeling of anti-Ly49C was performed according to manufacturer's protocol using the DIG Protein Labeling kit (Roche). To block nonspecific binding of antibodies to Fc receptors, all antibodies were diluted in the presence of mAb 2.4G2 (Fcγ II/III receptor; American Type Culture Collection).

Cytokine assays. For stimulation of NK cells, splenocytes (10^7 cells/ml) were incubated with equal volume of target cells (10^6 cells/ml) for 1 h, and then further incubated in the presence of an 833-fold dilution of stock GolgiPlug (BD Biosciences) for an additional 6–8 h, as previously described (17). Alternatively, spleen cells were incubated in 6-well plates coated with anti-NK1.1 mAb or anti-Ly49H mAb at a concentration of 5 μg/well. For IL-12 and -18 stimulation, splenocytes were incubated with 10 ng/ml IL-12 and 50 ng/ml IL-18 for 4 h in the presence of GolgiPlug. Splenocytes were stained for NK1.1, CD3, CD19, and Ly49H as described in the Flow cytometry section. Staining for Ly49C was done using digoxigenin-labeled anti-Ly49C followed by FITC-D1-22 (anti-digoxin; Sigma-Aldrich). To block nonspecific binding of antibodies to Fc receptors, all antibodies were diluted in the presence of mAb 2.4G2 (anti-Fcγ receptor II/III; American Type Culture Collection). Cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences), and then stained with either Alexa Fluor 488-XMG1.2 (anti-IFN-γ; BD Biosciences) or PE-Cy7-XMG1.2 (anti-IFN-γ; BD Biosciences) diluted in perm/wash buffer (BD Biosciences). Cells were analyzed using a FACSCalibur or FACSCanto cytometer (BD Biosciences) gating on NK1.1$^+$, CD3$^-$, CD19$^-$ populations.

Cytotoxicity assays. Generation of LAK cells, sorted Ly49H$^+$ and Ly49H$^-$ NK cells, and standard 4-h ^{51}Cr release assays were performed as previously described (6, 45). For sorting assays, LAK cells were sorted on day seven of culture by staining with biotinylated 3D10, followed by PE-streptavidin (BD Biosciences). After sorting, LAK cells were washed and cultured for 2 d before use in cytotoxicity assays.

Statistical analysis. Survival data were analyzed by the Mantel–Haenszel test, with death as the primary variable using Prizm software (GraphPad). The data in Fig. 2 were analyzed using the Mann-Whitney test with Prizm software. The remainder of the data were analyzed with Excel X for Mac (Microsoft). Error bars in the figures represent the SEM.

Online supplemental material. Fig. S1 depicts the construct used to generate the m157-Tg mouse, as well as identification of m157-Tg mice by PCR analysis of genomic DNA, expression of m157 on peripheral blood, and BM lymphocytes from the mouse. Fig. S2 compares splenocyte and NK cell counts from WT and m157-Tg mice. Fig. S3 demonstrates that the m157-Tg mouse can reject $\beta_2m^{-/-}$ BM cells. Fig. S4 demonstrates that IL-2 stimulation results in a normalization of Ly49H expression on m157-Tg NK cells. Fig. S5 demonstrates cell surface expression of Ly49H, c-Kit, Mac1, and CD43 on BM NK cells. Fig. S6 compares IFN-γ production after stimulation of WT and m157-Tg NK cells with IL-12 and -18. Fig. S7 compares production of IFN-γ by NK cells from WT or m157-Tg mice treated with poly I:C. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20072446/DC1.

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