Published in final edited form as:

Eur J Immunol. 2013 May ; 43(5): 1345–1355. doi:10.1002/eji.201243215.

Viral infection transiently reverses activation receptor mediated NK cell hyporesponsiveness in an MHC class I independent mechanism

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Summary

Continuous engagement of the Ly49H activating receptor with its ligand (m157) in a transgenic mouse expressing m157 (m157-Tg) results in hyporesponsiveness of Ly49H⁺ natural killer (NK) cells. The same interaction, during murine cytomegalovirus (MCMV) infection, leads to activation of Ly49H⁺ NK cells. MCMV infection results in decreased MHC class I expression on the infected cell as well as inflammatory responses, both of which do not take place in the uninfected m157-Tg mouse, potentially allowing for activation of NK cells in the context of MCMV infection. In this study, we demonstrated that viral infection transiently reverses activation receptor mediated NK cell hyporesponsiveness in an MHC class I independent mechanism. Furthermore, Ly49H⁺ NK cells in an MHC class I deficient environment remained hyporesponsive in the context of m157 expression, even when mature wildtype (WT) splenocytes were transferred into m157-Tg mice in the MHC class I deficient environment. However, the administration of cytokines TNF- α , IL-12 and IFN- β resulted in a partial recovery from activation receptor induced hyporesponsiveness. Thus, the release of the aforementioned cytokines during MCMV infection and not the downregulation of MHC class I expression appears to be responsible for partial resolution of Ly49H receptor induced NK cell hyporesponsiveness.

Keywords

NK cell; activating receptor; tolerance; NK cell ligand

Introduction

Natural killer (NK) cells play an important role in the host defense against viral pathogens, as well as the recognition of transformed cells. Unlike B or T cells, NK cells do not express a clonal receptor that undergoes recombination. Instead, NK cells can express several inhibitory and activating receptors on their cell surface. It is believed that a summation of the stimuli through these receptors results in the activation or inhibition of the NK cell. NK cells perform their function by releasing cytotoxic granules as well as secreting a number of cytokines, which results in the destruction of the target cell and the recruitment of other immune cells that will eventually shape the subsequent adaptive immune response.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

The Ly49H receptor is an activating receptor expressed on a subset of NK cells in the C57BL/6 mouse, which confers resistance to murine cytomegalovirus (MCMV) [1]. The Ly49H receptor specifically recognizes the MCMV-encoded protein m157 expressed on the cell surface of the MCMV-infected cell, resulting in an activating signaling cascade that is mediated primarily through the DAP12 adaptor protein [2-4]. Thus, in the context of MCMV infection, the m157/Ly49H interaction results in the activation of the NK cells.

Stimulation through NK activation receptors can function to increase as well as decrease NK cell function. Transgenic mouse models as well as in vitro studies have demonstrated that continuous engagement of the Ly49H activating receptor results in hyporesponsiveness of Ly49H expressing NK cells [5-7]. Using a transgenic mouse model expressing the MCMV encoded m157 in a ubiquitous manner (m157-Tg), we demonstrated that Ly49H⁺ NK cells from the m157-Tg mouse (where the Ly49H receptor was continuously engaged with m157 ligand) were hyporesponsive compared to Ly49H⁺ NK cells from non-transgenic (non-Tg) mice. These NK cells produced less interferon gamma (IFN- γ) and degranulated less well when stimulated through the Ly49H receptor as well as other ITAM-associated activating receptors [6]. NK cell hyporesponsiveness required signaling through the Ly49H receptor as mutations in the tyrosine amino acids that serve as phosphorylation sites in the DAP12 ITAM resulted in loss of the hyporesponsiveness to non-DAP12 mediated stimuli [5, 6]. Thus, in contrast to MCMV infection, the m157/Ly49H interaction in the m157-Tg mouse results in hyporesponsiveness of the Ly49H⁺ NK cells.

MCMV encodes three immunoevasins (*m04*-encoded gp34 glycoprotein, *m06*-encoded gp48, and *m152*-encoded gp40 glycoprotein) that modulate MHC class I expression and antigen presentation which likely helps decrease the ability of the adaptive immune system to recognize viral-infected cells [8-11]. In addition, acute MCMV infection results in an early systemic pro-inflammatory cytokine response including IFN- α/β , TNF- α and IL-12 [12-14]. Thus, downregulation of MHC class I molecules as well as onset of an inflammatory response are two major events that distinguish the m157/Ly49H interaction during MCMV infection from the same interaction that takes place in uninfected m157-Tg mouse.

These differences could explain how the m157/Ly49H interaction results in different NK cell functional outcomes in the two settings (m157-Tg mouse vs MCMV infection). We hypothesized that the m157/Ly49H interaction, in the context of down modulation of MHC class I (such as during MCMV infection) or in the presence of cytokines, results in activation of the Ly49H⁺ NK cells while the same interaction in the context of MHC class I expression (such as in the m157-Tg mouse) results in hyporesponsiveness of the Ly49H⁺ NK cells.

In this study, we demonstrate that viral infection transiently reverses activation receptor mediated NK cell hyporesponsiveness in an MHC class I independent mechanism. Furthermore, Ly49H⁺ NK cells, in an MHC class I deficient background, remain hyporesponsive in the context of m157 expression. In addition, upon transfer of mature wildtype (WT) splenocytes into m157-Tg mice, in both MHC class I sufficient and deficient backgrounds, the Ly49H⁺ cells became hyporesponsive. However, following cytokine administration, we observed a partial resolution of the hyporesponsiveness of the Ly49H⁺ NK cells from m157Tg mice. Thus, the data suggests that engagement of MHC class I molecules does not play a role in the induction of Ly49H mediated hyporesponsiveness seen in NK cells upon continuous engagement with m157, but cytokines can partially overcome this NK cell hyporesponsiveness.

Results

Ly49H-mediated NK cell hyporesponsiveness can be transiently reversed during MCMV infection

To test if viral infection could reverse activation receptor mediated NK cell hyporesponsiveness, we infected WT and m157-Tg with a Δ m157 MCMV. The Δ m157 MCMV is an MCMV isolate with a mutation in the m157 gene that prevents its expression following infection [15, 16]. We used the Δ m157 MCMV to prevent the preferential activation of Ly49H⁺ NK cells that is seen upon infection with wildtype MCMV. There was no downregulation of the Ly49H receptor in the WT mice following viral infection, since no m157 was being expressed (Fig. 1A). Prior to infection, a statistically significant difference was seen when we compared the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells from WT and m157-Tg mice (Fig 1B).

Upon infection of m157-Tg mice with the Δ m157 MCMV, we observed that the Ly49H⁺ NK cells harvested from m157-Tg mice at 60 and 108 hours post infection were no longer hyporesponsive compared to Ly49H⁻ NK cells from the same mouse following PK136 stimulation (Fig. 1A and B). At these time points, the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells was not statistically different between WT and m157-Tg mice (Fig. 1B).

At 132 and 180 hours post infection, however, the Ly49H⁺ NK cells from previously infected m157-Tg mice regained the hyporesponsive phenotype with a statistically significant difference between the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells and the ratio of the percentage of IFN- γ producing Ly49H⁻ NK cells from WT and m157-Tg mice (Fig. 1B). To confirm the status of viral infection we measured Δ m157 MCMV load at the different time points by real time PCR (Fig 1C). This suggests that activating receptor-mediated NK cell hyporesponsiveness can be transiently broken during viral infection.

Loss of MHC class I expression does not alter Ly49H-mediated NK cell hyporesponsiveness

The fact that MCMV expresses a number of immunoevasins to decrease MHC class I expression on infected cells, led us to hypothesize that differential MHC class I expression may determine if the m157/Ly49H interaction leads to the activation or hyporesponsiveness of the NK cell. To test this hypothesis, we bred the m157-Tg mouse onto the $\beta_2m^{-/-}$ background, allowing us to assess the function of the Ly49H⁺ cells in a mouse that expresses m157 but are deficient in expression of MHC class I molecules (H2K^b and H2D^b).

We confirmed that m157 was expressed at a similar level in m157-Tg and m157-Tg $\beta_2m^{-/-}$ mice and that H-2K^b and H-2D^b (MHC class I molecules expressed in C57BL/6 mice) were absent in the $\beta_2m^{-/-}$ background by flow cytometry (Supplemental Figure 1A). We also observed that expression of maturation (CD11b) and activation markers (CD69) was similar on NK cells from both WT and $\beta_2m^{-/-}$ mice. This was the case in both the presence and absence of m157 transgene expression (Supplemental Figure 1B). Thus, NK cells from an MHC class I deficient environment, in the presence or absence of the m157 transgene, expressed cell surface markers that suggested they were mature and nonactivated.

We next assessed if MHC class I expression plays a role in the induction of Ly49H-mediated NK cell hyporesponsiveness. Similar to the C57BL/6 background ($\beta_2m^{+/+}$), down regulation of the Ly49H receptor was seen in m157-Tg $\beta_2m^{-/-}$ mice when compared to $\beta_2m^{-/-}$ mice (Fig. 2A and C). To demonstrate NK cell hyporesponsiveness in the MHC class

In an MHC class I deficient environment, we stimulated splenic NK cells from m157-Tg $\beta_2m^{-/-}$ and $\beta_2m^{-/-}$ mice through the NK1.1 or Ly49D activating receptors. NK1.1 and Ly49D are Ly49H-independent activating receptors associated with the ITAM containing adaptor proteins FcR γ and DAP12 respectively [17]. Upon stimulation with either plate bound PK136 (anti-NK1.1) or 4E4 (anti-Ly49D), the Ly49H⁺ NK cells from m157-Tg $\beta_2m^{-/-}$ mice produced less IFN- γ than Ly49H⁺ NK cells from the same mice and when compared to Ly49H⁺ NK cells from $\beta_2m^{-/-}$ mice. Ly49H⁺ and Ly49H⁻ NK cells from $\beta_2m^{-/-}$ mice produced similar amounts of IFN- γ (Fig. 2A and C). There was also a statistically significant difference when we compared the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells from $\beta_2m^{-/-}$ and m157-Tg $\beta_2m^{-/-}$ mice (Fig. 2B and D). This was similar to the difference seen when comparing the same ratios between WT and m157-Tg mice (Fig 2B and D) and previously published data [5, 6].

These experiments demonstrate that in an MHC class I deficient environment, similar to an MHC class I sufficient environment, the continuous engagement of Ly49H with m157 results in a Ly49H-mediated hyporesponsiveness of the Ly49H⁺ NK cells. Furthermore, NK cell defects were seen upon stimulation through multiple Ly49H-independent activating receptors associated with different ITAM containing adaptor molecules. Thus, the absence of MHC class I expression does not effect Ly49H-mediated NK cell hyporesponsiveness in m157-Tg mice.

Ly49H-mediated NK cell hyporesponsiveness can be induced in mature WT NK cells transferred into an MHC class I deficient background

We have previously demonstrated that NK cell hyporesponsiveness can be mediated through continuous engagement of activating receptor in mature NK cells [5]. To address if this was still the case upon transfer into an MHC class I deficient background, we performed adoptive transfer of labeled mature WT splenic NK cells into both $\beta_2m^{-/-}$ and m157-Tg $\beta_2m^{-/-}$ recipients. Transfer of WT NK cells into m157-Tg $\beta_2m^{-/-}$ but not $\beta_2m^{-/-}$ recipient mice resulted in downregulation of the Ly49H receptor (Fig. 3A). At 24 as well as 72 hours post transfer, WT donor Ly49H⁺ NK cells transferred into m157-Tg $\beta_2m^{-/-}$ recipient mice produced less IFN- γ than those transferred into $\beta_2m^{-/-}$ mice when stimulated with plate-bound anti-NK1.1. In addition donor Ly49H⁺ NK cells from m157-Tg $\beta_2m^{-/-}$ recipients produced less IFN- γ than Ly49H⁻ NK cells from the same mouse (Fig 3A). Furthermore, a statistically significant difference was seen when we compared the ratio of the percentage of IFN- γ producing donor Ly49H⁺ NK cells to the percentage of IFN- γ producing donor Ly49H⁻ NK cells from recipient $\beta_2m^{-/-}$ and m157-Tg $\beta_2m^{-/-}$ mice (Fig. 3C). Similar results were seen upon transfer of WT mature NK cells into WT or m157Tg recipient mice at 24 hours and 72 hours post transfer (Fig. 3B and D). This confirms that NK cell hyporesponsiveness can be induced in mature NK cells through continuous engagement of activating receptor in an MHC class I deficient environment.

Ly49H-mediated NK cell hyporesponsiveness can be transiently reversed during MCMV infection in an MHC class I deficient environment

To test if viral infection could reverse activation receptor mediated NK cell hyporesponsiveness in an MHC class I deficient environment, we infected $\beta_2m^{-/-}$ and m157-Tg $\beta_2m^{-/-}$ with a Δ m157 MCMV. Again, there was no downregulation of the Ly49H receptor in the $\beta_2m^{-/-}$ following viral infection, since no m157 was being expressed (Fig. 4A). Prior to infection, a statistically significant difference was seen when we compared the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells from $\beta_2m^{-/-}$ and m157-Tg $\beta_2m^{-/-}$ mice (Fig 4B). Upon infection of m157-Tg $\beta_2m^{-/-}$ mice with the Δ m157 MCMV, we observed that the Ly49H⁺ NK cells harvested from m157-Tg $\beta_2m^{-/-}$ mice at 60 hours post infection were no longer

hyporesponsive compared to Ly49H⁺ NK cells from the same mouse following PK136 stimulation (Fig. 4A). At this time point, the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells was not statistically different between $\beta_2m^{-/-}$ and m157-Tg $\beta_2m^{-/-}$ mice (Fig. 4B). After 60 hours post infection, however, the Ly49H⁺ NK cells from previously infected m157-Tg $\beta_2m^{-/-}$ mice regained the hyporesponsive phenotype with a statistically significant difference between the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells and the ratio of the percentage of IFN- γ producing Ly49H⁻ NK cells from $\beta_2m^{-/-}$ and m157-Tg $\beta_2m^{-/-}$ mice. (Fig. 4B). To confirm the status of viral infection we measured Δ m157 MCMV load at the different time points by real time PCR (Fig. 4C). This suggests that activating receptor-mediated NK cell hyporesponsiveness can be transiently broken in an MHC class I deficient background during viral infection.

Cytokines can transiently reverse activating receptor mediated NK cell hyporesponsiveness

To test if we could reverse activating receptor induced hyporesponsiveness by inflammation in the absence of virus, we injected mice with 300 μ g of poly I:C and assessed for IFN- γ production by splenic NK cells following stimulation with plate bound PK136 at six hours post injection. Following treatment with poly I:C, the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells was statistically different between treated and untreated m157-Tg mice (Fig. 5A). Though the ratio increased following poly I:C injection, it did not reach levels seen in non-Tg mice, suggesting that inflammation induced through poly I:C treatment alone was not enough to completely reverse hyporesponsiveness.

As TNF- α , INF- β and IL-12 have been shown to play a role in the induction of IFN- γ production by NK cells following MCMV infection, we assessed if we could reverse hyporesponsiveness by the addition of these three cytokines in vitro. Similar to poly I:C injections, we were only able to partially restore Ly49H⁺ NK cell response to PK136 stimulation with any of the three cytokines. In each case, the ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells was statistically different between treated and untreated m157-Tg mice (Fig. 5B-C). We also combined the cytokines to determine if an additive effect could reverse hyporesponsiveness. We observed similar results in that the combination of cytokines only partially restored Ly49H⁺ NK cell response to plate bound PK136 stimulation in m157-Tg mice (Supplemental Figure 2). This data suggests that cytokines can partially reverse the hyporesponsiveness induced by continuous engagement of the activating receptor.

Discussion

It is clear from a number of in vitro and in vivo mouse models that continuous engagement of NK cell activating receptors renders NK cells hyporesponsive to further receptor-mediated stimulation [6, 7, 18-20]. In addition, a recent report demonstrated that NK cells were hyperresponsive in a mutant mouse where expression of NKp46 was lost, suggesting that engagement of NKp46 (an activating receptor) down-regulates NK cell responsiveness [21]. However, it still remains unclear as to the mechanism and the context in which the engagement of activating receptors results in the hyporesponsiveness, rather than activation, of the NK cells.

The engagement of m157 with the Ly49H activating receptor results in activation of the Ly49H⁺ NK cells in the C57BL/6 mouse following infection with MCMV [1, 22-24]. In fact, it is this interaction that is responsible for the genetic resistance of this strain of mice to MCMV infection [25, 26]. This same interaction, however, in the context of a transgenic

mouse in the C57BL/6 background that expresses the m157 protein, results in hyporesponsiveness rather than activation of the Ly49H⁺ NK cells.

In this study, we have provided evidence that viral infection can transiently reverse activating receptor induced NK cell hyporesponsiveness in an MHC class I independent mechanism. We demonstrate that NK cell impairment resulting from sustained engagement of the Ly49H activating receptor still occurs in the context of an MHC class I deficient background. Though we did see decreased IFN- γ production by both Ly49H⁺ and Ly49H⁻ NK cells from mice in an MHC class I deficient background as compared to those that did express MHC class I, there was a further decrease in IFN- γ production in the Ly49H⁺ population compared to the Ly49H⁻ population when the m157 transgene was expressed. This demonstrates that the m157/Ly49H interaction imparted further defects in addition to the hyporesponsiveness seen in NK cells due to the inability to be licensed by being in an MHC class I deficient environment [27].

Previous work has demonstrated that unlicensed mature NK cells from an MHC class I deficient background can acquire a licensed phenotype after transfer to an MHC class I sufficient background [28, 29]. These studies demonstrate that NK cells have the capacity to reset their responsiveness depending on the environment in which they are placed. Changes in NK cell function, though not statistically significant, were seen by about 4 days after adoptive transfer. However, maximal resetting of the NK cells did not happen until after about 4-7 days [28, 29]. In our experiments, we were able to see significant defects in Ly49H⁺ NK cells at 24 and 72 hours post transfer into an MHC class I-deficient environment that expresses m157. Thus, the effects of the m157/Ly49H interaction resulting in hyporesponsiveness of the Ly49H⁺ NK cells are likely taking place prior to resetting of the NK cells due to transfer into an MHC-deficient environment.

The fact that the normal Ly49H⁺ NK became hyporesponsive upon transfer into the m157-Tg $\beta_2m^{-/-}$ mice is a counterintuitive response, especially in light of data demonstrating that expression of m157 on RMA cells can overcome class I-mediated inhibition of NK cell cytotoxicity [30]. Normal Ly49H⁺ NK cells transferred into an MHC class I deficient mouse in which m157 is highly expressed would be in an environment where their activating receptors are engaged but the inhibitory receptors are no longer engaged. The summation of these stimuli imparted on the Ly49H⁺ NK cells would be expected to result in their activation. However, our results demonstrate the opposite, with the Ly49H⁺ NK cells in this context becoming hyporesponsive.

Previous studies have demonstrated that tolerance to “missing self” can be overcome by activation of NK cells through infection or cytokine stimulation [31-34]. The NK cells in these studies were hyporesponsive as a result of impaired engagement of MHC class I molecules with inhibitory receptors on the NK cell (“unlicensed” NK cells). In our studies, although the MHC class I deficient environment did not alter activation-receptor mediated NK cell hyporesponsiveness, MCMV infection was able to reverse NK cell hyporesponsiveness induced by continuous engagement of the Ly49H receptor. Thus, activation receptor mediated NK cell hyporesponsiveness, similar to hyporesponsiveness seen in “unlicensed” NK cells, was transiently reversed following MCMV infection.

In addition to alteration in MHC class I expression, MCMV infection has been reported to produce a number of pro-inflammatory cytokines including IFN- α/β , TNF- α and IL-12 [12-14]. It has been demonstrated that IL-12 levels increase following MCMV infection, with serum levels detectable at about 48 hours post infection and tapering off by about 72 hours [13]. Moreover, it has been demonstrated that IL-12 stimulates the production of IFN- γ by NK cells [12-14, 35]. This would suggest that increased levels of these cytokines

during acute MCMV infection could reverse hyporesponsiveness induced by continuous activation receptor engagement. In this study we demonstrate that the administration of the cytokines TNF- α , IFN- β , or IL-12 can partially reverse activation receptor mediated NK cell hyporesponsiveness.

Activating receptor mediated NK cell hyporesponsiveness appears to take place in human NK cells as well. Recently, it has been demonstrated, in freshly isolated human NK cells, that engagement of the KIR2DS1 decreases the responsiveness of the NK cells to target cell stimulation in donors homozygous for the human HLA-C of group 2 [36]. Furthermore, several studies suggest that this mechanism may play a role in survival and relapse rate following hematopoietic stem cell transfer (HSCT) [37, 38]. These studies provides evidence that activating receptor engagement can induce hyporesponsiveness in human NK cells and may play an important role in the outcome of HSCT.

Of note, KIR2DS1 receptors expressed on human NK cells signal through the same adaptor molecule (DAP12) as does the Ly49H receptor in C57BL/6 mice [39, 40]. This would suggest that mechanisms of activation receptor induced hyporesponsiveness could be similar in both the mouse and human. Thus, the m157-expressing transgenic mouse model is an ideal system to further study this mechanism.

The studies described in this report demonstrate that in both an MHC class I deficient and sufficient environment, the m157/Ly49H interaction results in the hyporesponsiveness of the Ly49H⁺ NK cells to Ly49H-independent activating receptor stimulation. MCMV infection, however, was able to transiently reverse the activation receptor mediated hyporesponsiveness. This appeared to be mediated at least in some part through the release of cytokines during infection as the administration of cytokines could partially reverse the activation receptor mediated NK cell hyporesponsiveness. Although these studies shed some light in regards to the context in which activating receptor mediated hyporesponsiveness takes place, further studies are required to understand the mechanism leading to the two different outcomes of activating receptor engagement - activation during viral infection but hyporesponsiveness in m157 transgene expression. Understanding the signaling cascade downstream of the activating receptor in the two situations may provide clues to understanding this complex phenomenon.

Material and Methods

Mice and infections

The m157-Tg mouse has been previously described [6]. C57BL/6. $\beta_2m^{-/-}$ mice were obtained from Charles River. The m157-Tg $\beta_2m^{-/-}$ mice were obtained by crossing m157-Tg mice with C57BL/6. $\beta_2m^{-/-}$ mice. Mice were maintained under specific pathogen-free conditions and used after they reached 8 weeks of age. Mice were infected intraperitoneally (i.p.) with 1×10^4 PFU/mouse from a salivary gland stock of Δ m157 MCMV (AT1.5), an m157-deficient isolate with a mutation in the m157 gene [15, 16]. The Δ m157 MCMV virus was a gift from A. French. For stimulation with poly I:C, mice were injected i.p. with 300 μ g of poly I:C (SIGMA) diluted in sterile phosphate buffered saline. The Animal Studies Committee at Washington University (St. Louis, MO) approved all animal studies.

Antibodies

The following antibodies were obtained from EBioscience or Biolegend: APC-PK136 (anti-NK1.1), PerCP-Cy5.5-145-2C11 (anti-CD3), Alexa488-XMG1.2 (anti-IFN- γ), PacBlue-XMG1.2 (anti-IFN- γ) and streptavidin-PE. The 3D10 (anti-Ly49H) and 6H121 (anti-m157) mAbs were purified from hybridomas by the Protein Production and Purification Core Facility of the RDCC and conjugated to biotin using EZ-Link Sulfo-NHS-LC-LC-Biotin

(Pierce) according to manufacturer's protocol. The 28-8-6 (anti-H-2K^b/H-2D^b) mAb was obtained from BD Pharmingen and conjugated to allophycocyanin (APC). Purified PK136 (anti-NK1.1) was purchased from BioXcell. The purified 4E4 antibody was a gift from W. Yokoyama.

Adoptive transfer experiments

Spleen cell suspensions were generated as previously described [6]. Donor splenocytes were labeled using the Vybrant CFDA SE Cell tracer kit (Molecular Probe) per the manufacturer's protocol. Briefly, splenocytes were washed with PBS and then resuspended in 1 μ m CFSE (diluted in PBS) at a concentration of 100×10^6 cells/ml for 10 minutes in the dark at room temperature. The reaction was stopped with R10 media. Cells were washed with PBS and then resuspended at 250×10^6 cells/ml. Prior to injection, cells were assessed for CFSE labeling as well as NK cell percentage by flow cytometry. Mice were injected intravenously in the tail vein with 200 μ l of labeled splenocytes (approximately 50×10^6 total splenocytes). Mice were then harvested at the indicated time points.

IFN- γ assays

Spleen cells suspensions were generated as previously described [6]. To coat plates, appropriate antibody was diluted to 2-4 μ g/ml in PBS. One ml of antibody (2-4 μ g) was placed in 6 well tissue culture plates (Techno Plastic Product) and incubated at 37°C for 90 minutes. After incubation, the plates were washed with PBS three times prior to use for stimulation assays. For stimulation of NK cells, 1 ml of splenocytes (10^7 cells/ml in R10) were incubated in 6 well plates coated with anti-NK1.1 mAb or anti-Ly49D mAb for 1/2 hr and then further incubated in the presence of a 833-fold dilution of stock brefeldin A (GolgiPlug, BD Pharmingen) for an additional 6-8 hours, as previously described [6]. For cytokine stimulation, the TNF- α (50 ng/ml) (Peprotech), IFN- β (1000 IU/ml) (PBL InterferonSource), and IL-12 (10 ng/ml) (Peprotech) were added individually or in combination at the time of plating. Splenocytes were stained for NK1.1, CD3 and Ly49H using the antibodies described above. To block nonspecific binding of antibodies to Fc receptors, all antibodies were diluted in the presence of mAb 2.4G2 (anti-Fc γ receptor II/III, ATCC). Cells were fixed and permeabilized with Cytotfix/Cytoperm kit (BD Pharmingen) and then stained with either Alexa488-XMG1.2 (anti-IFN- γ) or PacBlue-XMG1.2 (anti-IFN- γ) diluted in perm/wash buffer (BD Pharmingen). NK cells were analyzed using a FACSCalibur or FACSCanto cytometer (BD Biosciences) gating on the NK1.1⁺, CD3⁻ populations. The data were analyzed using FlowJo software (Tree Star).

Determination of MCMV load by quantitative real-time PCR

DNA samples were purified from MCMV infected mouse spleen fragments suspended in cell lysis buffer (50mM Tris, pH 7.5, 50mM EDTA, 0.5% SDS and 0.2M NaCl) containing 100 μ g/ml proteinase K followed by isopropanol (50% v/v) precipitation. They were resuspended in TE, pH 8.0 at a concentration of 50ng/ μ l. Oligonucleotide primers used for amplification of the IE1 gene were: forward primer 5'-TGGTGCTCTTTTCCCGTG-3' and reverse primer 5'-CCCTCTCCTAACTCTCCCTTT-3'. The sequence of the TaqMan probe was 5'-TCTCTTGCCCCGTCCTGAAAAACC-3'. For real-time PCR, the probe was labeled at the 5' end with the reporter dye FAM and at the 3' end with the quencher dye Iowa Black FQ. β -actin was included as a housekeeping gene control for normalization. Real time PCR was performed using TaqMan Universal Master Mix (Applied Biosystems). Standard curves for both MCMV-IE1 and β -actin amplification were generated by plotting average Ct values against the logarithm of target template molecules obtained from the plasmids, followed by a sum of least squares regression analysis. Target copy numbers in tissue samples were calculated using the equations obtained in regression analysis. Results

were expressed as a logarithm of the IE1/ β -actin copy number ratio that was further multiplied by 1000 [41].

Statistical analysis

The data were analyzed with Microsoft Excel X for Mac (Microsoft). Unpaired, two-tailed *t* test was used to determine statistically significant differences between experimental groups. Error bars in the figures represent the standard error of the mean (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Washington University Pilot and Feasibility program of the RDCC (NIH P30AR048335), an award from the Edward Mallinckrodt, Jr. Foundation, and NIH Grant R01AI089870 (all to S.K.T.). The authors would like to thank Dr. Anthony R. French (Washington University, St. Louis, MO) for providing the Δ m157 MCMV (AT1.5) and Dr. Wayne M. Yokoyama (Washington University, St. Louis, MO) for providing the 4E4 (anti-Ly49D) mAb. Experimental support for antibody production was provided by the Protein Production and Purification Core Facility of the Rheumatic Diseases Core Center (NIH P30AR048335).

Abbreviations

MCMV	Murine Cytomegalovirus
WT	wild type
Tg	transgenic
β_2m	beta-2-microglobulin

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FIGURE 1. MCMV infection transiently reverses Ly49H receptor-mediated NK cell hyporesponsiveness

(A) Representative dot plots demonstrating IFN- γ production by freshly isolated splenocytes from WT and m157-Tg stimulated with plate bound PK136 at 0, 60, 108, 132 and 180 hours post i.p. infection with a Δ m157 MCMV. The dot plots were gated on NK cells (NK1.1⁺, CD3⁻ cells). The numbers represent the percentage of IFN- γ producing Ly49H⁺ or Ly49H⁻ NK cells. (B) The ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells at various times post infection from WT mice (n=3 at 0 h, n=6 at 60 h, n= 3 at 108 h, n=5 at 132 h, n= 4 at 180 h) and m157-Tg mice (n=5 at 0 h, n=6 at 60 h, n=3 at 108 h, n=5 at 132 h n= 4 at 180h) stimulated with plate bound PK136. (C) Δ m157 MCMV splenic loads at various time points post viral infection from WT mice (n= 6 at 60 h, n= 3 at 108 h, n= 5 at 132 h, n= 4 at 180 h) and m157-Tg mice (n=6 at 60 h, n=3 at 108 h, n=5 at 132 h n= 4 at 180h). The results are presented as the mean $\pm$ SEM. *p<0.05, **p<0.0005.

FIGURE 2. MHC class I deficient environment does not alter Ly49H-mediated NK cell hypo-responsiveness to NK1.1 or Ly49D stimulation

(A) Representative dot plots demonstrating IFN-γ production by freshly isolated splenocytes from WT, m157-Tg, β₂m^{-/-} and m157-Tgβ₂m^{-/-} mice stimulated with plate bound anti-NK1.1 mAb (PK136). The dot plots were gated on NK cells (NK1.1⁺, CD3⁻ cells). The numbers represent the percentage of IFN-γ producing Ly49H⁺ or Ly49H⁻ NK cells. (B) The ratio of the percentage of IFN-γ producing Ly49H⁺ NK cells to the percentage of IFN-γ producing Ly49H⁻ NK cells from WT (n=5), m157-Tg (n=5), β₂m^{-/-} (n=14) and m157-Tgβ₂m^{-/-} (n=12) mice stimulated with plate bound PK136. (C) Representative dot plots demonstrating IFN-γ production by freshly isolated splenocytes from WT, m157-Tg, β₂m^{-/-} and m157-Tgβ₂m^{-/-} mice stimulated with plate bound anti-Ly49D mAb (4E4). The numbers represent the percentage of Ly49H⁺ or Ly49H⁻ NK cells producing IFN-γ. The dot plots were gated on NK cells (NK1.1⁺, CD3⁻ cells). The numbers represent the percentage of IFN-γ producing Ly49H⁺ or Ly49H⁻ NK cells. (D) The ratio of the percentage of IFN-γ producing Ly49H⁺ NK cells to the percentage of IFN-γ producing Ly49H⁻ NK cells from WT (n=5), m157-Tg (n=5), β₂m^{-/-} (n=7) and m157-Tgβ₂m^{-/-} (n=6) mice stimulated with plate bound 4E4. The results are presented as the mean ± SEM. *p<0.0005.

FIGURE 3. Mature donor WT Ly49H⁺ NK cells display hyporesponsive phenotype within 24 hours of transfer into m157-Tg $\beta_2m^{-/-}$ mice or m157Tg mice

(A&B) Representative dot plots demonstrating IFN- γ production by freshly isolated WT donor splenocytes stimulated with plate bound PK136 at 24 and 72 hours post transfer into (A) $\beta_2m^{-/-}$ recipient (WT $\rightarrow$ $\beta_2m^{-/-}$) and m157-Tg $\beta_2m^{-/-}$ recipient (WT $\rightarrow$ m157-Tg $\beta_2m^{-/-}$) or (B) WT recipient (WT $\rightarrow$ WT) and m157-Tg recipient (WT $\rightarrow$ m157-Tg). The dot plots were gated on donor NK cells (NK1.1⁺, CD3⁻, CFSE⁺ cells). The numbers represent the percentage of IFN- γ producing Ly49H⁺ or Ly49H⁻ NK cells. (C&D) The ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells from (C) WT $\rightarrow$ $\beta_2m^{-/-}$ (n=8 at 24 h, n=9 at 72 h) and WT $\rightarrow$ m157-Tg $\beta_2m^{-/-}$ (n=7 at 24 h, n=8 at 72 h) or (D) WT $\rightarrow$ WT (n=5 at 24 h, n=5 at 72 h) and WT $\rightarrow$ m157Tg (n=5 at 24 h, n=7 at 72 h). The results are presented as the mean $\pm$ SEM.

*p<0.005, **p<0.005.

FIGURE 4. MCMV infection transiently reverses Ly49H receptor-mediated NK cell hyporesponsiveness in MHC class I deficient environment

(A) Representative dot plots demonstrating IFN- γ production by freshly isolated splenocytes from $\beta_2m^{-/-}$ and m157-Tg $\beta_2m^{-/-}$ stimulated with plate bound PK136 at 0, 60, 108, 132, and 180 hours post i.p. infection with a Δ m157 MCMV. The dot plots were gated on NK cells (NK1.1⁺, CD3⁻ cells). The numbers represent the percentage of IFN- γ producing Ly49H⁺ or Ly49H⁻ NK cells. (B) The ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells at various times post infection from $\beta_2m^{-/-}$ mice (n=6 at 0 h, n=3 at 60 h, n=4 at 108 h, n=3 at 132 h, n=4 at 180h) and m157-Tg $\beta_2m^{-/-}$ mice (n=7 at 0 h, n=6 at 60 h, n=5 at 108 h, n=4 at 132 h, n=4 at 180h) stimulated with plate bound PK136. (C) Δ m157 MCMV splenic loads at various time points post viral infection from $\beta_2m^{-/-}$ mice (n=3 at 60 h, n=4 at 108 h, n=3 at 132 h, n=4 at 180 h) and m157-Tg $\beta_2m^{-/-}$ mice (n=6 at 60 h, n=5 at 108 h, n=4 at 132 h, n=4 at 180 h). The results are presented as the mean $\pm$ SEM. *p<0.05, **p<0.0005.

FIGURE 5. Cytokines can partially reverse activating receptor mediated NK cell hyporesponsiveness

The ratio of the percentage of IFN- γ producing Ly49H⁺ NK cells to the percentage of IFN- γ producing Ly49H⁻ NK cells stimulated with plate bound PK136 (A) at 6 hours after poly I:C injection (n= 3 for WT without poly I:C, n= 8 for WT with poly I:C, n= 3 for m157-Tg without poly I:C, n= 7 for m157-Tg with poly I:C). (B) with and without TNF- α (n= 5 for WT without cytokine, n= 5 for WT with cytokine, n= 6 for m157-Tg without cytokine, n= 6 for m157-Tg with cytokine). (C) with and without IFN- β (n= 5 for WT without cytokine, n= 5 for WT with cytokine, n= 6 for m157-Tg without cytokine, n= 6 for m157-Tg with cytokine). (D) with and without IL-12 (n= 5 for WT without cytokines, n= 5 for WT with cytokine, n= 6 for m157-Tg without cytokine, n= 5 for m157-Tg with cytokine). The results are presented as the mean $\pm$ SEM. **p<0.005.