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*J Immunol* 2011; 186:2765-2771; Prepublished online 24 January 2011; doi: 10.4049/jimmunol.1003046

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Supplementary Material

http://www.jimmunol.org/content/suppl/2011/01/24/jimmunol.1003046.DC1

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Activation Receptor-Induced Tolerance of Mature NK Cells In Vivo Requires Signaling through the Receptor and Is Reversible

Fred D. Bolanos and Sandeep K. Tripathy

NK cell responses are determined by signals received through activating and inhibitory cell surface receptors. Ly49H is an NK cell-specific activating receptor that accounts for the genetic resistance to murine CMV (MCMV). The Ly49H receptor has been shown to interact with two adaptor proteins (DAP12 and DAP10). In the context of MCMV infection, interaction of m157 (the MCMV-encoded ligand for Ly49H) with Ly49H results in activation of Ly49H-expressing NK cells. Chronic exposure of Ly49H with m157, however, induces tolerance in these same cells. The mechanism of this tolerance remains poorly understood. Using a transgenic mouse model, we demonstrate that induction of tolerance in Ly49H+ NK cells by chronic exposure to m157, in vivo, requires signaling through the Ly49H adaptor protein DAP12, but not the DAP10 adaptor protein. Furthermore, mature Ly49H-expressing NK cells from wild-type mice can acquire a tolerant phenotype by 24 h posttransfer into a transgenic C57BL/6 mouse that expresses m157. The tolerant phenotype can be reversed, in vivo, if tolerant NK cells are transferred to mice that do not express the m157 protein. Thus, continuous activating receptor engagement can induce a transient tolerance in mature NK cells in vivo. These observations provide new insight into how activating receptor engagement shapes NK cell function and have important implications in how NK cells respond to tumors and during chronic viral infection. The Journal of Immunology, 2011, 186: 2765–2771.
Multiple transgenic (Tg) mouse models have demonstrated that continuous expression of ligands for NK cell activating receptors results in NK cell defects in vivo (18, 21, 22). Initial studies addressing the mechanism in which chronic activating receptor engagement results in NK cell dysfunction have all been in vitro and involved using NK cells that had been stimulated with cytokines (a necessity when culturing NK cells in vitro) (12, 20). To better understand the mechanism and extent of activating receptor-induced tolerance in vivo, we took advantage of our previously described m157-Tg model system (18). Using this system, we demonstrate that Ly49H activating receptor-induced NK cell tolerance in vivo requires signaling through the receptor via the DAP12 adaptor protein. Additionally, tolerance can be induced in mature NK cells in vivo, demonstrating that activating receptor-induced tolerance is not developmentally regulated. Finally, we demonstrate that activating receptor-induced tolerance can be reversed in vivo.

Materials and Methods

Mice

The m157-Tg mouse has been previously described (18). Mice expressing a nonfunctional mutated form of DAP12 (DAP12ki), as well as the DAP10 knockout mice (DAP10ko), have both been previously described (23, 24). All mice used in these experiments were C57BL/6 background. The m157-Tg mice were crossed with DAP12ki and DAP10ko mice to generate mice that were heterozygous at the DAP12 and DAP10 locus, respectively. These mice were mated with DAP12ki or DAP10ko mice to generate the m157-Tg mice in the homozygous DAP12ki and DAP10ko backgrounds. The Tg mice were maintained on the DAP12ki and DAP10ko background by mating the m157-Tg mice in the DAP12ki and DAP10ko backgrounds with non-Tg DAP12ki and DAP12ko mice, respectively. Mice were maintained under specific pathogen-free conditions and used after they reached 8 wk of age. The Animal Studies Committee at Washington University (St. Louis, MO) approved all animal studies.

Abs

The following Abs were obtained from eBioscience or BD Pharmingen: allophycocyanin-PK136 (anti-NK1.1), PerCP-Cy5.5-145-2C11 (anti-CD3), Alexa 488-XMG1.2 (anti–IFN-γ), PacBlue-XMG1.2 (anti–IFN-γ), and FITC-1D4B (anti-CD107a). Biotinylated 3D10 (anti-Ly49H) was purified from hybridomas by the Rheumatic Diseases Core Center Ab Production core and conjugated to biotin using EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce) according to the manufacturer’s protocol. Purified 4E4 (anti-Ly49D) and PK136 (anti-NK1.1) were obtained from hybridomas by the Rheumatic Diseases Core Center Ab Production core.

Adoptive transfer experiments

Spleen cell suspensions were generated by harvesting spleens into R10 media (RPMI 1640, 10% FCS) through a 70-μm nylon cell strainer (BD Falcon). Following passage through the strainer, splenocytes were centrifuged and RBCs were lysed by resuspending pelleted cells in 1 ml RBC lysing solution (Sigma-Aldrich) and incubating for 5 min at room temperature. The cells were then washed twice with R10, counted, and resuspended in R10 at 10^6 cells/ml. Donor splenocytes were labeled using the Vybrant CFDA SE Cell Trace Kit (Molecular Probes) per the manufacturer’s protocol. Briefly, splenocytes were washed with PBS and then resuspended in 1 μM CFSE (diluted in PBS) at a concentration of 100 × 10^6 cells/ml for 15 min in the dark at room temperature. The reaction was stopped by washing 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. In all injections NK cells made up 2–4% of the injection, cells were assessed for CFSE labeling as well as NK cell percentage.

Cytokine assays

Spleen cells suspensions were generated as described above. To coat plates, appropriate Ab was diluted to 2 μg/ml in PBS. One milliliter Ab (2 μg) was placed in 6-well tissue culture plates (Techno Plastic Products) and incubated at 37°C for 90 min. After incubation, the plates were washed with PBS three times prior to use for stimulation assays. For stimulation of NK cells, 1 ml splenocytes (10^7 cells/ml in R10) was incubated in 6-well plates coated with anti-NK1.1 mAb or anti-Ly49D mAb for 1 h and then further incubated in the presence of an 833-fold dilution of stock GolgiPlug (BD Pharmingen) for an additional 6–8 h, as previously described (25). Stimulation with RMA and RMAm157 cells was performed as previously described (18). Splenocytes were stained for NK1.1, CD3, and Ly49H using the Abs described above. To block nonspecific binding of Abs to Fc receptors, all Abs were diluted in the presence of mAb 2.4G2 (anti-Fc receptor II/III; American Type Culture Collection). Cells were fixed and permeabilized with a Cytofix/Cytoperm kit (BD Pharmingen) and then stained with either Alexa 488-XMG1.2 (anti–IFN-γ; BD Pharmingen) or PacBlue-XMG1.2 (anti–IFN-γ; BD Pharmingen) diluted in Perm/Wash buffer (BD Pharmingen). Cells were analyzed using a FACSCalibur or FACSCanto cytometer (BD Biosciences) gating on NK1.1^−, CD3^+ populations.

Degranulation assays

Spleen cell suspensions (at a concentration of 10^6 cells/ml) were generated as described above. YAC target cells were resuspended at a concentration of 10^6 cells/ml in R10 containing 2% monensin (eBioscience). Equal volumes (100 μl each) of spleen cells and YAC cells were incubated in 96 V-bottom wells (Nunc) for 2 h at 37°C. Following incubation, the cells were stained for NK1.1, CD3, Ly49H, and CD107a using the Abs described above.

Statistical analysis

The data were analyzed with Microsoft Excel X for Macintosh. An unpaired, two-tailed t test was used to determine statistically significant differences between experimental groups. Error bars in the figures represent the SEM.

Results

Signaling through the Ly49H receptor is necessary to induce Ly49H-mediated NK cell tolerance

Cell surface expression of the Ly49H activating receptor as well as signaling through the receptor requires association of Ly49H receptor with the adaptor molecule DAP12. Upon engagement of the Ly49H activating receptor, the DAP12 adaptor molecule is phosphorylated, which begins an intracellular signaling cascade resulting in the activation of the NK cell.

Recent work has demonstrated that continuous engagement of NK cell activating receptors results in NK cell tolerance both in vivo and in vitro. Although in vitro studies suggest that signaling through activating receptors is important for tolerance to be induced, it remained unclear whether this was the case in vivo (12, 20). To determine whether signaling through the Ly49H receptor (not just receptor engagement) was important to induce tolerance in the NK cells in vivo, we bred the m157-Tg mouse onto a DAP12ki background. The DAP12ki mouse is a DAP12 loss-of-function mutant where the wild-type (WT) C-terminal Y_{75}R_{86} amino acid stretch (YSDLNTRQYR) of the DAP12 protein is replaced with G_{75}I_{86} (GLQFEIDDEKKRNISI). This mutation results in the loss of tyrosine residues, whose phosphorylation is important for signaling mediated by DAP12 (23).

The breeding of the m157-Tg mouse onto the DAP12ki background resulted in a mouse that ubiquitously expressed the m157 protein but also contained NK cells in which the Ly49H activating receptor was associated with a mutant form of the DAP12 protein that could not be phosphorylated and thus would not allow for signal transduction upon engagement of the Ly49H receptor. Although the DAP12ki mutant does not allow for signaling through the Ly49H receptor, it does allow for expression of the receptor on the NK cell surface. Similar to the downregulation of the Ly49H receptor in m157-Tg mice in the C57BL/6 background, we observed decreased levels of the Ly49H receptor in m157-Tg mice in the DAP12ki backgrounds, suggesting that engagement with the m157 ligand was taking place (Fig. 1A).

As previously demonstrated, upon stimulation of NK cells through a Ly49H-independent manner with plate-bound anti-
NK1.1 mAb, Ly49H+ NK cells produce less IFN-γ as compared with Ly49H- NK cells in m157-Tg mice in the C57BL/6 background (18). Ly49H+ NK cells and Ly49H- NK cells from non-Tg mice in the C57BL/6 background produced similar levels of IFN-γ. Interestingly, Ly49H+ NK cells from both m157-Tg and non-Tg mice in the DAP12ki background produced similar amounts of IFN-γ to Ly49H- NK cells (Fig. 1B). This demonstrates that in the absence of signaling through the Ly49H receptor, tolerance is no longer established in the NK cells despite the fact that activating receptor engagement is still taking place. Thus, DAP12 signaling is required for Ly49H-mediated NK cell tolerance.

In addition to IFN-γ production, we assessed degranulation of NK cells upon stimulation with YAC target cells (another Ly49H-independent stimulation). Degranulation of NK cells was determined by the appearance of cell surface LAMP-1 (CD107a) following stimulation with YAC target cells. Degranulation of Ly49H+ NK cells from m157-Tg mice was significantly decreased compared with non-Tg mice in the C57BL/6 background. Similar to IFN-γ production, degranulation was not altered by the presence of the m157 transgene in the DAP12ki background (Supplemental Fig. 1). This further demonstrates the importance of DAP12 signaling in the induction of Ly49H-mediated tolerance.

Signaling through DAP10 is not important to induce Ly49H-mediated NK cell tolerance

Recent work suggests that the adaptor molecule DAP10 can also associate with the Ly49H activating receptor (12–14). To determine whether interaction and signaling through DAP10 plays a role in the induction of Ly49H-mediated NK cell tolerance, the m157-Tg mice were bred to the DAP10ko background. Molecular confirmation of the m157-Tg mice in the DAP10ko background was carried out by PCR (data not shown) as previously described (24). Similar to the C57BL/6 and DAP12ki background, downregulation of the Ly49H receptor was seen in m157-Tg in the DAP10ko background when compared with non-Tg mice from a similar background (Fig. 1A). Upon stimulation, the Ly49H+ NK cells from m157-Tg mice in the DAP10ko background produced decreased amounts of IFN-γ as compared with non-Tg mice in the DAP10ko background (Fig. 1B). This demonstrates that in the absence of the DAP10 adaptor protein, the continuous engagement of Ly49H with its ligand results in a Ly49H-mediated tolerance of the NK cells. Thus, DAP10 is not required for Ly49H-mediated NK cell tolerance.

Ly49H-mediated NK cell tolerance can be induced in mature NK cells

To determine whether receptor engagement/signaling during NK cell development was required for the induction of Ly49H-mediated NK cell tolerance, we performed adoptive transfer of labeled mature splenic NK cells into both m157-Tg and WT recipients. Transfer of WT NK cells into m157-Tg but not WT recipient mice resulted in downregulation of the Ly49H receptor (Fig. 2A). By 7 d posttransfer, Ly49H+ NK cells from donor NK cells transferred into m157-Tg mice produced less IFN-γ than did Ly49H- NK cells. Ly49H+ and Ly49H- NK cells from donor NK cells transferred into non-Tg mice produced similar levels of IFN-γ.
Ly49H-mediated NK cell tolerance occurs quickly following adoptive transfer of mature NK cells

To better understand the time course in which Ly49H-mediated NK cell tolerance can be induced, we examined donor WT NK cells at 6 and 24 h posttransfer into WT or m157-Tg recipient mice. At 6 h posttransfer, there was no difference in IFN-γ production by donor WT NK cells transferred into either WT or m157-Tg recipients upon stimulation with plate-bound PK136. Furthermore, there was no baseline IFN-γ production or difference in NK cell activation marker expression (CD69) or NK cell maturation marker (Mac1) in the two groups of mice, even though downregulation of the Ly49H receptor was noted (Supplemental Fig. 2). Twenty-four hours after transfer of donor NK cells from the C57BL/6 and DAP10ko background, differences in IFN-γ production could be detected in Ly49H+ NK cells upon transfer into m157-Tg mice (Fig. 4). No difference in IFN-γ production was seen at 24 h when NK cells from DAP12ki mice were used as the donors. Additionally, at 24 h, there was no baseline production of IFN-γ by donor WT NK cells upon transfer into m157-Tg recipients. Finally, there was no difference in NK cell activation marker (CD69) or NK cell maturation marker (Mac1) expression in the WT donor NK cells upon transfer into m157-Tg or WT recipient mice (Supplemental Fig. 3).

Ly49H-mediated NK cell tolerance is reversible in vivo

To determine whether the Ly49H-mediated induced tolerance was fixed, we adoptively transferred splenocytes from m157-Tg donor mice into m157-Tg or WT recipient mice. Seven days after transfer, we assessed donor NK cells for the expression of Ly49H and for their ability to produce IFN-γ following stimulation through the NK1.1 activating receptor. By 7 d, donor m157-Tg cells transferred into WT recipient mice expressed normal levels of Ly49H on their cell surface (Fig. 5A). Additionally, m157-Tg donor NK cells transferred into WT recipient mice produced significantly more IFN-γ as compared with identical donor cells transferred into m157-Tg recipient mice (Fig. 5B). To address kinetics of this gain of function, we assessed donor m157-Tg NK cells at 24 and 72 h after transfer. By 24 h, donor m157-Tg NK cells transferred into WT recipient mice were expressing almost normal levels of Ly49H as compared with donor m157-Tg NK cells transferred into m157-Tg recipients. Additionally, donor m157-Tg NK cells transferred into WT recipients were starting to produce more IFN-γ (Fig. 2B; see also Ref. 18). Decreased IFN-γ production by Ly49H+ NK cells was seen upon transfer into m157-Tg mice when NK cells were stimulated with either plate-bound anti-NK1.1 or anti-Ly49D (Fig. 2A, 2B). This demonstrates that NK cell tolerance can be mediated through continuous engagement of activating receptor in mature NK cells.

To determine whether signaling through either DAP12 or DAP10 adaptor proteins is important for the induction of Ly49H-mediated NK cell tolerance seen in adoptively transferred mature NK cells, we transferred donor mature splenic NK cells from DAP12ki and DAP10ko mice into non-Tg or m157-Tg recipient mice. Donor Ly49H+ NK cells from both DAP12ki and DAP10ko backgrounds demonstrated downregulation of the Ly49H receptor upon transfer into m157-Tg recipient mice as compared with transfer into WT recipient mice (Fig. 3A). Unlike donor cells from the C57BL/6 and DAP10ko background, DAP12ki Ly49H+ donor NK cells produced similar levels of IFN-γ when transferred into m157-Tg or non-Tg mice (Fig. 3B). This result, along with the previous results, clearly demonstrate that DAP12-mediated signaling through the Ly49H receptor, and not engagement alone, is necessary to induce Ly49H-mediated NK cell tolerance.
γ upon stimulation through the NK1.1 activating receptor than those transferred into m157-Tg recipients. Similar results were seen at 72 h (Supplemental Fig. 4). Thus, upon removal of activating receptor stimulation NK cells regain normal function, suggesting that Ly49H-mediated NK tolerance is not a fixed defect in the NK cells.

Discussion

The chronic engagement of the Ly49H activating receptor with m157 results in the impairment of NK cell function in vitro and in vivo (18, 19). In the studies described in this article, we demonstrate that NK cell impairment resulting from sustained engagement of Ly49H activating receptor in vivo requires signaling through the DAP12 adaptor molecule and is independent of DAP10. Additionally, we demonstrate that Ly49H-mediated NK cell tolerance can be established in mature NK cells and that the Ly49H-mediated NK cell tolerance is reversible in vivo.

The use of an in vivo transgenic mouse model offers advantages over in vitro models to study activation receptor-induced NK cell hyporesponsiveness. Primarily, NK cell activation assays in these experiments were performed on freshly isolated NK cells without the need for additional cytokines necessary for growing NK cells in vitro. Although it is necessary to use cytokines to grow in vitro, it is unclear whether these lymphokine-activated killer cells truly represent NK cell function in a mouse or human. Additionally, freshly isolated murine NK cells are poor effector cells when used in killing assays. To generate more efficient killing, NK cells are grown in high levels of IL-2 for extended periods of time. In these experiments we focused on IFN-γ production and degranulation because such assays could be performed reproducibly with freshly isolated NK cells. The use of freshly isolated splenocytes likely represents true NK cell function and physiology better than cytokine-stimulated cells in vitro.

Studies of transgenic mice expressing ligands (Rae-1ε, MICA) for the NKG2D activation receptor provide additional evidence for NK cell tolerance due to engagement of an activation receptor (21, 22). In addition to decreased levels of NKG2D receptor levels on the NK cell surface, sustained expression of Rae-1ε resulted in a defect in natural cytotoxicity to Rae1-expressing targets, as well as more general impairment in NK cell function, such as reactivity against MHC class I-deficient targets (22). Similarly, sustained MICA expression resulted in defects in NK cell cytotoxicity against MICA-expressing target cells (21). These results are similar to the Ly49H downregulation and the defects in the Ly49H+ NK cells that we observed in the m157-Tg mice (18).

Following engagement of the Ly49H receptor with m157, there is a decrease in the level of Ly49H receptor on the cell surface of the NK cell (7, 18). It remained unclear whether the engagement of the receptor alone or whether signaling through the receptor was necessary for activation receptor-induced tolerance to occur. Our studies clearly demonstrate that signaling through activating
In agreement with these findings, our studies also demonstrate that mature NK cells can reset their functional activity when exposed to a different environment; specifically, mature NK cells can acquire a tolerant phenotype upon transfer into mice that express activating receptor ligands. Additionally, even though donor WT NK cells appeared to engage ligand as early as 6 h posttransfer into m157-Tg recipient mice (as demonstrated by the downmodulation of the Ly49H receptor), they did not produce IFN-γ at baseline and there was no difference in the expression of activation markers on donor WT NK cells that were transferred into either WT or m157-Tg mice. This suggests that the donor Ly49H+ NK cells are not becoming activated upon transfer to m157-Tg recipient mice. Instead, the Ly49H+ NK cells that had matured in an m157-free environment became less responsive to stimulation after transfer into m157-Tg mice. This demonstrates that activation receptor-induced hypo-responsiveness is not developmentally regulated and can be induced by activating receptors that do not recognize “self” proteins.

Not only can the hypo-responsiveness be induced in mature splenic NK cells, but it is also reversible in vivo. When splenocytes from m157-Tg mice were transferred into WT recipient mice, Ly49H expression levels increased on the cell surface of the NK cells and they regained function. This recovery appeared to occur within 24 h posttransfer. This is in agreement with previously published in vitro data (12, 18, 20). This also suggests that trans interactions of the Ly49H receptor with a ligand on another cell are significantly more important than cis interactions in the induction of NK cell tolerance.

The fact that mature NK cells can become hypo-responsive upon engagement of an activating receptor is quite intriguing. Recent studies suggest that NK cells have an ability to adapt to previous stimuli and change subsequent responses (29, 30). Activating receptor-induced tolerance may represent another adaptive response of the NK cell. The induced hypo-responsiveness may be an adaptive response that provides a control mechanism to limit tissue damage by NK cells during responses to infection. However, it is possible that such adaptive responses may result in NK cells adapting to a tumor or a chronic infection rather than attacking it.

There is evidence in both mouse and human models suggesting that induced NK cell hypo-responsiveness plays a role in the pathogenesis of disease. For example, most NK cells in patients with acute myeloid leukemia, unlike healthy individuals, display low levels of natural cytotoxicity receptors and have an associated functional defect (31). Similarly, patients with HIV infection have NK cells that display low natural cytotoxicity receptor levels and have also decreased function (32, 33). The basis for the decrease in natural cytotoxicity receptor on the NK cells in these situations remains unclear, but it is possible that the continuous engagement of the receptors with their ligand is responsible for the low levels of the receptor and associated functional defects. In fact, recent work demonstrating that human NK cells expressing an activating receptor (KIR2DS1) together with its HLA class I molecule ligand (HLA-C2) are hypo-responsive to stimulation with cellular targets (34) suggests that continuous activating receptor engagement in human NK cells can indeed result in their functional defect.

A large number of tumor cells express NKG2D ligands constitutively (35–38). The fact that NKG2D ligand-expressing tumors progress suggests that NKG2D function may be impaired during tumor progression. In vitro studies have demonstrated that chronic engagement of cell-bound NKG2D ligand can result in NK cell hypo-responsiveness (12, 20). Furthermore, analysis of lung adenocarcinoma-infiltrating NK cells revealed decreased cytotoxicity associated with low NKG2D (39). These findings, along with...
our adoptive transfer studies, suggest that normal NK cells could be made hypofunctional by the engagement of an activating receptor in a setting where activation of the NK cell would be the desired outcome. These issues become extremely relevant when one considers using NK cell-based therapies in the treatment of cancer and other diseases. Interestingly, recent work has demonstrated that in a transgenic mouse model where NK cells were continuously stimulated through an NKG2D receptor ligand (Rae-1), NKG2D-independent function remained intact in vivo. In particular, response to MCMV infection was unaltered in the Rae-1 Tg mice (40). It is possible that in the context of viral infection, the inflammatory milieu and released cytokines can break activating receptor-induced NK cell tolerance. Clearly, further studies are required to better understand the factors in vivo that determine the outcome of activating receptor engagement (activation versus induced hyporesponsiveness).

Acknowledgments
We thank Wayne M. Yokoyama (Washington University) for helpful discussions and for providing the 4E4 (anti-Ly49D), 3D10 (anti-Ly49H), and PK136 (anti NK1.1) mAbs. The DAP12ki mice were a gift from Eric Vivier (Centre National de la Recherche Scientifique-INSERM, Université de la Méditerranée, Marseille, France). We are grateful to Marco Colonna and Susan Gilfillan (Washington University) for providing the DAP10ki mice. We thank Anthony French, Rodney Newberry, Julie Elliot, Megan Cooper, and Deborah Lenschow for comments on this manuscript.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental figure 1. DAP12 required for degranulation defect seen in tolerant NK cells. (A) Representative dot plots demonstrating CD107a (LAMP-1) expression by freshly isolated splenocytes from WT, m157-Tg, DAP12ki or m157-TgDAP12ki mice stimulated with YAC cell targets. The numbers represent the percentage of Ly49H⁺ or Ly49H⁻ NK cells expressing CD107a. The dot plots were gated on NK cells (NK1.1⁺, CD3⁻ lymphocytes). (B) The ratio of the percentage CD107a expressing Ly49H⁺ NK cells to the percentage of CD107a expressing Ly49H⁻ NK cells in non-Tg and m157-Tg mice in the C57BL/6 and DAP12ki. The graph represents the mean ± the SEM. Ratios were calculated after stimulation with YAC target cells. The number of mice used in each group: WT=11, m157Tg=10, DAP12ki=6 and m157TgDAP12ki=6.

Supplemental figure 2. Assessment of donor NK cells 6 hours post transfer of WT splenocytes into WT or m157Tg mice. (A) Representative dot plots demonstrating IFN-γ production by freshly isolated donor splenocytes stimulated with PBS or plate bound anti-NK1.1 mAb at 6 hours post transfer. The numbers represent the percentage of Ly49H⁺ or Ly49H⁻ NK cells producing IFN-γ. The dot plots were gated on donor NK cells (NK1.1⁺, CD3⁻, CFSE⁺ 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→WT (n=4) or WT→m157-Tg (n=6) mice following stimulation with plate bound anti-NK1.1 mAb. The results are presented as the mean ± SEM. (C) Assessment of NK cell activation markers (CD69) and maturation marker (Mac1) on cell surface of donor NK cells at 6 hours post transfer.

Supplemental figure 3. Assessment of donor NK cells 24 hours post transfer of WT splenocytes into WT or m157Tg mice. (A) Representative dot plots demonstrating IFN-
γ production by freshly isolated donor splenocytes stimulated with PBS or plate bound anti-NK1.1 mAb at 24 hours post transfer. The numbers represent the percentage of Ly49H⁺ or Ly49H⁻ NK cells producing IFN-γ. The dot plots were gated on donor NK cells (NK1.1⁺, CD3⁻, CFSE⁺ 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→WT (n=6) or WT→m157-Tg (n=8) mice following stimulation with plate bound anti-NK1.1 mAb. The results are presented as the mean ± SEM. (C) Assessment of NK cell activation markers (CD69) and maturation marker (Mac1) on cell surface of donor NK cells at 6 hours post transfer.

Supplemental figure 4. Kinetics of the reversal of tolerance upon transfer of m157-Tg NK cells into WT mice. (A) Representative dot plots demonstrating IFN-γ production by freshly isolated donor splenocytes stimulated with plate bound anti-NK1.1 mAb at 24 and 72 hours post transfer. The dot plots were gated on donor NK cells (NK1.1⁺, CD3⁻, CFSE⁺ cells). The numbers represent the percentage of Ly49H⁺ or Ly49H⁻ NK cells producing IFN-γ. The dot plots were gated on donor NK cells (NK1.1⁺, CD3⁻, CFSE⁺ 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 m157-Tg→WT (n=6 at 24 hr and n=5 at 72 hr) or m157-Tg→m157-Tg (n=3 at both 24 and 72 hr) mice following stimulation with plate bound anti-NK1.1 mAb at either 24 or 72 hours post transfer. The results are presented as the mean ± SEM.
Supplemental figure 1.

A

No target | YAC target
---|---
Ly49H  
13.0
4.5
9.3
12.0
9.8
9.5

CD107a

B

WT
m157Tg
DAP12ki
m157TgDAP12ki

Ly49H+/Ly49H- CD107a expression

WT | m157Tg | DAP12ki | m157TgDAP12ki
---|---|---|---
1.2 | 0.4 | 0.8 | 0.6
0.8 | 0.4 | 0.8 | 0.6

Mean ± SEM
Supplemental figure 2.

A

WT → WT
WT → m157Tg

Ly49H

PBS
PK136

19
17
18

IFN-γ

B

Ly49H⁺/Ly49H⁻ IFN-γ production

WT
m157Tg

recipient

C

WT → WT
WT → m157Tg

Ly49H

CD69

Ly49H

Mac1
Supplemental figure 3.

A

Ly49H

WT → WT

WT → m157Tg

IFN-γ

28

16

PK136

28

26

B

Ly49H+Ly49H IFN-γ production

p < 1 x 10^-5

WT

m157Tg

recipient

C

Ly49H

WT → WT

WT → m157Tg

CD69

Ly49H

Mac1
Supplemental figure 4.

A

24 hrs post transfer

m157-Tg → m157-Tg

m157-Tg → WT

Ly49H

11.0

24.2

IFN-γ

72 hrs post transfer

m157-Tg → m157-Tg

m157-Tg → WT

Ly49H

7.0

12.0

IFN-γ

B

24 hrs post transfer

Ly49H+Ly49H− IFN-γ production

m157Tg WT

p = 0.04

72 hrs post transfer

Ly49H+Ly49H− IFN-γ production

m157Tg WT

p = 0.06

recipient