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Acquisition of Activation Receptor Ligand by Trogocytosis Renders NK Cells Hyporesponsive

Cathrine A. Miner, Tusar K. Giri, Claire E. Meyer, Mark Shabovich, and Sandeep K. Tripathy

Because NK cells secrete cytotoxic granules and cytokines that can destroy surrounding cells and help shape the subsequent immune response, they must be kept under tight control. Several mechanisms, at different levels, are in place to control NK cell function. In this study, we describe a novel mechanism regulating NK cell function in which NK cells acquire ligands for activating receptors from target cells by trogocytosis, rendering the NK cells hyporesponsive. In this model, murine NK cells acquire m157, the murine CMV-encoded ligand for the Ly49H-activating receptor, from target cells both in vitro and in vivo. Although acquisition of m157 requires cell-to-cell contact, it does not require the expression of the Ly49H receptor by the NK cell. Acquired m157 protein is expressed on the NK cell surface with a glycosylphosphatidylinositol linkage and interacts with the Ly49H receptor expressed on the NK cell. This interaction results in blocking the Ly49H receptor that prevents the NK cells from recognizing m157-expressing targets and continuous engagement of the Ly49H-activating receptor, which results in the hyporesponsiveness of the Ly49H⁺ NK cell to stimulation through other activating receptors. Thus, NK cell acquisition of a ligand for an activation receptor by trogocytosis renders them hyporesponsive. This mechanism, by which mature NK cell function can be altered, has important implications in regard to how NK cells respond to tumors in specific microenvironments as well as the use of expanded NK cells in treating various malignancies. The Journal of Immunology, 2015, 194: 1945–1953.
formation of the IS allows for transfer of molecules from one cell to another. This has been demonstrated in T cells, B cells, and NK cells (27–30). The transfer of proteins has been demonstrated to have functional consequences on the recipient cells. For instance, the incubation of NK cells with MHC class I polypeptide-related sequence MIC B (MICB)–expressing targets resulted in the exchange of NK 2, member D (NKGD2), and MICB proteins. This exchange was associated with a reduction in the cytotoxicity of these NK cells upon subsequent encounters with MICB-expressing target cells (19). Another study demonstrated that MICA could be transferred from the target cell to the NK cell, and the acquired MICA protein was expressed on the cell surface such that it could interact with the NKG2D receptor and influence the function of other NK cells (31). More recent studies suggest that NK cells that acquire ligands for activating receptors by trogocytosis become viable targets for nearby NK cells (32), providing yet another mechanism of controlling NK cell function once they have been activated.

In this study, we demonstrate that NK cells quickly acquire the m157 ligand from target cells and express them on their cell surface as a GPI-linked protein. The acquisition of m157 from the target cells is independent of expression of Ly49H by the NK cell. Cell-to-cell contact, however, is necessary for transfer of m157 to occur. Upon transfer to the NK cell, m157 can interact with Ly49H expressed on the cell surface of the recipient NK cell. Furthermore, we demonstrate that the acquisition of m157 by NK cells makes them functionally defective both in vitro and in vivo. Specifically, they are less efficient at killing m157-expressing target cells, and they are hyporesponsive when stimulated through another activating receptor. We propose a model in which the acquisition of m157 by trogocytosis allows for the interaction of m157 and Ly49H on the NK cell surface. This interaction not only has a blocking effect such that the NK cells can no longer detect m157 on targets, but it also provides a mechanism by which the continuous engagement of the Ly49H receptor with m157 can occur. This results in the NK cells becoming hyporesponsive secondary to the continuous engagement of the activating receptor.

Materials and Methods

Mice and cell lines

The m157-transgenic (Tg) mouse has been previously described (8). The DAP12ki mice, obtained from E. Vivier (Centre d’Immunologie de Marseille-Luminy, Marseille), as well as the B6.BXD8 mice, obtained from W. Yokoyama (Washington University), have both been previously described (33, 34). The CD45.1+ mice (B6.SJL-Pgk-Cre; Pep3b/BoyJ) were purchased from The Jackson Laboratory. 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) reviewed and approved all animal studies. The RMA (Rauscher virus-induced murine T cell lymphoma), RMAm157 (RMA cells that stably express m157), RMA-S (RMA cells that express markedly reduced levels of Ly49H) (19), rescued as described above, were maintained for an additional 3–4 d before use. This protocol yielded lymphokine-activated killer (LAK) cells in which 60–90% of the cells were NK1.1+, CD3+. Abs

The following Abs were obtained from eBioscience or BioLegend: allophycocyanin–PK136 (anti-NK1.1), PerCP-Cy5.5–145-2C11 (anti-CD3), PerCP-Cy5.5–145-1D3 (anti-CD19), Alexa488–XMGl2.1 (anti–IFN–γ), PacBlue–XMGl2.1 (anti–IFN–γ), FITC–104 (anti-CD45.2), FITC–KH95 (anti–H2-D), 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 Rheumatic Disease Core Center at Washington University and conjugated to biotin using EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce), according to manufacturer’s protocol. Purified PKJ36 (anti-NK1.1) was purchased from BioXcell.

In vitro trogocytosis assays

LAK cells (1 × 10^5) were cocultured in 96-well, flat-bottom plates with either RMA or RMAm157 cells at a 1:1 ratio and harvested at the indicated time points. For studies using fresh NK cells, spleen-cell suspensions were prepared, as previously described. Splenocytes (1 × 10^6) were cocultured with RMA, RMAm157, BaF, or BaFm157 cells (5 × 10^3) in 96-well, flat-bottom plates for 2 h. All cells were harvested; stained for NK1.1, CD3, CD19, and m157; and acquired using a FACSCalibur machine (BD Biosciences). The data were analyzed using FlowJo software (Tree Star).

Transwell assays

LAK cells were plated into 24-well cell culture insert companion plates (Corning). RMAm157 cells were plated into Transwell inserts (0.4 μm; Corning) atop the LAK cells so that media exchange was allowed but cell-to-cell contact was not. Cells were incubated for 30 min, and LAK cells were harvested from the plates. Cells were stained and assessed for m157 expression on NK1.1+ CD3+ cells. Cells were acquired using a FACS Calibur machine (BD Biosciences). Flow cytometry data were analyzed by FlowJo software (Tree Star).

Adoptive transfer experiments

Spleen-cell suspensions were generated from CD45.2+ donor mice, as described above. Cells were washed with PBS and then resuspended at 250 × 10^6 cells/ml. Recipient mice (CD45.1+) were injected i.v. in the tail vein with 200 μl splenocytes (~50 × 10^6 total splenocytes). Mice were then harvested at the indicated time points. Donor NK cells were identified as NK1.1+, CD3+, CD19+, and CD45.2+.

Phosphatidylinositol-specific phospholipase C treatment

After a 30- to 40-min incubation of NK cells with RMAm157 cells, the cells were harvested, washed, and incubated with either 1 ml PBS or phosphatidylinositol-specific phospholipase C (PI-PLC; 1.25 U/ml; Molecular Probes) diluted in PBS for 20 min on ice. After washing, cells were stained for m157 or Ly49H, as well as NK1.1 and CD3. Cells were acquired using a FACS Calibur machine (BD Biosciences). Flow cytometry data were analyzed by FlowJo software (Tree Star).

In vitro killing assay

LAK cells were labeled with CFSE (1 μM; Invitrogen) and then cocultured with either RMA or RMAm157 cells at a 1:1 ratio for 30 min. CFSE+ LAKs were sorted purified by FACS Aria II (BD Biosciences) and used as effector cells at 3:1 or 10:1 E:T ratios. The RMAm157 or RMA-S target cells (1 × 10^5) were cocultured with the RMA or RMAm157 “dressed” LAK cells for 2 h in 96-well U-bottom plates. Cells were stained for NK1.1 and propidium iodide (Sigma-Aldrich). The target cells were identified as NK1.1+, CFSE–, and assessed for propidium iodide incorporation. The cells were acquired using a FACS Calibur machine (BD Biosciences). Flow cytometry data were analyzed by FlowJo software (Tree Star).

IFN-γ assays

Spleen-cell suspensions were generated, as described above. To coat plates, PK136 mAb (anti-NK1.1) was diluted to 2–4 μg/ml in PBS. A total of 1 ml Ab (2–4 μg) was placed in six-well tissue culture plates (Techno Plastic Product) and incubated at 37°C for 90 min. After incubation, the plates were washed with PBS to remove unbound antibody. For stimulation of NK cells, 1 ml splenocytes (10^6 cells/ml in R10) were incubated in six-well plates coated with anti-NK1.1 mAb for 30 min and then further incubated in the presence of a 333-fold dilution of stock
brefeldin A (GolgiPlug; BD PharMingen) for an additional 6–8 h. Cells were harvested and stained for the surface markers NK1.1, CD3, CD45.1, and Ly49H. Cells were then fixed and permeabilized using Cytofix/Cytoperm solution (BD PharMingen) and stained for intracellular IFN-γ. The cells were acquired using a FACS Canto machine (BD Biosciences). Flow cytometry data were analyzed by FlowJo software (Tree Star).

**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 SEM.

**Results**

NK cells rapidly acquire m157 on their cell surface when incubated with m157-expressing target cells, and m157 is expressed on the cell surface as a GPI-linked protein

To determine whether m157 could be acquired from target cells, IL-2-activated NK cells from C57BL/6 mice were incubated in vitro with RMA or RMAm157 cells. At various time points, the NK cells were assessed by flow cytometry for the presence of m157 on their cell surface using the gating strategy shown in Supplemental Fig. 1A. As demonstrated in Fig. 1A, the NK cells acquired m157 rapidly after coculture with RMAm157 with 50% of the NK cells expressing the m157 protein on their cell surface by 30 min. The percentage of NK cells expressing m157 was maximal by 2 h. No m157 was detected on the NK cells when they were incubated with RMA cells. We also assessed the mean fluorescence intensity (MFI) of m157 on NK cells incubated with RMAm157 cells. Similar to the increase in the percentage of NK cells that expressed m157, we observed that the MFI of the m157 on NK cells was increased when they were incubated with RMAm157 cells (Fig. 1B). There was no increase in the MFI of m157 when NK cells were incubated with RMA cells.

The m157 protein is expressed on the surface of MCMV-infected cells as a GPI-linked protein (16). To determine whether the acquired m157 on dressed NK cells was also anchored to the cell surface by GPI linkage, we treated dressed NK cells with PI-PLC, an enzyme that cleaves GPI-linked proteins. Upon treatment with PI-PLC, the percentage of NK cells that expressed m157 on their cell surface decreased significantly compared with those treated with just PBS as a control (Fig. 1C). In addition, the MFI of the m157 on dressed NK cells decreased upon treatment with PI-PLC compared with PBS (Fig. 1D). This demonstrates that the m157 present on the dressed NK cells is anchored on the cell surface as a GPI-linked protein.

**Acquisition of m157 by NK cells requires cell-to-cell contact**

To determine whether cell-to-cell contact was required for transfer of m157 from target cells to IL-2-activated NK cells, we incubated the NK cells with RMAm157 in the presence or absence of transwells and assessed the percentage of NK cells that acquired m157 as well as the MFI of m157 by flow cytometry. As seen in Fig. 1E, in the presence of a transwell, which allows for secreted or shed proteins to cross between cells, but does not allow cell contact, acquisition of m157 by NK cells is essentially abolished when compared without the transwell. Furthermore, there was a significant decrease in the MFI of m157 on the dressed NK cells in the presence of the transwell (Fig. 1F). This demonstrates that the acquisition of m157 by the NK cells is not due to m157 being secreted or shed by target cells and then picked up by the NK cell, but rather acquisition of the protein by a mechanism that requires cell-to-cell contact.

**Acquisition of m157 by NK cells is independent of the Ly49H receptor**

To determine whether the Ly49H receptor is required for the acquisition of m157 onto the NK cells, we used IL-2-activated NK cells that were isolated from B6.BXD8 mice. These mice, as previously described, are identical to C57BL/6 mice, except that their NK cells do not express the Ly49H receptor (34). The NK cells from the B6.BXD8 mice, unlike WT NK cells, did not express Ly49H (Supplemental Fig. 1B). Similar to WT NK cells, upon incubation with RMAm157 cells, we observed rapid accumulation of m157 on the surface of these NK cells. This was not seen when the NK cells were incubated with RMA cells (Fig. 2A).

In addition, the transferred m157 on the cell surface of B6.BXD8 NK cells was attached by GPI linkage, as the percentage of NK cells expressing m157 as well as the MFI of m157 on the dressed NK cells is anchored on the cell surface as a GPI-linked protein (16). To determine whether the acquisition of m157 by NK cells is cell-to-cell contact dependent, we performed the MFI of target cells and then picked up by the NK cell, but rather acquisition of the protein by a mechanism that requires cell-to-cell contact. In addition, we transferred m157 on the cell surface of B6.BXD8 NK cells was attached by GPI linkage, as the percentage of NK cells expressing m157 as well as the MFI of m157 on the dressed NK cells is anchored on the cell surface as a GPI-linked protein (16). To determine whether the acquisition of m157 by NK cells is cell-to-cell contact dependent, we performed the MFI of target cells and then picked up by the NK cell, but rather acquisition of the protein by a mechanism that requires cell-to-cell contact.

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Acquired m157 can interact with Ly49H on the NK cell surface

To determine whether acquired m157 interacts with the Ly49H receptor expressed on the cell surface of IL-2–activated NK cells, we used a flow cytometry–based cell surface–staining approach. The engagement of Ly49H with m157 decreases the detectable level of Ly49H on the cell surface of NK cells when assessed by flow cytometry (8, 35). This could be secondary to down-modulation of the receptor in response to engagement of ligand or the fact that the m157/Ly49H interaction prevents efficient binding of the 3D10 mAb to Ly49H. If Ly49H interacts with m157 on the cell surface, we hypothesized that removal of acquired m157 would result in higher detectable levels of Ly49H on the Ly49H+ NK cells. This suggests that the amount of protein acquired by the NK cells corresponds to levels of the proteins expressed on the target cell.

Acquisition of m157 occurs in vivo with fresh NK cells

The previous experiments described in this study used LAK cells, which are NK cells that have been grown in culture for 1 wk in media containing IL-2. Although this enriches and amplifies the number of NK1.1+, CD3− NK cells, fresh NK cells are functionally different from fresh NK cells. To determine whether fresh NK cells can acquire m157 from target cells, we incubated splenocytes from non-Tg mice with RMA or RMAm157 cells (Fig. 3D, 3E). This suggests that the m157 acquired via trogocytosis can interact with the Ly49H receptor on the NK cell surface.

Acquired m157 can interact with Ly49H on the NK cell surface

(4) B6.BXD8 LAKs were incubated with RMAm157 cells for 2 h in the presence (open histogram) and absence (dashed histogram) of PI-PLC. (5) Representative histogram depicting sorted m157 dressed LAKs that were assessed for Ly49H levels in the presence (open histogram) and absence (dashed histogram) of PI-PLC. (E) MFI of Ly49H on Ly49H+ sorted dressed LAKs in the absence (black, n = 6) or presence (white, n = 6) of PI-PLC. All data are presented as the mean ± SEM. *p < 0.0005.

Ly49H on the cell surface, treatment with PI-PLC (which decreased m157 levels on the cell surface through cleavage of its GPI linkage) increased the level of Ly49H detected on the Ly49H+ NK cells (Fig. 3D, 3E). This suggests that the m157 acquired via trogocytosis can interact with the Ly49H receptor on the NK cell surface.
line (Fig. 4B). To assess whether signaling through the Ly49H receptor was necessary for acquisition of m157, we incubated splenocytes from B6.BXD8 DAP12ki or WT mice with the target cells. The DAP12ki mice, which have been previously described, contain mutations in the tyrosine residues within DAP12 that prevent phosphorylation and thus prevent signaling through the Ly49H receptor (33). As shown in Fig. 4B, NK cells from both the B6.BXD8 and DAP12ki mice were able to acquire m157 on their cell surface from the different target cells, demonstrating that neither signaling through the receptor, nor the receptor itself, was required for trogocytosis of m157.

To determine whether our in vitro findings also apply in vivo, we adoptively transferred splenocytes from WT and B6.BXD8 mice into m157-Tg or non-Tg mice and assessed by flow cytometry for the acquisition of m157 by the donor NK cells 24 h posttransfer. As can be seen in Fig. 4C, adoptively transferred NK cells from both WT and B6.BXD8 mice were able to acquire m157 on their cell surface from the different target cells, demonstrating that neither signaling through the receptor, nor the receptor itself, was required for trogocytosis of m157.

To establish whether the acquisition of m157 onto NK cells resulted in functional defects, we assessed killing of m157-expressing target cells. Following incubation of IL-2–activated NK cells with RMA or RMAm157 cells, the NK cells were sorted from the target cells. The sorted RMA dressed and sorted RMAm157 dressed NK cells, as well as NK cells from B6.BXD8 mice were incubated with RMAm157 target cells at 3:1 and 10:1 ratios. Death of the target cells was determined by propidium iodide incorporation into dead cells using flow cytometry (Fig. 5A). At both ratios, the RMAm157 dressed NK cells were significantly worse at killing RMAm157 cells as compared with RMA dressed NK cells, although both were more effective than NK cells from the B6.BXD8 mice (Fig. 5B). Background levels of cell death were noted when RMA cells were used as the target, which were similar to the B6.BXD8 killing of RMAm157 cells (data not shown). In addition, we assessed whether coculture with RMAm157 would impact IL-2–activated NK cell killing of other tumor targets. Sorted RMAm157 dressed IL-2–activated NK cells killed RMA-S targets as well as, if not better, than sorted RMA dressed IL-2–activated NK cells (Supplemental Fig. 3). This demonstrates that m157 dressed NK cells are less effective at killing m157-expressing target cells than non-m157 dressed NK cells and the defect is specific for m157-expressing targets.

**FIGURE 4.** Acquisition of m157 occurs with fresh NK cells and in vivo. (A) WT splenocytes were incubated with RMA (○) or RMAm157 (●) cells and assessed by flow cytometry for the percentage of NK (NK1.1+, CD3−) cells that were positive for m157 on their cell surface at various time points. (B) Splenocytes from B6.BXD8 DAP12ki, or WT mice were incubated with RMA (black), RMAm157 (white), BaF (gray), or BaFm157 (hatched) cells for 2 h and assessed by flow cytometry for the percentage of NK (NK1.1+, CD3−) cells that were positive for m157 on their cell surface (n = 6 for all groups except for WT with RMA or RMAm157, where n = 3). (C) Adoptive transfer of 50 million WT or B6.BXD8 donor splenocytes into non-Tg (black) and m157-Tg (white) recipient mice. Donor NK cells were assessed by flow cytometry for the acquisition of m157 on their cell surface (n = 5–17). (D) WT and B6.BXD8 donor NK cells were obtained 24 h posttransfer into m157-Tg recipient mice (n = 4–5). The percentage of NK cells expressing m157 was assessed by flow cytometry before (black) and after treatment with PI-PLC (white). All data are presented as the mean ± SEM. *p < 0.005.
Expression of the Ly49H receptor is required to induce NK cell hyporesponsiveness

Although Ly49H is not necessary for the acquisition of m157 from target cells, we wanted to determine whether the receptor was required for NK cell defects in the production of IFN-γ upon stimulation through the NK1.1-activating receptor. We injected m157-Tg or non-Tg recipient mice with either non-Tg WT donor splenocytes, non-Tg B6.BXD8 donor splenocytes, or non-Tg DAP12ki splenocytes. Days postinjection, splenocytes were harvested and stimulated through the NK1.1 receptor and the donor NK cells were assessed for IFN-γ production. The WT, B6.BXD8 and DAP12ki donor NK cells all acquired m157 on their cell surface when injected into m157-Tg recipient mice (Fig. 6A). Of note, the level of m157 expression on the surface of m157-Tg recipients as compared with non-Tg recipients (Fig. 6B).

However, although both donor non-Tg B6.BXD8 and DAP12ki NK cells acquired m157 when injected into a m157-Tg recipient, they produced similar levels of IFN-γ as those injected into a non-Tg recipient (Fig. 6B). In addition, we separated the NK cell populations from WT and DAP12ki donors into Ly49H⁺ and Ly49H⁻ NK cells and assessed IFN-γ production from these groups individually upon transfer into non-Tg or m157-Tg recipient mice. Similar to the bulk NK cell population, we only observed IFN-γ production defects in the Ly49H⁺ NK cell population upon transfer into m157-Tg mice (Fig. 6C). Our data demonstrate that NK cells that interact with m157 through the Ly49H receptor on their cell surface are least effective at producing IFN-γ upon stimulation through the NK1.1 receptor. Thus, it appears that acquisition of target cell membrane proteins by NK cells can alter their function (Fig. 7).

Discussion

Continuous engagement of activating receptors on NK cells renders them hyporesponsive when stimulated through other activating receptors, a process that occurs both in vitro and in vivo (10, 11, 35–37). We have demonstrated that Ly49H⁺ NK cells from WT mice, upon transfer into a m157-Tg environment, become hyporesponsive when stimulated through other activating receptors (35). It remained unclear how the Ly49H receptor on transferred NK cells remained continuously engaged with m157, resulting in a hyporesponsive donor NK cell.

In this study, we have provided evidence that NK cells can acquire ligands for activating receptors (in this case m157) on their cell surface upon incubation with cells expressing the ligand. The
H2-D\textsuperscript{d} expression on the NK cell surface, Ly49A is efficiently receptors (Ly49A) on the NK cell surface has been documented another mechanism to control NK cell function following activation. the NK1.1 receptor. The acquisition of ligand appears to be yet a GPI-linked protein. More importantly, we demonstrated that the interaction of the Ly49H receptor on the NK cell surface.

acquisition occurred quickly in vitro with both LAK cells as well as fresh NK cells and required cell-to-cell contact. The acquired m157 was anchored to the surface of the NK cell by GPI linkage. The acquisition of m157, however, did not require signaling through the Ly49H receptor. In fact, it did not even require the expression of the Ly49H receptor by the NK cell. Transfer of the m157 also occurred in vivo, as we observed transfer of m157 onto donor NK cells upon adoptive transfer of donor splenocytes into m157-Tg recipients, but not WT recipients.

Although it is clear that m157 can be acquired by NK cells in the absence of the Ly49H receptor, the in vitro data using fresh NK cells suggest that NK cells from B6.BXD mice acquire less m157 than WT NK cells (Fig. 4B). However, upon injection in vivo, we observe that B6.BXD8 NK cells acquire more m157 (Fig. 4C). We believe that the in vivo interaction represents a more natural interaction for the NK cell and its target than in vitro studies in which cell densities can play a major role. One explanation as to why B6.BXD8 NK cells appear to acquire more m157 in vivo could be that, in WT mice, Ly49H interacts with the acquired m157 and prevents binding of the 6H121 (anti-m157) mAb, thus limiting detection of m157 on the cell surface. In B6.BXD8 NK cells, the interaction of acquired m157 with Ly49H does not occur, so interactions with the anti-m157 mAb are not hindered, allowing for better detection of m157 on the cell surface. The in vivo pattern of staining suggests that acquired m157 interacts with the Ly49H receptor on the NK cell surface.

Upon acquisition of m157 by the NK cells, we demonstrated that the Ly49H receptor (when present) can interact with the acquired protein. More importantly, we demonstrated that the interaction of the acquired m157 with the Ly49H receptor resulted in impaired function of the Ly49H\textsuperscript{+} NK cells, most likely by two mechanisms: blocking the Ly49H receptor from recognizing other targets and continuous engagement of the Ly49H receptor, which imparts a functional defect in IFN-\gamma production upon stimulation through the NK1.1 receptor. The acquisition of ligand appears to be yet another mechanism to control NK cell function following activation.

Of note, cis interactions of MHC class I with receptors on the NK cells surface have been shown to alter NK cell function. For example, the interaction of MHC class I (H2-D\textsuperscript{d}) with inhibitory receptors (Ly49A) on the NK cell surface has been documented and appears to alter NK cell function (38–40). In the absence of H2-D\textsuperscript{d} expression on the NK cell surface, Ly49A is efficiently recruited to the NK cell synapse by the H2-D\textsuperscript{d} expressed in trans on target cells. However, Ly49A at the NK cell synapse is reduced when NK cells express both H2-D\textsuperscript{d} and Ly49A (40). The coexpression of Ly49A and H2-D\textsuperscript{d} on the NK cell membrane is believed to sequester the Ly49A receptors, restricting the pool of Ly49A available for functional interaction with MHC class I ligands on target cells. This results in decreased inhibitory capacity of Ly49A in the context of H2-D\textsuperscript{d} expression. In addition, cis interactions of Ly49C receptor with MHC class I have been shown to block the ability of the Ly49C receptor to bind a mutant form of m157, making mice susceptible to MCMV expressing the mutant m157 (41). Finally, studies suggest that the education or licensing of NK cells requires both cis and trans recognition of MHC class I molecules (42).

Although the Ly49H receptor is not required for the acquisition of m157, when the receptor is present on the NK cell, it appears to be able to interact on the cell surface with the acquired m157. This is suggested by the increase in detectable levels of Ly49H on the cell surface of m157-dressed NK cells upon treatment with PI-PLC (which removes m157 from the cell surface) and the blocking of the ability of m157-dressed NK cells to kill RMAm157 targets. The ability of the Ly49H to interact with the acquired m157 in cis would provide an explanation of how continuous engagement of the Ly49H receptor could take place on NK cells, particularly in adoptive transfer experiments in which we observe that donor WT NK cells acquire m157 on their cell surface as well as a hyporesponsive phenotype upon transfer into a m157-Tg mouse. Regardless of whether cis or trans interactions are taking place, continuous engagement of Ly49H and m157 results in a hyporesponsive NK cell. We propose a model in which m157, acquired via trogocytosis from the target cell, interacts with Ly49H on the same NK cell, resulting in blocking as well as continuous engagement of the receptor (Fig 7).

Our finding that the Ly49H receptor is not necessary for the acquisition of m157 from target cells is novel and differs from a recent paper showing that trogocytosis of Rae-1 by NK cells was decreased if the NKG2D receptor was blocked with Ab or if signaling through the NKG2D receptor was disrupted by using NK cells that did not express adaptor molecules for the receptor (32). In our system, this was not the case, as trogocytosis of m157 was still observed by B6.BXD8 NK cells (where the Ly49H receptor is absent) and by DAP12ki NK cells (where signaling through the receptor is disrupted). The fact that NK cells from B6.BXD8 mice can still acquire m157 from target cells suggests that receptors and ligands other than Ly49H and m157 may be responsible for the formation of the IS between the NK cell and targets. Alternatively, it is possible that an IS does not need to form in order for trogocytosis to occur. Regardless of which receptors are responsible for forming an IS between the NK cell and target or whether the formation of an IS is important at all, the expression of Ly49H is not necessary for the acquisition of m157 by the NK cell.

The fact that acquisition of m157 occurs on the surface of NK cells in the absence of the Ly49H receptor also demonstrates that the acquisition of m157 by NK cells was not the result of a trans interaction (between the Ly49H on the NK cell and m157 on the target cell), in which the NK cell “rips” the m157 off the target cell. In addition, it also suggests that the exchange of membrane proteins most likely takes place between NK cells and target cells under normal conditions. However, functional consequences to the NK cell, in our experiments, required the presence of the Ly49H receptor on the NK cell. It is possible that a number of NK cells are involved in trogocytosis of membrane and associated proteins from different cells, but display no functional consequence because receptors for the ligands that are acquired are not present on.
the recipient NK cell. In addition, the amount of ligand taken up on the NK cell membrane is 1- to 2-log fold lower than is seen on the target cell, and, in order for functional consequences to occur, the receptor must be present at a high enough level on the NK cell to be able to engage the acquired ligand. Thus, it is possible that trogocytosis may function to decrease NK cell immune responses in situations in which ligands from target cells are taken up in large enough quantities by NK cells expressing an activating receptor that can bind the ligand.

During the process of immune surveillance, NK cells form an IS where they contact the target cell. It has been demonstrated that, upon IS formation, activating receptors can undergo ligand-induced downmodulation as a form of negative feedback regulation. NK cells have been shown to acquire a number of proteins from target cells following formation of the immunological synapse. These include ligands for the NKG2D receptor MICA, MICB, Rae-1, and the chemokine receptor CCR7 (19, 29, 32, 43). The acquisition of ligands from target cells can have a profound effect on the function of the NK cell, including downregulation or blocking of the activating receptor. Such alterations can make the NK cell less efficient at killing targets recognized by the downregulated or blocked receptor (19). In addition, acquisition of chemokine receptors (CCRs) can alter cell trafficking; acquisition of CCR7 by NK cells has been described, resulting in increased homing to the lymph node in athymic nude mice (43). In fact, it has been suggested that trogocytosis could be a method to transiently modify expanded NK cells without using gene transfer in adoptive immunotherapy for cancer.

NK cells can also acquire immunomodulatory molecules from tumor cells that alter NK cell function. For example, it has been demonstrated that HLA-G1, commonly expressed by tumor cells in vivo and whose expression on tumor cells confers resistance to killing in vitro, can be acquired by NK cells through trogocytosis. NK cells that acquired HLA-G1 stopped proliferating, were no longer cytotoxic, and were capable of inhibiting cytotoxic functions of other NK cells (44). This suggests that NK cells can acquire proteins from the cell surface of tumor cells, resulting in modification of their function.

It has also been shown that interaction of NK cells with cancer cells results in the downmodulation of NKG2D. This has been shown with cervical cancer models in which NKG2D was downmodulated, as well as with melanoma models in which NKG2D as well as other activating receptors, including NKP30 and NKP44, were downmodulated (45–47). The downmodulation of the receptors was associated with impairment of NK cell–mediated cytolytic activity. A more recent study described the induction of NKG2D ligands on the surface of healthy myeloid cells by lactate dehydrogenase-5 secreted from glioblastoma cells. This resulted in the downmodulation of the NKG2D receptor on the NK cells as well as their impairment in killing the tumor (48). Trogocytosis of ligands from target cells to NK cells in tumor-specific environments could provide an explanation for how the receptors are downmodulated as well as how the NK cells become impaired. Recent studies also suggest that the ligands acquired by NK cells by trogocytosis make them targets for other NK cells in a process termed fratricide. Fratricide of NK cells appears to provide another level of control of NK cells (32). Clearly, NK cells have evolved a number of ways to control their function. Most likely this is a way to limit collateral damage during NK cell function. In addition to allowing for fratricide, our model suggests that ligand acquired on the NK cells by trogocytosis binds in cis to the receptor, resulting in blocking the receptor from recognizing target cells, and allows for the continuous engagement of the receptor, resulting in a hyporesponsive NK cell.

Clearly, NK cell trogocytosis of ligands from target cells can profoundly alter NK cell function. This has important implications regarding how NK cells respond to tumors. NK cells may acquire, through trogocytosis, tumor-expressed ligands for NK cell–activating receptors, impairing the NK cells’ ability to destroy the tumor and possibly allowing for tumor growth and metastasis. In addition, trogocytosis of ligands may play a role in the use of expanded NK cells in immunotherapy for cancer. Modulation of NK cell function by trogocytosis could provide both helpful and harmful effects in regard to tumor immunosurveillance. More work needs to be done to understand the situations in which trogocytosis occurs, which proteins can be transferred by trogocytosis, and the signaling mechanisms that take place that render the NK cell dysfunctional following trogocytosis. Such understanding might make it possible to better use NK cells in future immunotherapy protocols.

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Disclosures
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References
Supplemental Figure 1. Gating strategy used to assess trogocytosis by LAK cells. (A) Representative dot plots and histograms depicting gating on NK1.1⁺, CD3⁻ LAK cells and assessment of m157 on these populations following incubation with RMA or RMAm157 cells. (B) Representative dot plots and histograms depicting gating on NK1.1⁺, CD3⁻ LAK cells from B6.BXD8 and WT mice as well as assessment of Ly49H on these populations.
Supplemental Figure 2. Acquisition of other membrane proteins by LAK cells. (A)

Representative histogram showing CD45.2 expression on RMA cells (tinted histogram), WT CD45.1 LAKs (open histogram with solid line) and CD45.1 LAK cells that had been incubated with RMA cells for 2 hours (open histogram with dashed line). (B) MFI of CD45.2 expression on CD45.1 LAKs (LAK; black, n=5), CD45.1 LAKs incubated with RMA cells (dLAK; hatched, n=5) and RMA cells (RMA; white, n=5). *p<0.0005. (C) Representative histogram showing H2-D\(^b\) expression on RMA cells (filled histogram), BALB/c LAKs (open solid histogram) and BALB/c LAK cells that had been incubated with RMA cells for 2 hours (open dashed histogram). (D) MFI of H2-D\(^b\) expression on BALB/c LAKs (LAK; black, n=10), BALB/c LAKs incubated with RMA cells (dLAK; hatched, n=10) and RMA cells (RMA; white, n=10). **p<0.02.
Supplemental Figure 3. The m157-dressed NK cells efficiently kill RMA-S target cells. WT IL-2 stimulated NK cells (LAKs) were incubated with RMA or RMAm157 cells and then sorted to obtain “dressed” LAKs. The sorted LAK cells were used as effector cells at 3:1 and 10:1 E:T ratios. (A) Representative histogram to evaluate killing of RMA-S target cells based on propidium iodide (PI) incorporation assessed by flow cytometry. The numbers in the histogram are the percentage of cells present in the PI+ and PI- gates. (B) Percentage of RMA-S target cells killed at two different E:T ratios. Effector cells were sorted RMA- “dressed” LAKs (black, n=8), sorted RMAm157-“dressed” LAKs (white, n=8) or unsorted B6.BXD8 LAKs (hatched, n=8). All data are presented as the mean+/- SEM.