MHC class I–deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I–sufficient environment

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MHC class I–deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I–sufficient environment

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In MHC class I–deficient hosts, natural killer (NK) cells are hyporesponsive to cross-linking of activation receptors. Functional competence requires engagement of a self–major histocompatibility complex (MHC) class I–specific inhibitory receptor, a process referred to as “licensing.” We previously suggested that licensing is developmentally determined in the bone marrow. In this study, we find that unlicensed mature MHC class I–deficient splenic NK cells show gain–of–function and acquire a licensed phenotype after adoptive transfer into wild-type (WT) hosts. Transferred NK cells produce WT levels of interferon–γ after engagement of multiple activation receptors, and degranulate at levels equivalent to WT NK cells upon coinoculation with target cells. Only NK cells expressing an inhibitory Ly49 receptor specific for a cognate host MHC class I molecule show this gain–of–function. Therefore, these findings, which may be relevant to clinical bone marrow transplantation, suggest that neither exposure to MHC class I ligands during NK development in the BM nor endogenous MHC class I expression by NK cells themselves is absolutely required for licensing.

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donor cells in to that of host WT NK cells (Fig. 1 A). In contrast, NK cells in WT hosts produced IFN-γ.

We found that donor-derived MHC class I–deficient splenocytes were activated in vitro by anti-NK1.1 cross-linking, and labeled them into MHC class I–sufficient WT (C57BL/6) or control hosts. At day 7, host spleens were harvested and bulk splenocytes were activated in vitro by anti-NK1.1, and analyzed for intracellular IFN-γ production versus Ly49C expression. We previously hypothesized licensing to be a developmental process because Ly49 receptors are first expressed at an immature stage during NK cell maturation in the BM (Kim et al., 2002). Thereafter, developing NK cells undergo constitutive proliferation that appears to be influenced by MHC class I and the relevant Ly49 receptor (Kim et al., 2005). However, it is not known if licensing can occur outside the context of development within the BM, or whether the unlicensed phenotype is fixed.

Herein, we performed adoptive transfer of peripheral, hyporesponsive NK cells from MHC class I–deficient donors to MHC class I–sufficient hosts, resulting in the generation of functional donor NK cells. Expression of a host MHC class I ligand specific for a donor cell inhibitory NK cell receptor was necessary for the observed gain of function, which is highly suggestive of licensing as a mechanism. We conclude that the unlicensed phenotype is not fixed in apparently mature peripheral NK cells, and that licensing may not be exclusively a developmental process in the BM.

RESULTS AND DISCUSSION

MHC class I–deficient NK cells gain function after transfer to MHC I–sufficient hosts

To examine potential for gain of function by hyporesponsive, peripheral MHC class I–deficient NK cells, we harvested and labeled β2m−/− splenocytes with CFSE and injected them into MHC class I–sufficient WT (C57BL/6) or control β2m−/− hosts. At day 7, host spleens were harvested and bulk splenocytes were activated in vitro by anti-NK1.1 cross-linking, and then analyzed for intracellular IFN-γ. Surprisingly, we found that donor-derived MHC class I–deficient β2m−/− NK cells in WT hosts produced IFN-γ at a level equivalent to that of host WT NK cells (Fig. 1 A). In contrast, β2m−/− donor cells in β2m−/− hosts produced IFN-γ at a frequency identical to host-derived β2m−/− NK cells (Fig. 1 A). This was equivalent to the hyporesponsive production of IFN-γ by NK cells from unmanipulated β2m−/− control animals (unpublished data). Therefore, adoptive transfer of hyporesponsive splenic NK cells appeared to result in the acquisition of activation receptor responses that correlated with host NK cell responsiveness.

It may seem surprising that donor-derived MHC-deficient cells could be detected because WT host NK cells can eliminate MHC class I–deficient BM and splenocyte grafts (Bix et al., 1991; Höglund et al., 1991). However, transfer of a sufficiently large number of cells can result in BM graft acceptance (Cudkowicz and Bennett, 1971; Koh et al., 2005), and...
we suspect the same process may be occurring here. Regardless, host NK cells did not demonstrate increased responsiveness as compared with NK cells from unmanipulated controls (Fig. 1 B) and did not display markers associated with activation (not depicted). Thus, we found no evidence for host NK cell activation that may have accounted for the phenotypic change in the donor NK cells.

It was possible that the gain of function observed for transferred MHC class I–deficient cells could be caused by their coincubation with WT splenocytes during in vitro stimulation. To test this possibility, we mixed freshly isolated βm−/− and WT splenocytes at various ratios and stimulated them under the conditions used for the analyses of adoptively transferred cells. Regardless of the presence of WT cells in co-culture, βm−/− NK cells retained their hyporesponsive phenotype after receptor cross-linking (Fig. S1). These results indicate that the gain of function of the βm−/− NK cells after adoptive transfer to WT hosts is imparted during residence in the MHC class I–sufficient environment, not during co-culture and stimulation.

As we and others have recently shown that previously activated NK cells exhibit a memory-like property of enhanced cytokine production upon restimulation (Cooper et al., 2009; Sun et al., 2009), we sought to determine if our current results could reflect prior donor NK cell activation. Although kinetic studies revealed that donor NK cells gained function at WT levels by day 4 after transfer and that their phenotype was stable thereafter (Fig. S2), donor βm−/− NK cells did not spontaneously produce IFN-γ or display markers of activation, such as CD69, at any time (Fig. S3 and not depicted). Prior studies also showed that previously activated NK cells displaying the memory-like phenotype preferentially proliferate upon transfer (Cooper et al., 2009; Sun et al., 2009). In contrast, donor βm−/− NK cells did not proliferate after residence in WT hosts, as indicated by a lack of CFSE dilution (Fig. S3 A). Collectively, these data indicate that βm−/− NK cells were not activated after adoptive transfer to WT hosts, suggesting that acquisition of functional competence is unrelated to a memory-like phenotype.

Gain of function is restricted to cells expressing inhibitory Ly49 receptor specific for host MHC class I

We next examined if the NK cells acquiring function had properties of licensed NK cells. Such cells would be expected to express a receptor specific for an MHC class I allele in the WT host. In particular, Ly49C is an inhibitory NK cell receptor with an H2b ligand, H2Kb (Kim et al., 2005). We hypothesized that if transfer of hyporesponsive βm−/− NK cells into an H2b environment results in licensing, we would observe a higher frequency of IFN-γ+ donor NK cells among the Ly49C+ population than the Ly49C− population. Indeed, transferred βm−/− Ly49C+ NK cells made IFN-γ at a significantly higher frequency than Ly49C− NK cells (p = 0.02; Fig. 1 B). Additionally, similar results were observed after stimulation through another NK cell activation receptor, Ly49H (Fig. S4). Therefore, adoptive transfer of MHC-deficient splenocytes into MHC class I–expressing hosts, there is a selective gain of function in NK cells expressing an inhibitory receptor capable of interacting with host MHC class I.

To extend these findings to other inhibitory Ly49 receptors and MHC ligands, we transferred βm−/− NK cells into KODO (Kb−/−Db−/−) D8 hosts, which lack H2b MHC class Ia heavy chains and display Tg expression of H2Dd, a ligand for Ly49A (Karlishofer et al., 1992). After transfer, we observed a gain in IFN-γ production among Ly49A+ MHC class I–deficient NK cells (Fig. 2 A), consistent with enhanced responsiveness of Ly49A+ NK cells in H2Dd–expressing mice (Kim et al., 2005). Thus, we confirmed that there was a selective gain of function for transferred NK cells expressing another inhibitory receptor able to bind to a different host MHC class I molecule.

We consistently observed that Ly49C− NK cells in WT hosts, and Ly49A− NK cells in KODO D8 hosts, trended toward greater IFN-γ production than their counterparts from MHC class I–deficient hosts (Fig. 1 and Fig. 2 A). Although these findings were not statistically significant, we hypothesized that expression of other self-MHC–specific receptors may contribute to this effect. To investigate this hypothesis, we transferred βm−/− NK cells into SCT-Kb Tg TKO hosts, which express only a single MHC class I molecule (SCT-Kb)
that binds only one NK cell receptor, Ly49C (Kim et al., 2005). After adoptive transfer, donor Ly49C+ NK cells gained the ability to produce IFN-γ at levels equivalent to that of host Ly49C+ cells (Fig. 2B). However, there was no increase in IFN-γ production by either donor or host Ly49C− NK cells. Therefore, the interaction between an inhibitory Ly49 receptor and its cognate self–MHC class I ligand is necessary for the gain of function observed in hyporesponsive MHC class I–deficient NK cells after their adoptive transfer to MHC class I–sufficient hosts, consistent with licensing.

Gain of function extends to NK cell degranulation
MHC class I–deficient NK cells are defective in their cytokine production capacity and in their ability to kill targets as compared with WT NK cells (Bix et al., 1991; Höglund et al., 1991). As killing is dependent on degranulation, we exposed transferred NK cells to sensitive targets and assessed the level of surface CD107a (LAMP-1), a marker for NK cell degranulation (Fig. 3). Additionally, NK cells were assessed after transfer into Jinx mice, which have a mutation in Unc13d (Crozat et al., 2007). The Jinx mice provide the baseline level of CD107a staining because their NK cells are unable to degranulate. After transfer to MHC class I–expressing hosts, β2m−/− NK cells gained the ability to degranulate at a level equivalent to that of host and control WT NK cells in response to YAC targets (Fig. 3). In contrast, β2m−/− NK cells adoptively transferred to β2m−/− hosts show defective degranulation, comparable to Jinx NK cells. Furthermore, as with cytokine production, we found no increase in the ability of freshly isolated β2m−/− NK cells to degranulate in response to target cells when coincubated with WT NK cells in the degranulation assay (Fig. S5). Collectively, these data indicate that the gain of function (degranulation) observed in MHC class I–deficient cells is acquired after their residence in the WT host environment.

Specific decrease in inhibitory receptor MFI on MHC class I–deficient cells after transfer
Previous studies indicate that splenic NK cells from β2m–deficient animals are mature and indistinguishable from peripheral NK cells in WT mice (Huntington et al., 2007; Joncker and Raulet, 2008; Kim et al., 2002; Sun and Lanier, 2008). Nevertheless, one potential mechanism for the gain of function by transferred β2m−/− NK cells could be differentiation of the NK cells after transfer. To further evaluate this possibility, the surface phenotype of β2m−/− donor cells was assessed after recovery from WT hosts at 7 d after adoptive transfer, a time when donor cells possess the capacity for normal cytokine production and degranulation (Fig. S2 and Fig. 3). These experiments indicate that, regardless of the genotype of the host or donor, all NK cells appeared phenotypically indistinguishable in most aspects. For example, markers such as NK1.1, CD94, NKG2A, CD69, DX5, and 2B4 showed no difference in expression between β2m−/− and WT NK cells, before or after adoptive transfer (Fig. 4). Other markers, such as Nkp46, NKG2D, and CD11b were expressed at a slightly higher level on WT NK cells when compared with β2m−/− NK cells, and their expression slightly increased on β2m−/− NK cells after their transfer to WT hosts. Therefore, there were minimal changes overall in the expression of most surface molecules after adoptive transfer of β2m−/− cells to WT hosts.

In contrast to the minimal changes in expression of most surface markers, we found a distinct and reproducible decrease of Ly49C mean fluorescence intensity (MFI) on MHC class I–deficient cells after adoptive transfer to WT C57BL/6 hosts (Fig. 5A). This effect was observed for multiple systems in which the inhibitory Ly49 receptors had a known MHC class I ligand in the host (Fig. 5, A–C). In particular, decreased
Ly49C MFI was specifically observed in cells transferred to the SCT-Ko Tg TKO mice (Fig. 5 B), whereas MFI of Ly49A, a receptor for which no MHC class I ligand is present in this host, remained unchanged (Fig. 5 D). However, Ly49A MFI decreased on cells transferred into KODO D8 mice that express the ligand for Ly49A (Fig. 5 C). Thus, decreased Ly49 MFI appears to reflect a specific interaction of an inhibitory receptor on a donor NK cell with a host MHC class I molecule.

Herein, we found that introduction of MHC class I-deficient NK cells into an MHC class I–sufficient host results in their acquisition of function, as demonstrated by their increased capacity for cytokine production and degranulation upon activation receptor cross-linking. The requirement that MHC class I–deficient NK cells express an inhibitory receptor specific for host MHC class I to become functional can be interpreted as evidence that these cells become licensed in the MHC class I–sufficient host. Importantly, these experiments suggest that licensing of NK cells can occur after their egress from the BM, after the time at which they are believed to have become fully mature. Thus, although it is possible that licensing may occur during NK cell development in vivo, these results demonstrate that licensing is not exclusively a developmental process in the BM.

In the current experiments, adoptive transfer of NK cells from an MHC-deficient background was performed without host irradiation and subset depletion, thereby minimizing the impact of experimental manipulation on host and donor NK cells. Because WT host NK cells can reject MHC class I–deficient grafts (Bix et al., 1991; Höglund et al., 1991), it is possible that host NK cells could have been activated and influenced the function of transferred donor cells. However, we found no evidence of any host NK cell activation. Most importantly, restriction of the gain of function to only those NK cells bearing an inhibitory receptor specific for self-MHC, as was observed, would not be expected if NK cells had been activated by a broad stimulus, such as cytokine stimulation. Thus, it appears that acquisition of activation receptor responsiveness by the MHC-deficient NK cells is a cell-intrinsic property, consistent with a licensed phenotype.

NK cell receptor–ligand interactions can occur in either trans (on different cells) or in cis (on the same NK cell). Importantly, the licensed phenotype of the donor MHC-deficient NK cells in the MHC-sufficient environment indicates that NK cell-intrinsic expression of MHC class I is not strictly a requirement for licensing. This seems to be consistent with trans effects and contradictory to studies suggesting that cis interactions are necessary for education (Chalifour et al., 2009). However, numerous works indicate that NK cells can acquire
plasma membrane components from other cells, in a process termed trogocytosis (Carlin et al., 2001; Sjöström et al., 2001; Zimmer et al., 2001). This may result in the accumulation of enough cognate MHC ligand on the surface of βm−/− donor NK cells sufficient to induce the licensing state by cis interactions. Previous studies have suggested that the decreased MFI in this context can be caused, at least in part, by cis interactions (Zimmer et al., 2001; Anderson et al., 2007). Consistent with this possibility, we found a correlation between decreased MFI for a Ly49 receptor specific for host MHC and the licensed phenotype. Importantly, a similar decrease in inhibitory receptor MFI was reported after adoptive transfer of WT splenocytes to hosts with Tg MHC I-expression (Käse et al., 1998), supporting our observations and suggesting that the phenomenon of decreased inhibitory receptor MFI after exposure to MHC I is not an irregularity of MHC I-deficient cells. Regardless of the mechanism, our data indicate that NK cells may not need to produce their own MHC ligand to be licensed.

In summary, our data indicate that NK cell function in respect to host MHC environment is more plastic than originally anticipated, as in vivo exposure of a peripheral cell to the presence of an inhibitory ligand can alter the functional capacity of the NK cell. As such, these results may have important clinical implications. In particular, patients undergoing BM transplantation experience greater donor-mediated anti-leukemic effect when donor NK cells express KIRs specific for HLA lacking in the patient (for review see Grzywacz et al., 2008). Therefore, within the context of the transfer of peripheral NK cells, such as NK immunotherapy, donor NK cells unlicensed by HLA alleles absent in the donor may become licensed by host HLA alleles, leading to a beneficial gain in reactivity of donor NK cells against host tumors lacking HLA expression. Perhaps prospective pairing of donor KIR, along with donor and host HLA to anticipate gain of function by otherwise unlicensed donor NK cells may enhance patient outcomes.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6 Ly5.1 congenic mice and βm−/− mice were purchased from The Jackson Laboratory. βm−/− Ly5.1 mice were generated by crossing βm−/− mice with Ly5.1 mice (both from the Jackson Laboratory). H2Kb−/−H2Db−/−H2Dd− expressing mice (KODO D8) were generated by crossing H2K−/−H2D−/− mice (Taconic Farms) with mice expressing a Dd-transgene (D8), provided by D. Margulies (National Institute of Allergy and Infectious Diseases, Bethesda, MD). TKO K−/−D−/−βm−/− mice with Tg expression of K−/−OVA MHC class I as a single protein (SCT-K Tg TKO mice) were a gift from T. Hansen (Washington University, Saint Louis, MO). Inx mice, harboring a mutation in Ifnγ (UNC1D3), were provided by B. Beutler (Scirpis Research Institute, La Jolla, CA) and have been previously described (Crozat et al., 2007). Genotyping was performed by the Speed Congenics core facility, supported by the Rheumatic Disease Core Center grant to Washington University. Animals were housed in specific pathogen–free facilities at Washington University in Saint Louis, and all mice were used between 6 and 14 wk of age. Experiments were conducted in accordance with the animal protocol approved by the Animal Studies Committee at Washington University in Saint Louis.

Antibodies and flow cytometry. The following antibodies and reagents were purchased from BD: anti-CD3 (145-2C11), anti-Ly-5.1 (A20), anti-Ly-5.2 (104), anti-Ly-5.11 (PK136), anti-Ly-9A (JR9 and A1), anti-CD8 (H12F3), streptavidin APC (SA-APC), SA-PerCP-Cy5.5, and SA-PE-Cy7. The following antibodies were purchased from ebioscience: anti-NKp46 (29A1.4), anti-IFN-γ (XMGI1.2), anti-K-0VYA (eBio25-D1.16), anti-CD94 (18D3), anti-NKG2D (CSX), anti-CD11b (M1/70), anti-CD127 (A7R34), anti-CD49 (DX5), and anti-CD107a (eBio1D4B). Anti-Ly49C (4LO33) and anti-Ly49H were purified in our laboratory from hybridoma supernatants. (The 4LO33 hybridoma was generously provided by Suzanne Lemieux, Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Quebec, Canada.) All surface staining was performed on ice in staining buffer (3% FCS, 0.1% Na2HPO4 in PBS) containing anti-FeR antibodies (2.4G2). Samples were collected on a FACSCanto (BD) using FACSdiva software (BD), and data were analyzed using FlowJo (Tree Star, Inc.).

Adoptive transfer and co-culture. For adoptive transfer experiments, donor splenocytes were purified from βm−/− mice, red-blood cell lysed, and labeled with 1 µM CFSE (CellTrace; Invitrogen). Cells were washed twice in PBS and resuspended at a 2.5 × 106 splenocytes/ml PBS. 200 µl of the cell suspension was injected into the lateral tail vein of age- and sex-matched recipient mice. For co-culture experiments, freshly isolated RBC-lysed splenocytes from C57/BL/6 Ly5.1 and βm−/− donors were plated in 6-well plates at ratios indicated in the figures, with 1.0 × 106 cells/ml of R.10 media (RPMI, 10% FCS, 1-glutamine, and pen-strep)/well. For the kinetic analysis, a single donor population was prepared and injected into multiple recipients, which were analyzed on varying days after transfer using the same set of reagents.

In vitro stimulation and intracellular cytokine staining. Splenocytes were harvested from adoptive-transfer recipients at day 7 after transfer and stimulated with anti-NK1.1 (or anti-Ly49H) mAb as previously described (Kim et al., 2005). In brief, 107 splenocytes were plated into 6-well plates, that were either precoated with antibody (5 µg/ml; 1.5 h at 37°C), contained media alone, or contained media with 0.5 µg phorbol myristate acetate (Sigma-Aldrich) and 4 µg ionomycin (Calbiochem). Cells were incubated for 1 h at 37°C in 5%CO2, Brefeldin A (GolgiPlug; BD) was added, and cells were incubated for an additional 7 h. Cells were then fixed and permeabilized (Cytofix/Cytoperm; BD), followed by antibody staining for intracellular IFN-γ, as previously described (Kim et al., 2005).

Degranulation assay. Splenocytes were harvested from adoptive-transfer recipients at day 7 after transfer, and NK cells were purified using magnetic separation columns (MACS NK isolation kit; Miltenyi Biotech.). Separation purity was determined via flow cytometry (gating on CD3−NK1.1− cells), and NK cells were plated with target cells at a ratio of 1:1 (E:T) in 96-well V-bottom plates. Anti-CD107a antibody and monensin (ebioscience) were added to each well before incubation. Plates were incubated for 2 h at 37°C, after which surface staining for flow cytometry was performed as described in Antibodies and flow cytometry.

Statistical analysis. Data are presented as mean ± SD. Statistics were generated using unpaired Student’s t test within GraphPad Prism software (GraphPad), and P values <0.05 were considered significant.

Online supplemental material. Fig S1 shows MHC class I-deficient and WT NK cells retain distinct cytokine production phenotypes during splenocyte co-culture. Fig. S2 shows MHC class I−/− NK cells gain function ~72 h after transfer into WT hosts. Fig. S3 shows that MHC class I−/− NK cells do not proliferate and do not constitutively produce IFN-γ after adoptive transfer into WT hosts. Fig. S4 shows selective gain-of-function by MHC class I−/− NK cells expressing an inhibitory receptor specific for host-MHC class I after activation through an alternate stimulus. Fig. S5 shows that MHC class I−/− and WT NK cells retain distinct degranulation phenotypes during splenocyte co-culture. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100986/DC1.

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