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Skewing of the NK Cell Repertoire by MHC Class I via Quantitatively Controlled Enrichment and Contraction of Specific Ly49 Subsets

Petter Brodin,1 Tadepally Lakshmikanth, Klas Kärre, and Petter Höglund1

A major task for the immune system is to secure powerful immune reactions while preserving self-tolerance. This process is particularly challenging for NK cells, for which tolerizing inhibitory receptors for self-MHC class I is both cross-reactive and expressed in an overlapping fashion between NK cells. We show in this study that during an education process, self-MHC class I molecules enrich for potentially useful and contract potentially dangerous NK cell subsets. These processes were quantitatively controlled by the expression level of the educating MHC class I allele, correlated with susceptibility to IL-15 and sensitivity to apoptosis in relevant NK cell subsets, and were linked to their functional education. Controlling the size of NK cell subsets with unique compositions of inhibitory receptors may represent one mechanism by which self-MHC class I molecules generate a population of tolerant NK cells optimally suited for efficient missing self-recognition. The Journal of Immunology, 2012, 188: 2218–2226.

Natural killer cell function is controlled by a balance between inhibitory and activating receptors. Activating receptors recognize a variety of ligands, some of which are induced by cellular stress (1). Murine Ly49 receptors and human killer Ig-like receptors (KIR) are the most well-studied inhibitory receptors. These recognize MHC class I molecules and prevent NK cell activation upon engagement. The NKG2A receptor recognizes nonclassical MHC class I molecules (Qa-1 in mice and HLA-E in humans) containing peptides derived from classical MHC class I molecules (2). Thus, two parallel layers of MHC class I-mediated protection of normal cells exist (1). When such cells downregulate MHC class I expression, the protective self-ligand is lost for both layers, allowing the NK cell to mediate missing self-recognition (3, 4).

To secure self-tolerance, yet allow for missing self-recognition when necessary, the NK cell system must be educated by self-MHC class I. This process includes two effects. The first controls development of functional potency in NK cells, resulting in a capacity to react against normal cells lacking MHC class I expression (3–13). This effect depends on the expression of inhibitory Ly49/KIR or NKG2A receptors on NK cells, because NK cells lacking such receptors, or expressing inhibitory receptors for which no self-MHC class ligand is present, are hyporesponsive (11–13). Furthermore, functional education by MHC class I does not work as an on/off switch. Instead, an individual NK cell dynamically determines its threshold for activation based on the strength of inhibitory input (14–18).

A second effect by MHC class I molecules on NK cells is an influence on the frequency distribution of Ly49/KIR subsets. Specifically, it has been suggested that MHC class I expression decreases the frequency of cells expressing several self-specific receptors (14, 19–21), whereas cells expressing only one such receptor were increased in frequency (21, 22). Such repertoire effects have been interpreted as a means of limiting the frequency of NK cells that would be inhibited too easily and at the same time enriching for the most useful NK cells (i.e., those expressing single Ly49 receptors). However, most of these data were generated with limited resolution of individual NK cell subsets in combination with complex MHC genotypes, making quantitative studies of NK cell education of individual NK cell subsets difficult. This limitation becomes even more pronounced when considering our recent data suggesting that Ly49 receptors may have a broader specificity than what was previously thought (21). The link between functional education and repertoire effects also requires further studies, including the effect of self-MHC class I on potentially dangerous NK cells expressing activating receptors for self-MHC class I.

We dissect in this study NK cell education in transgenic mice expressing high or low levels of a single MHC class I allele, H2Dd. Using these mice in combination with advanced flow cytometry, we are able to analyze precisely the quantitative effects of MHC class I on NK cell function as well as on the development of the NK cell receptor repertoire. We find that processes resembling both positive and negative selection shape the NK cell repertoire,
leading to an accumulation of potentially useful and contraction of potentially dangerous NK cell subsets. Both processes are regulated in a quantitative manner by MHC class I, operate on both inhibitory and activating Ly49 receptors, and are linked to a dose-dependent MHC class I influence on functional responsiveness in the selected subsets. Selected subsets showed changes in sensitivity to IL-15, affecting proliferation and resistance to apoptosis, which supports, but does not prove, a model in which inhibitory receptor engagement control mechanisms affect expansion of selected subsets. We propose that inhibitory signals optimize the repertoire of NK cells to fit the MHC class I environment and that this mechanism has developed alongside functional education to enrich for the most optimally tuned NK cell subsets for missing self-recognition.

Materials and Methods

Mice

All mice were bred and maintained at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, in cages with standard bedding. The mice received food and water ad libitum and were used for experiments at the age of 8–12 wk. B6.Kb Dd−/− (de facto MHC class I deficient) and B6.Kb Dd+/+ mice expressing Dd only (B6.Kb cocyanin, anti-CD107a (1D4B)-FITC or -PE, anti–IFN-γ) were generated as described (14). Dd+/+ mice were generated by crossing Dd+/+ and MHC class II congenic (e.g., Dd+/+ on the C57BL/6 background) were generated as described (14). Dd+/− mice were generated by crossing Dd+/− and MHC class II+/− mice. MHC class I molecules are denoted by their allele (e.g., Dd+/−, Dd+/+) names throughout the paper.

In vivo target cell rejection assay

Target cells and syngeneic control cells were mixed in a 1:1 ratio after being labeled with either 3.0 or 0.3 μM CFSE (Molecular Probes/Invitrogen, Stockholm, Sweden) for 10 min at 37°C. The differently labeled populations could be distinguished by flow cytometry and the ratio between them measured. This ratio corresponds to the degree of target cell rejection in vivo over time (14).

Abs and flow cytometric analysis

Single-cell suspensions of splenocytes were prepared by gently mashing spleens against a 40-μm pore-cell strainer. Erythrocytes were lysed by adding 9/10 H2O followed by immediate addition of 1/10 10 mM EDTA. FcRs were blocked by incubation with anti-FcγRIII (2.4G2). Data were acquired using an LSRII special order system (BD Biosciences, Mountain View, CA) and analyzed using FlowJo software (Tree Star).

The following Abs were used: anti-NK1.1 (PK136)-allophycocyanin-Cy7 and anti-Ly49A (YE1/48)–biotin from BioLegend; anti-CD3ε (145-2C11)–PE-Cy5, anti-CD19 PerCP-Cy5.5, anti-Ly49G2 (4D11)-allophycocyanin, anti-CD117a (1D4B)-FITC or -PE, anti–IFN-γ (XMG1.2)-Alexa 700, anti–H2Dd (34-5.8S)-PE, anti-Ly49I (YLI-90)-PE, and anti-Ly49D (4E5) purified were purchased from BD Pharmingen (Stockholm, Sweden) and conjugated to Qdot 605 using conjugation kit (Molecular Probes). NKG2A/C/E (20d5) was conjugated to Pacific Blue using a conjugation kit (Molecular Probes/Invitrogen). Streptavidin Qdot 565 was used with anti-Ly49A biotin, and dead cells were excluded using the Vivid Aqua reagent, a fixable dye binding intracellular amines specifically in dead and dying cells due to its inability to penetrate the cell membrane of healthy cells (Invitrogen). Anti-Ly49C (4L03311) hybridoma was a kind gift from Suzanne Lemieux (Institut Armand Frappier) and David Raulet (University of California Berkeley) and was used together with anti-mouse IgG3 (Southern Biotechnology Associates). NKp46 (NCR1)-purified and anti-Mipi-α (clone)–FITC/PE were from R&D Systems (Abingdon, U.K.). Anti–p-STAT5 (pY694) and anti-mouse STAT5 (catalog number 610191) were both from BD Biosciences. Rabbit anti-him Ab (1036-1) was from Epitomics, and secondary anti-rabbit APC (111-136-144) was from Jackson ImmunoResearch Laboratories.

Annexin V staining

For Annexin V staining ex vivo, cells were first stained with Abs to surface markers in FACS buffer, followed by incubation in Annexin V buffer (BD Biosciences), and followed by Annexin V FITC according to manufacturer’s protocol (BD Biosciences).

Measurement of responsiveness by Nkp46 cross-linking

Single-cell suspensions were prepared from naive splenocytes as described above. Cells were added to plates precoated for 2 h at 37°C with 10 μg/ml anti-Nkp46 (Mar-1) Ab (R&D Systems). After 1 h, 1.4 μM monomer/multimer Stop (BD Pharmingen) and 2 μl brefeldin A (Sigma-Aldrich, Stockholm, Sweden) were added per well containing a total of 200 μl RPMI 1640 media. For degranulation experiments, anti-CD107a (LAMP-1) was also included during the stimulation (5 μg/ml) (23). For negative controls, cells were incubated in PBS-coated wells, and for positive controls, cells were stimulated with 20 ng/ml PMA plus 1 μg/ml ionomycin (Sigma-Aldrich). Plates were incubated at 37°C for 10 h followed by surface staining as described above. For IFN-γ and Mipi10a detection, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) for 2–5 min on ice, followed by intracellular staining with anti–IFN-γ-Ax700 and anti-Mipi10a FITC.

Statistical calculations

All comparisons between multiple groups such as Ly49/NKG2A subsets and the three different strains of mice were done using a one-way ANOVA with Bonferroni’s multiple comparisons correction in cases with p < 0.05. Correlations were calculated using the Pearson’s product moment correlation coefficient, which is preferred over linear regression when both the x and y variables are experimentally measured without any manipulation. Calculations were performed using the Prism 5 program (GraphPad).

IL-15 stimulation, p-STAT5 detection, and CFSE dilution experiments

Naive and viable NK cells were sorted using a MoFlo XDP system (Beckman Coulter) based on the expression of Ly49A. Cells were then placed in 37°C RPMI 1640 media without cytokine supplements for 2 h before stimulation. Thereafter, cells were washed and stimulated for 0–40 min with 100 ng/ml in 37°C. Stimulation was broken by fixation using 2% paraformaldehyde for 10 min in 37°C and permeabilized using carefully added (dropwise) ice-cold MeOH (total of 200 μl) for 1 h on ice. Cells were then stained using an anti–p-STAT5 Ab (pY694).

For CFSE dilution experiments, cells were labeled with 3 μM CFSE (Molecular Probes/Invitrogen) for 10 min in 37°C, washed in PBS 10% FCS, and placed in 10 ng/ml IL-15 for 3 d and CFSE dilution assessed by flow cytometry. For intracellular staining of Bim upon cytokine deprivation, whole spleen populations were placed in culture for 11 h without any cytokine supplementation and subsequently fixed using the Cytofix/ Cytoperm kit (BD Biosciences) according to the manufacturer’s protocol and intracellularly stained using anti-Bim primary Ab (Epitomics) for 45 min on ice, followed by staining using a secondary anti-rabbit APC Ab (Jackson ImmunoResearch Laboratories) for 15 min in room temperature.

Results

The expression level of H2Dd tunes the strength of missing self-rejection and controls functional responsiveness of Ly49A+ NK cells

To establish a rigorous model for assessing quantitative influences of MHC class I molecules on NK cell education, we created transgenic mice expressing the MHC class I allele H2Dd in a hemizygous (Dd+/−) or homozygous (Dd+/+) fashion. The expression of only one MHC class I allele was instrumental to avoid influences of other MHC class I molecules on inhibitory receptors under study, which could otherwise blur the results. Dd+/− mice showed a 54 ± 17% reduction in Dd expression at the cell surface (Fig. 1A) and displayed a markedly reduced capacity to reject MHC-deficient cells compared with Dd+/+ mice (Fig. 1B).

The inhibitory receptor Ly49A binds Dd with high affinity (24, 25), and Ly49A+ NK cells require interactions with Dd for efficient functional maturation (11, 12, 16). To test if the level of Dd expression would affect function of Ly49A+NK cells, we developed a polychromatic flow cytometry panel (Supplemental Fig. 1) that allowed the identification of NK cells expressing Ly49A but none of the inhibitory receptors Ly49C, Ly49I, Ly49G2, and

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FIGURE 1. Dose-dependent influence of MHC class I on NK cell-mediated function. (A) Surface Dd expression level on total splenocytes stained with an anti-Dd Ab. One representative experiment of at least 10 is shown. (B) Rejection of CFSE-labeled MHC−/− splenocytes in vivo over 1, 2, and 4 d as measured by peripheral blood sampling. Mean and SD, n = 5 mice/strain in three independent experiments are shown. Statistical differences in target cell rejection between undepleted Dd+/− and Dd+/+ mice. (C) Frequencies of cells within the indicated subset, responding to NKp46 cross-linking with the production of IFN-γ, Mip1α, or CD107a. Colors indicate frequencies of cells responding with one, two, or three simultaneous effector responses. Mean values of three to six experiments with one mouse in each. Statistical differences shown between triple-responding subsets. (D) Percent Ly49A-sp (Ly49C−, -G2−, -I−, NKG2A+) NK cells responding with CD107a, Mip1α, or IFN-γ. White bars, MHC−/−; light grey bars, Dd+/−; and dark grey bars, Dd+/+. (E) Geometric mean fluorescence intensity (GeoMFI) for indicated effector responses by Ly49A-sp NK cells after NKp46 cross-linking. Mean values of three to six experiments with one mouse in each. *p < 0.05, **p < 0.01, ***p < 0.001.

NKG2A (Ly49A–single-positive [sp] NK cells). The panel also allowed simultaneous measurements of degranulation (CD107a) and cytokine (IFN-γ) and chemokine (Mip1α) production, which allowed the identification of NK cells producing multiple, simultaneous effector responses, a hallmark of highly efficient lymphocytes (16, 17, 26, 27). To stimulate NK cells, we used an Ab against the activating receptor NKp46, which was similarly expressed in all mouse lines (Supplemental Fig. 2).

The responsiveness of the total NK cell population (CD19−CD3ε−NK1.1+) was similar in Dd+/− and Dd+/+ mice, but both strains differed from MHC−/− mice (Fig. 1C). Ly49A+ NK cells were more likely to respond with three effector responses simultaneously in Dd+/− as compared with Dd+/+ mice, an effect that was even more pronounced in Ly49A-sp NK cells (Fig. 1C). When analyzed individually, all three effector functions in Ly49A-sp NK cells differed between Dd+/− and Dd+/+ mice but were most pronounced for the frequency of IFN-γ–producing cells (Fig. 1D) and for the amount of CD107 that reached the surface during degranulation (Fig. 1E). Thus, the expression level of a single MHC class I allele directly determines the strength of NK cell-mediated function in vivo and the extent of responsiveness of NK cells bearing a receptor for this MHC class I allele in vitro.

Ly49A−sp NK cells are enriched in the NK cell repertoire of Dd+/− mice

MHC class I molecules have been proposed to not only provide signals for functional maturation, but also to make the NK cell repertoire more useful by selecting against cells expressing more than one inhibitory receptor for self-MHC class I (19, 20, 28). With the use of an extended flow cytometry panel (Fig. 2A), we corroborate and extend this notion by reporting that the expression of Dd skewed the repertoire toward a reduced frequency of NK cell subsets expressing three to five inhibitory receptors compared with MHC−/− mice (Fig. 2B, 2C). More surprisingly, our data revealed a corresponding increase in Ly49 subsets expressing one to two receptors, including Ly49A-sp NK cells (Fig. 2B, 2C).

The expression level of Dd affected the frequency of Ly49A−sp NK cells (Fig. 2D) but did not change the frequency of Ly49C-sp, Ly49G2-sp, Ly49I-sp, and NKG2A-sp NK cells (Fig. 2E). We conclude that out of five single receptor-expressing subsets, one was responsive to a gene-dose effect of Dd (Ly49A-sp), two subsets were responsive to Dd as such but were insensitive to the quantitative input (Ly49C-sp, Ly49G2-sp), and two subsets were not increased in response to Dd, irrespective of expression level (Ly49I-sp, NKG2A-sp).

The phenotypes of NK cells in MHC−/−, Dd+/−, and Dd+/+ mice were similar for most cell-surface markers (Supplemental Fig. 2). However, when we looked at markers for NK cell maturation (killer cell lectin-like receptor G1 [KLRG1], CD27, and Mac-1) we found that these varied in a direction that suggests a more mature phenotype than NK cells from MHC-expressing mice (Fig. 3A–C). The association between KLRG1 expression and the extent of Dd input was particularly striking in the Ly49A-sp subset (Fig. 3B, 3C).
Enrichment of Ly49A-sp NK cells correlates with increased sensitivity to IL-15, proliferation, and a higher resistance to apoptosis

We hypothesized that the difference in frequency of Ly49A-sp NK cells between $D^{d+}$/+ and $D^{d+}$/2 mice could be reflected by a skewed balance between proliferation and apoptosis in this subset. Ly49A-sp cells displayed a lower frequency of Annexin V+ cells in $D^{d+}$/+ mice compared with $D^{d+}$/2 mice, even if the difference was not statistically significant (Fig. 3D).

This result implied that cellular turnover may differ between Ly49A-sp NK cells in MHC$^{-/-}$, $D^{d+}$/-, and $D^{d+}$/+ mice. We therefore asked if Ly49A-sp NK cells would differ in their sensitivity to the growth factor IL-15, known to prevent apoptosis in NK cells (29). First, we tested the expression levels of all components of the IL-15 receptor complex on Ly49A-sp NK cells and found them to be similar in all mice (Supplemental Fig. 3A). Next, we sorted Ly49A+ NK cells from MHC$^{-/-}$, $D^{d+}$/-, and $D^{d+}$/+ mice, stimulated them with IL-15, and measured phosphorylation of the IL-15–responsive transcription factor Stat5 using flow cytometry. After the addition of IL-15, NK cells from all three mice responded with Stat5 phosphorylation (Fig. 4A), but the level of upregulation was stronger in Ly49A+ NK cells from $D^{d+}$/+ (1.7-fold) than from $D^{d+}$/2 (1.3-fold) and MHC$^{-/-}$ (1.2-fold) mice (Fig. 4B). There were no differences in the total amounts of Stat5 protein (data not shown). When Ly49A-sp NK cells were cultured in a low-dose of IL-15 (10 ng/ml) for 3 d, there was a larger expansion among Ly49A-sp NK cells from $D^{d+}$/+ mice as compared with the same cells from $D^{d+}$/- and MHC$^{-/-}$ mice, supporting an enhanced sensitivity to IL-15 in these cells (Fig. 4C, Supplemental Fig. 3B).
Cytokine deprivation of NK cells induces apoptosis through the upregulation of the proapoptotic factor Bim (29). To test the expression of this proapoptotic molecule in our system, we placed splenocytes from MHC\(^{-/-}\), D\(^{d+/+}\), and D\(^{d+/+}\) mice in vitro for 11 h without cytokine supplement and measured Bim expression in the presence of self-ligand. Uptregulation of Bim was more pronounced in MHC\(^{-/-}\) than in MHC\(^{d+/+}\) mice, a tendency for intermediate levels in D\(^{d+/+}\) mice (Fig. 5A). In contrast, when excluding cells expressing inhibitory Ly49 and/or NKG2A receptors in addition to Ly49D, a contraction of the Ly49D-sp subset was seen in D\(^{d}/\) -expressing mice. There was a tendency toward a less prominent contraction in D\(^{d+/+}\) versus D\(^{d+/+}\) mice, but this difference was not statistically significant (Fig. 5B). Ly49D-sp cells showed a high degree of apoptosis in all mice, but the frequency of Annexin V\(^+\) cells ex vivo was higher in D\(^{d+/+}\) than in MHC\(^{d+/+}\) mice, with a tendency for intermediate levels in D\(^{d+/+}\) mice (Fig. 5C). Overall, these data suggest apoptosis as one important mechanism for contraction of a potentially dangerous subset, reminiscent of negative selection of T cells in the thymus.

Negative selection of Ly49D-sp cells in D\(^{d}\) mice is prevented by coexpression of some, but not all, inhibitory receptors

We next asked if inhibitory or activating signals would dominate in regulating subset sizes within the repertoire. Coexpression of Ly49D with Ly49A, Ly49C, or Ly49G2, respectively, prevented contraction induced by Ly49D engagement (Fig. 6A). Instead, these double-positive (dp) subsets were enriched in a similar way as subsets expressing these inhibitory receptors alone (Fig. 2D, 2E). Neither Ly49I nor NKG2A coexpression was sufficient to prevent contraction of Ly49D-sp cells (Fig. 6A). As expected from the pattern of expansion and contraction, coexpression of Ly49A counteracted Bim upregulation upon cytokine deprivation in Ly49AD-dp NK cells from D\(^{d+/+}\) mice, whereas coexpression of Ly49D did not (Fig. 6B). We conclude that coexpression of some inhibitory Ly49 receptors override Ly49D-mediated induction of apoptosis and rescues Ly49D\(^{+}\) NK cells from negative selection in the presence of self-ligand.

Positive and negative selection of Ly49A-sp and Ly49D-sp NK cells, respectively, are associated with positive and negative tuning of NK cell responsiveness in these subsets

Ly49A-sp cells were enriched for in D\(^{d+/+}\) and D\(^{d+/+}\) mice and also exhibited an increased cumulative functional responsiveness in these mice (Figs. 1, 2). The opposite was true for the Ly49D-sp subset, which showed a decrease in responsiveness in parallel with a drop in subsets frequency (Fig. 7, Supplemental Fig. 4). These parallel processes, visualized in pie charts in which subset size and effector responses are plotted in an integrative fashion (Fig. 7). Ly49AD-dp NK cells exhibited enhanced responsiveness in D\(^{d+/+}\) as compared with D\(^{d+/+}\) and MHC\(^{d+/+}\) mice as expected. Interestingly, the responsiveness of this subset was slightly higher than that seen for Ly49A-sp cells, possibly indicating that the coexpression of an activating receptor together with the inhibitory Ly49A receptor can provide additional signals leading to optimized responsiveness of NK subset (30).

Discussion

Early work on the impact of self-MHC class I on the Ly49 repertoire came to the paradoxical conclusion that NK cells expressing the self-specific Ly49A receptor were less frequent in mice expressing D\(^{d}\), a ligand for this receptor (20, 28, 31). The decrease was primarily seen in NK cells coexpressing Ly49A with Ly49G2, another D\(^{d}\)-specific receptor (20, 31), which led to the interpretation that NK cells expressing too many self-specific inhibitory receptors were disfavored in the repertoire. Our results are in
agreement with this early conclusion, but reveal several important novel concepts. First, based on the high resolution of our flow cytometry panel, we can conclude that only NK cells expressing three or more inhibitory receptors are contracted in the repertoire in response to MHC class I and that NK cells expressing one to two receptors, which include combinations of two strong self-receptors, are not contracted. Secondly, most NK cell subsets expressing one to two inhibitory receptors are in fact increased in frequency. This fits with the general idea and corresponds to recent data in a model of KIR-transgenic mice (22) that MHC education does not merely act to restrict the frequency of NK cells with multiple self-receptors, but also to enrich for NK cells with few inhibitory receptors. These are the potentially the most useful ones in the repertoire (at least from a missing self-perspective), because they are less likely to be overinhibited by self-MHC.

Thirdly, although the expression of Dd as such led to an enrichment of Ly49A-sp, Ly49C-sp, and Ly49G2-sp NK cells, only Ly49A-sp NK cells were sensitive to the gene dose effect of Dd. This unexpected finding indicates that several mechanisms may control repertoire formation in response to MHC class I. It has been proposed that interactions between MHC class I and Ly49 receptors can occur in trans (between NK cells and other cells) as well as in cis (in the NK cell membrane itself) (32, 33). One possibility is that the general shift in the repertoire toward few receptors results from MHC interactions in trans by the educating environment. This effect may be triggered by low levels of self-MHC class I expression that may affect several different Ly49 receptors (Ly49A, Ly49C, and Ly49G2 in this study) during NK cell development (20, 21, 28, 31, 34). On top of this general effect, which we have noted also in three additional MHC class I single mice (data not shown), a speculative idea is that the special interaction that occur between the Dd ligand and the Ly49A receptor in cis provides an additional survival benefit to this NK cell subset. One could speculate that the cis interaction may be more quantitative compared with the trans interaction, thus being more directly susceptible to variations in the level of self MHC class I. An additional explanation could be that Ly49 receptors may be functionally heterogeneous in terms of biological properties independent from MHC interactions, such as additional binding partners in the cell membrane, localizations to distinct membrane microdomains, or the signaling cascades they trigger, a potential diversity we know very little about at present. Thirdly, it is possible that the binding strength between individual Ly49 receptors and the Dd ligand influences these diverse outcomes.

Finally, we found that NK cells expressing only one activating receptor to self-MHC, Ly49D, in the presence of its ligand Dd, was contracted in the repertoire. This process resembles negative se-

FIGURE 4.  MHC class I dose-dependent education regulates the sensitivity of NK cells to IL-15. (A) p-Stat5 staining on sorted Ly49A+ NK cells from MHC2−/−, Dd+/+, and Dd+/− after 15 min of IL-15 stimulation (100 ng/ml). (B) Kinetics of p-Stat5 upregulation in the same experiment as in (A), over the indicated time points. One representative experiment of two. (C) CFSE dilution in sorted Ly49A+ NK cells from the indicated strains, cultured for 3 d in low dose (10 ng/ml) of IL-15. One representative experiment of three. (D) Intracellular bim staining (geometric mean fluorescence intensity [GeoMFI]) on y-axis and frequency of Ly49A-sp (Ly49C−, -D−, -G2−, -I−, NKG2A−) NK cells on x-axis after 11 h of cytokine deprivation ex vivo. One representative experiment of two is shown. The figure shows r and p values from the Pearson’s correlation test. *Statistical differences in bim GeoMFI between MHC2−/− and Dd+/+, p < 0.05.
lection of T cells and is likely necessary to avoid autoreactivity of this subset. Importantly, the coexpression of an inhibitory receptor with affinity for Dd rescued those cells from contraction, which is reminiscent of a previously proposed mechanism for self-tolerance in Ly49D-expressing NK cells in H2b mice (35).

The Ly49A-sp subset was always the most responsive in our assays and also the one that was most enriched for. This suggests that positive selection and functional responsiveness may be at least partially linked processes. For Ly49A-sp NK cells, this correlation may serve to secure optimal responsiveness in an NK cell subset of particular importance for missing self-responses in Dd-expressing mice. Data on Ly49D-sp NK cells, in which subset contraction and functional depression occurred in parallel, also support such a link. However, additional complexities reside in the system, as our preliminary data suggest that some contracted subsets show enhanced functionality, and some expanded subsets do not (data not shown). One such complexity may be related to homeostatic mechanisms acting to keep the size of the total NK cell population within a narrow range. Thus, of the two endpoints of education we discuss in this paper, subset skewing is a zero-sum game in which an increase in one or several subset must be accompanied by a decrease in other subsets, whereas responsiveness can theoretically increase in all subsets. One possibility is therefore that inhibitory input affects cellular responsiveness with a linear correlation, whereas the influence on subset expansion is a bell-shaped curve in which very high inhibition leads to a reduced subset size. Such a mechanism would decrease the number of overinhibited NK cells in the particular MHC environment, cells that might be less useful in the repertoire. At present, we believe that our data allow for the conclusion that the overall NK cell activity in a mouse strain is determined by the net outcome of functional education and subset skewing, two quantitative processes controlled by the strength of input from MHC class I.

A recent study proposed a link between NK cell activation via the adaptor DAP10 and IL-15 receptor signaling (36). Our findings suggest that signals via ITIM-coupled Ly49/NKG2A receptors may modulate the strength of IL-15R signaling in a similar way.

![FIGURE 5. Ligand-dependent contraction of an NK cell subset expressing the autoreactive activating Ly49D receptor but no inhibitory receptors.](image)

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**FIGURE 5.** Ligand-dependent contraction of an NK cell subset expressing the autoreactive activating Ly49D receptor but no inhibitory receptors. (A) Percent Ly49D+ NK cells among total CD3+CD19−NK1.1+ NK cell populations from MHC−/−, Dd+/-, and Dd+/+ or nonligand MHC class I-expressing K b mice. Mean of at least three experiments; n = 10–12/strain. (B) Percent Ly49D-sp (Ly49A+/-, -C-/-, -G2-/-, -I-/-, NKG2A−) cells from MHC−/−, Dd+/-, Dd+/+, and K b mice, respectively. Mean of at least three experiments; n = 9–12/strain. (C) Percent Annexin V+ among Ly49D-sp (Ly49A+/-, G2−/−, I−/−, NKG2A−) NK cells from the indicated strain, as stained directly ex vivo. Mean of three experiments; n = 6/strain. *p < 0.05, **p < 0.01, ***p < 0.001.

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**FIGURE 6.** Coexpression of inhibitory receptors prevents contraction of Ly49D-sp cells. (A) Frequency of splenic NK cells expressing Ly49D together with one inhibitory receptor (indicated) from MHC−/−, Dd+/-, Dd+/+, and K b mice. Summary of at least three experiments; n = 9–12/strain. (B) Intracellular bim staining (geometric mean fluorescence intensity [GeoMFI]) on y-axis and frequency of Ly49AD-dp (C + G2−I−NKG2A−) and Ly49ID-dp (A + C + G2−NKG2A−) on x-axis after 11 h of cytokine deprivation. One representative experiment of two is shown with n = 3–5/strain. *p < 0.05, **p < 0.01, ***p < 0.001.
and propose that this link explains the difference in frequency of Ly49A-sp NK cells in mice with high and low expression of D\(^\beta\). A direct link between the extent of inhibitory receptor signaling and NK cell homeostasis (37) would secure that NK cells with an optimal Ly49 receptor expression (in relation to the self-MHC setup) would have a survival benefit in a situation of limited IL-15 availability. However, it is also possible that signaling via the IL-15 receptor itself is a primary education event that controls Ly49 receptor expression, perhaps by influencing NK cell differentiation, rather than being regulated by inhibitory receptor signaling. More work will be needed to differentiate between these models and clarify the role of IL-15 in formation of the NK cell repertoire in vivo.

The quantitative control of NK cell function by the expression level of a single MHC class I allele was predicted from previous reports by us and others (14, 16–18). A recent study in which the level of a single MHC class I allele was predicted from previous repertoire in vivo. 

A recent paper based on mathematical modeling showed that inhibitory receptors could mediate NK cell activation under conditions of low ligand strength (30). Together, these studies suggest an unexpected complexity in inhibitory signaling. More work in this exciting area may uncover new principles in NK cell signaling that could clarify the presently paradoxical role of inhibitory receptors in NK cell education. Whatever these mechanisms are, we conclude from the current study that they affect function and also selection of NK cell subsets, possibly based on their potential usefulness in the NK cell repertoire.

A recent study required a combination of single MHC class I-inbred mice and high-dimension flow cytometry, a reductionistic approach that could clarify the presently paradoxical role of inhibitory receptors in NK cell education. Whatever these mechanisms are, we conclude from the current study that they affect function and also selection of NK cell subsets, possibly based on their potential usefulness in the NK cell repertoire.

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**Disclosures**

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**References**