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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 Tadeially Lakshminanth, 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: 000–000.

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).

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Abbreviations used in this article: dp, double-positive; KIR, killer Ig-like receptor; KLRG1, killer cell lectin-like receptor G1; sp, single-positive.

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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.Kb2Dd+/+ (depicted as MHC class I), mice expressing Dd only (B6.Kb2Dd−/+), and B6.Kb2Dd+/− were used with anti-Mip1α biotin, and dead cells were excluded using a conjugation kit (Molecular Probes/Invitrogen). Streptavidin Qdot-565 Probes). NKG2A/C/E (20d5) was conjugated to Pacific Blue using a conjugation kit (Molecular Probes Sweden) and conjugated to QDot-605 using conjugation kit (Molecular Probes). Ly49D (4E5) purified were purchased from BD Pharmingen (Stockholm, Sweden) and followed by Annexin V FITC according to manufacturer’s protocol (BD Biosciences).

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 PBS. All surface staining was done on ice in FACS buffer (PBS with 0.5% FBS and 0.02 M 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).

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 monoensine/Go6976 (BD Pharmingen) and 2 μM 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 Mip1α 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 Alexa700 and anti-Mip1α FITC.

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% parformaldehyde 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 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 level of H2Dd tunes the strength of missing self-recognition.

Axinn V staining

For Axinn V staining ex vivo, cells were first stained with Abs to surface markers in FACS buffer, followed by incubation in Axinn V buffer (BD Biosciences), and followed by Axinn V FITC according to manufacturer’s protocol (BD Biosciences).
FIGURE 1. Dose-dependent influence of MHC class I on NK cell-mediated function. (A) Surface D^d expression level on total splenocytes stained with an anti-D^d 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 D^d/- and D^d/+ mice. (C) Frequencies of cells within the indicated subset, responding to NKp46 cross-linking with the production of IFN-γ, Mip1α, or CD107α. 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, D^d/-; and dark grey bars, D^d/+. (E) Percent Ly49A-sp (Ly49C-, NKG2A-) 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 (CD107α) 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^- CD3e^- NK1.1^-) was similar in D^d/- and D^d/+ mice, but both strains differed from MHC^-/- mice (Fig. 1C). Ly49A^- NK cells were more likely to respond with three effector responses simultaneously in D^d/- 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 D^d/- and D^d/+ 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 D^d-expressing 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 D^d 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 D^d 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 D^d (Ly49A-sp), two subsets were responsive to D^d as such but were insensitive to the quantitative input (Ly49C-sp, Ly49G2-sp), and two subsets were not increased in response to D^d, irrespective of expression level (Ly49I-sp, NKG2A-sp).

The phenotypes of NK cells in MHC^-/-, D^d/-, and D^d/+ 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 D^d 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 Dd+/+ and Dd+/- 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 Dd+/+ mice compared with Dd+/- 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<sup>-/-</sup>, Dd+/-, and Dd+/+ mice. 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 Dd<sup>+/+</sup> mice compared with the same cells from Dd<sup>-/-</sup> and MHC<sup>-/-</sup> 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$^{-/-}$, Dd$^{-/-}$, and Dd$^{+/+}$ mice in vitro for 11 h without cytokine supplement and measured Bim expression in Ly49A-sp NK cells (Fig. 4D). Directly ex vivo, Bim was similarly expressed (data not shown). Altogether, our results suggest that signaling from MHC class I receptors quantitatively correlates with a higher sensitivity to IL-15 stimulation and a higher resistance to apoptosis, possibly via effects on Bim upregulation.

**Negative selection of NK cells expressing the activating Dd-binding receptor Ly49D in the absence of coexpressed inhibitory receptors**

Our observations so far identified a process resembling positive selection of presumably useful NK cell subsets within the NK cell repertoire. To investigate if changes in the opposite direction would occur for potentially dangerous NK cells, we studied NK cells expressing the Dd$^{+/-}$ binding activating receptor Ly49D in the same system. No differences in the total frequency of Ly49D-expressing NK cells were seen in these mice compared with MHC$^{-/-}$ 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 Dd$^{+/-}$-expressing mice. There was a tendency toward a less prominent contraction in Dd$^{+/+}$ versus Dd$^{+/+}$ 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 Dd$^{+/+}$ mice than in MHC$^{-/-}$ mice, with a tendency for intermediate levels in Dd$^{+/-}$ 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.

**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 Dd$^{+/+}$ and Dd$^{+/-}$ 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, are 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 Dd$^{+/-}$ as compared with Dd$^{+/+}$ and MHC$^{-/-}$ 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 Dd$^-$, a ligand for this receptor (20, 28, 31). The decrease was primarily seen in NK cells coexpressing Ly49A with Ly49G2, another Dd-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 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.

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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-
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 H2d 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. (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 Kb 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 Kb mice, respectively. Mean of at least three experiments; n = 9–12/strain. (C) Percent Annexin V− among Ly49D-sp (Ly49A−C−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.

**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 Kb 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 Dd. 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 expression of Dd, similar to our mice, presented a different responsiveness of NK cells in mice with hemi- or homozygous reports by us and others (14, 16–18). A recent study in which the signaling. More work will be needed to differentiate between these pathways associated with NK cell activation (48). In addition, the simultaneous detection of multiple effector responses is important to detect the quantitative differences between NK cells educated in Dd+/+ as compared with Dd−/− mice. Although subtle, these differences have strong functional consequences for the global NK cell-mediated responses in vivo, as shown in this study for the 50% reduced missing self-responses in Dd+/+ as compared with Dd+/+ mice (Fig. 1B).

Functional education of human NK cells by HLA class I is well established (13, 15, 39–42), and a quantitative influence of HLA on NK cell responsiveness may explain, for example, how a single nucleotide polymorphism associated with a higher HLA-C expression levels is beneficial in acute HIV infection (43, 44). The role of HLA class I molecules on the human NK cell repertoire is less clear, but is implied from studies showing that specific compound genotypes of KIR and HLA may provide protection or susceptibility to infections, cancers, and successful pregnancy (45). Evidence for control of NK cell subset frequencies by HLA also exist (46) but appear to be less strong than in the mouse. At this stage, it should be emphasized that the selection events described in this study required a combination of single MHC class I-inbred mice and high-dimension flow cytometry, a reductionistic system that cannot be reproduced in outbred humans (47). The potential differences between the KIR/HLA and Ly49/H2 systems should benefit from a more thorough comparison using similar analysis platforms.

Several models have been proposed for how NK cells are educated by MHC class I, including our own metaphor of NK cell education as a rheostat (11, 12, 16–18, 42). The most important issue to resolve in the future is which signaling pathways connect Ly49 receptor signaling to functional education and repertoire formation. Recent evidence suggests that ITIM-mediated signaling leads to active phosphorylation of components in signaling pathways associated with NK cell activation (48). In addition, 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.

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Disclosures
The authors have no financial conflicts of interest.

References


Boolean exclusion

Total Ly49A⁺

 NK1.1

Ly49A, NKG2A

Ly49C

Ly49D

Ly49G2

Ly49A-sp

Functional responses in the subset

IFNγ

Mip1α

CD107a

CD107a

IFNγ

FSC-H

SSC

FSC-A

Vivid

CD3ε

Brodin et al, Figure S1
Gated on CD3- NK-marker pos (NKp46 or NK1.1)

Brodin et al, Figure S2
Expression of the IL-15 receptor complex in Ly49A-sp cells

Gated on Ly49A⁺/C⁻/G⁻/NKG2A⁻ NK cells

% Ly49A⁺ NK cells in division in 3 days in low dose IL-15 (10ng/ml)

Brodin et al, Figure S3
Ly49D-sp NK cells

Brodin et al, Figure S4
**FIGURE S1.** Flow panel for functional experiments and individual responses by Ly49A-sp cells. Representative staining for the identification of Ly49A-sp (Ly49C-, D-, G2-, I-, NKG2A-) splenic NK cells with simultaneous detection of functional markers CD107a, Mip1α and IFNγ after NKp46 cross-linking. Figure shows the gating strategy for Boolean gating for the identification of Ly49A-sp cells as well as the response of these cells to NKp46 cross-linking. Black dots in functional plots are stimulated sample and background contour plot is unstimulated (PBS) control.

**FIGURE S2.** Phenotype of MHC/-, Dd/- and Dd/+ NK cells. Representative staining of 15 surface proteins for phenotypic comparisons between NK cells as defined by CD38-NK1.1+ (or NKp46+ for NK1.1 staining).

**FIGURE S3. (A)** Surface expression of IL-15 receptor complex is independent of NK cell education. Representative staining for IL-15 components: CD122, CD132 and IL-15Rα chains on Ly49A-sp (Ly49C-, D-, G2-, I-, NKG2A-) splenic NK cells stained *ex vivo.* (B) Proliferation of Ly49A+ NK cells in low-dose IL-15. Summary of %Ly49A+ NK cells in division on day three in low dose IL-15(10ng/ml). One-way ANOVA with Bonferroni’s correction for multiple testing. P-values: ** <0.01.

**FIGURE S4.** Reduced responsiveness in Ly49D-sp cells in Dd/+ mice. Mean % Ly49D-sp NK cells responding to NKp46 cross-linking with 1,2, or 3 (IFNγ, Mip1α, CD107a) simultaneous effector responses *in vitro.* Mean values of 4-6 experiments, n=2-5/strain. P-value: * <0.05.