

NK Cell Responsiveness Is Tuned Commensurate with the Number of Inhibitory Receptors for Self-MHC Class I: The Rheostat Model¹

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Inhibitory receptors that engage self-MHC class I molecules enable NK cells to detect disease-associated loss of MHC class I on surrounding cells. Previous studies showed that some NK cells lack all receptors for self-MHC class I, yet fail to exhibit autoimmunity because they are generally hyporesponsive to stimulation. We asked whether NK cells exist in only two states, responsive and hyporesponsive, corresponding to cells that express or fail to express inhibitory receptors for self-MHC class I. The alternative model is that NK cells vary continuously in their responsiveness, based on variations in the number of different inhibitory and stimulatory receptors they express, which is known to vary. In this study, we show in the murine system that NK cell responsiveness increases quantitatively with each added self-MHC-specific inhibitory receptor. Genetic analysis demonstrated that interactions of each of the receptors with self-MHC class I were necessary to observe augmented responsiveness. These findings suggest that NK cell responsiveness is comparable to a rheostat: it is tuned to an optimal set point depending on the inhibitory and stimulatory interactions encountered in the normal environment, so as to ensure self-tolerance and yet optimize sensitivity to changes in normal cells. *The Journal of Immunology*, 2009, 182: 4572–4580.

Natural killer cells represent a first line of defense against infections and developing malignancies (1–4). The finding that NK cells preferentially attack target cells that lack cell surface MHC class I molecules was the basis of the “missing-self” hypothesis, which states that NK cells protect the host from cells that lack critical markers of normalcy: self-MHC class I proteins (5, 6). NK cells express several families of inhibitory receptors specific for MHC class I molecules and various stimulatory receptors with diverse specificities. The net balance of activating and inhibitory signals resulting from interactions with the various ligands presented on a given target cell determines whether the NK cell becomes activated, produces inflammatory cytokines (including IFN- γ and TNF- α), and/or kills the target cell (7–9).

The NK cell population consists of many subpopulations expressing various combinations of the available inhibitory receptors (9–11). The variegated expression pattern of the inhibitory receptors confers each NK cell with a distinct pattern of reactivity, enabling some of them to attack cells lacking one MHC class I mol-

ecule and not another. However, it presents a major challenge as well: because the expression of each inhibitory receptor is determined by a probabilistic gene expression mechanism (9, 12–14), some NK cells should arise during development that lack inhibitory receptors specific for self-MHC class I molecules. Such NK cells have the potential to be autoreactive. How is NK cell self-tolerance established under these circumstances?

It was previously thought that NK cell self-tolerance ultimately depends on the expression at the surface of all mature NK cells of at least one inhibitory receptors specific for self-MHC class I (9, 15–18). Reconciling this model with the random expression of inhibitory receptors required that NK cells that lack receptors specific for self-MHC class I are eliminated during development. An alternative possibility was suggested by the finding that animals or individuals that lacked expression of MHC class I proteins acquired NK cells in normal numbers and these NK cells were self-tolerant (19–24). Hence, NK cells can be self-tolerant despite the absence of MHC class I molecules that can engage inhibitory receptors on the NK cells.

A substantial advance in understanding NK cell tolerance was the finding that some NK cells present in normal mice do not, in fact, express inhibitory receptors for self-MHC class I molecules (25). Such NK cells were shown to be mature in terms of marker expression yet failed to kill or respond to self-target cells. A clue to the basis of self-tolerance of these cells was their reduced response to a variety of stimuli, including tumor cell lines and cross-linking of stimulatory receptors using plate-bound Abs (25). Previous studies had shown that NK cells from MHC class I-deficient mice were similarly hyporesponsive (21, 26). It was proposed that these NK cells are self-tolerant due to impaired or dampened stimulatory signaling (25, 27). A separate study independently reached similar conclusions by using gene transfer to show that NK cells with inhibitory receptors for a transgenic MHC class I molecule exhibited high functional activity, whereas NK cells that lacked the receptor

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showed low functional activity (28). Studies of NK cells from healthy humans yielded similar results (29–31).

These studies led to the notion that some NK cells are hypo-responsive, whereas others, which express inhibitory receptors for self-MHC class I, are all similarly responsive. We questioned this binary model because NK cells are known to vary in the number and affinity of self-MHC-specific inhibitory receptors they express. Published reports showed that expression of two inhibitory receptors led to stronger effector cell inhibition by target MHC class I molecules than expression of only one (32), and different levels of inhibition resulted depending on the MHC class I molecule engaged by a given receptor (33). Hence, the extent of inhibitory receptor engagement accompanying interactions with normal cells is expected to vary considerably. Furthermore, NK cells vary in their expression of stimulatory killer Ig-like receptor or Ly49 receptors that bind to ligands on normal cells. As a result of these variations, developing NK cells will vary considerably in the balance of stimulatory and inhibitory receptor engagement that occurs when they encounter normal cells. The question arises whether NK cells exist only in homogenous highly responsiveness and hypo-responsiveness sets or instead vary continuously in their responsiveness. When a developing NK cell receives only a small excess of stimulation over inhibition, is it dampened to the same degree as a NK cell that receives a large excess of stimulation or is it dampened to a lesser extent, commensurate with the modest excess of stimulation that must be countered in order for the cell to be rendered self-tolerant?

A previous report demonstrated that as the number of different expressed MHC class I alleles increases in a mouse strain, the overall capacity for missing self-recognition by the NK cell population also increases (34). In the present study, we addressed how NK cell responsiveness is impacted by variations in the number of Ly49 and/or CD94/NKG2A inhibitory receptors that can engage self-MHC class I molecules. We show a striking continuum in the responsiveness of the NK cells, in which expression of a greater number of inhibitory receptors for self-MHC resulted in greater inherent responsiveness. Hence, NK cell responsiveness is adjusted along a spectrum depending on the stimulatory and inhibitory interactions that NK cells encounter in a given host.

Materials and Methods

Mice and cell lines

Mice were bred in the animal facility at the University of California according to institutional guidelines. C57BL/6J (designated as B6) mice (H-2^b) were obtained from The Jackson Laboratory. Congenic B6 mice expressing Ly5.1 (B6-Ly5.2/Cr) were obtained from Charles River Laboratories. β_2 -microglobulin ($\beta_2m^{-/-}$), $K^b^{-/-}$, $D^b^{-/-}$, and $K^bD^b^{-/-}$ mice on the B6 background were described previously (19, 20, 35, 36). B6 $\beta_2m^{-/-}$ Ly-5.1 mice were derived in our facilities.

The mouse lymphoma YAC-1 cell line (TIB-160; American Type Culture Collection) was cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (Omega Scientific), 100 U/ml penicillin (Invitrogen Life Technologies), 100 μ g/ml streptomycin (Invitrogen Life Technologies), 0.2 mg/ml L-glutamine (Sigma-Aldrich), 50 μ M 2-ME (EMD Biosciences), and 20 mM HEPES (Fisher).

Abs and flow cytometric analysis

Abs against NK1.1 (PK136), CD3 ϵ (145-2C11), NKG2A/C/E (20d5), Ly49I (YLI-90), CD45.1 (A20), CD45.2 (104), IFN- γ (XMG1.2), CD107a (eBio1D48), and NKG2D (MI6) (37) were purchased from eBioscience and BD Pharmingen. Purified NKp46 (29A1.4) (38) was conjugated using a Alexa Fluor 647 Protein Labeling Kit from Molecular Probes. The 4LO3311 hybridoma (anti-Ly49C) was a gift from S. Lemieux (INRS, Institut Frappier, Laval, Quebec, Canada) (39), and the mAb was purified

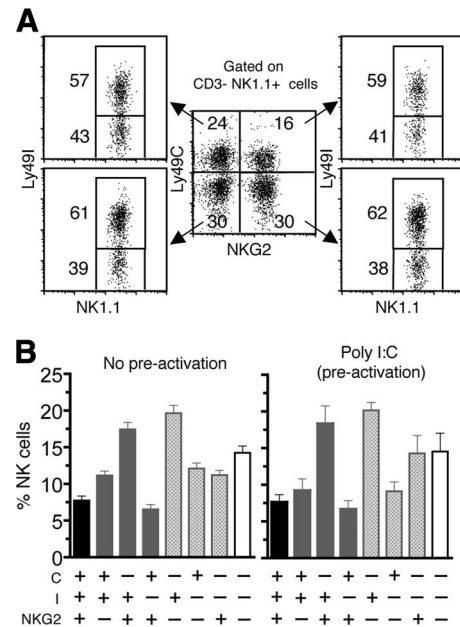


FIGURE 1. The self-MHC class I-specific inhibitory receptor repertoire in B6 mice. Using flow cytometry, spleen NK cells from B6 were simultaneously analyzed for expression of Ly49C (C), Ly49I (I), or NKG2A/C/E (NKG2). A, Representative flow cytometry analysis showing the eight different NK cell subsets expressing none, any one, any pairs, or all of the self-MHC-specific inhibitory receptors. B, Percentages of the above defined NK subsets in splenocytes from B6 mice that had been previously treated or not with poly(I:C). Data represent the mean \pm SD ($n = 5$). Similar results were obtained in at least two other experiments, whether NK cells were gated as NKp46⁺ cells or CD3⁻NK1.1⁺ cells.

from CELLline classic 1000 (Integra Biosciences) supernatant, then conjugated to biotin according to standard methods. Biotin-conjugated mAb were detected with streptavidin-PE-Texas Red (BD Pharmingen) or streptavidin-Pacific Blue (Invitrogen Life Technologies). Depleting Abs against NK1.1 (PK136), Ly49C and Ly49I (SW5E6), and NKG2A (16a11) were purified from CELLline classic 1000 supernatants. Before staining, cells were preincubated for 20 min with 2.4G2 hybridoma supernatant to block Fc γ R/III receptors. Flow cytometry was performed on a FC500 Dual Laser System Machine (Beckman Coulter) or a BD Biosciences LSR II cytometer, and data were analyzed with the FlowJo software (Tree Star).

In vitro NK cell stimulation and functional assays

Mice were either not pretreated or were injected i.p. with 200 μ g of poly(I:C) (Sigma-Aldrich) 1.5 days before harvesting spleens for NK cell

Table I. Percentages of NK cell subsets expressing self-MHC class I-specific inhibitory receptors in B6 mice^a

	No Preactivation		Poly(I:C) (preactivation)	
	Observed ^b	Predicted ^b	Observed	Predicted
C ⁺ I ⁺ NKG2 ⁺	7.7 \pm 0.6 ^c	9.0 \pm 0.5	7.6 \pm 1.0	8.6 \pm 1.5
C ⁺ I ⁺ NKG2 ⁻	11.1 \pm 0.6	12.0 \pm 0.7	9.2 \pm 1.6	9.6 \pm 1.5
C ⁻ I ⁺ NKG2 ⁺	17.4 \pm 1.0	15.0 \pm 1.0	18.4 \pm 2.4	17.5 \pm 1.8
C ⁺ I ⁻ NKG2 ⁺	6.5 \pm 0.6	7.1 \pm 0.5	6.7 \pm 1.2	6.8 \pm 0.8
C ⁻ I ⁻ NKG2 ⁻	17.4 \pm 1.0	20.0 \pm 0.9	20.2 \pm 1.1	19.7 \pm 1.3
C ⁺ I ⁻ NKG2 ⁻	12.1 \pm 0.8	9.4 \pm 0.8	9.1 \pm 1.3	7.7 \pm 1.1
C ⁻ I ⁺ NKG2 ⁺	11.2 \pm 0.7	11.8 \pm 0.6	14.3 \pm 2.5	14.1 \pm 1.8
C ⁻ I ⁻ NKG2 ⁻	15.7 \pm 0.8	14.2 \pm 0.9	14.5 \pm 2.5	16.0 \pm 2.7

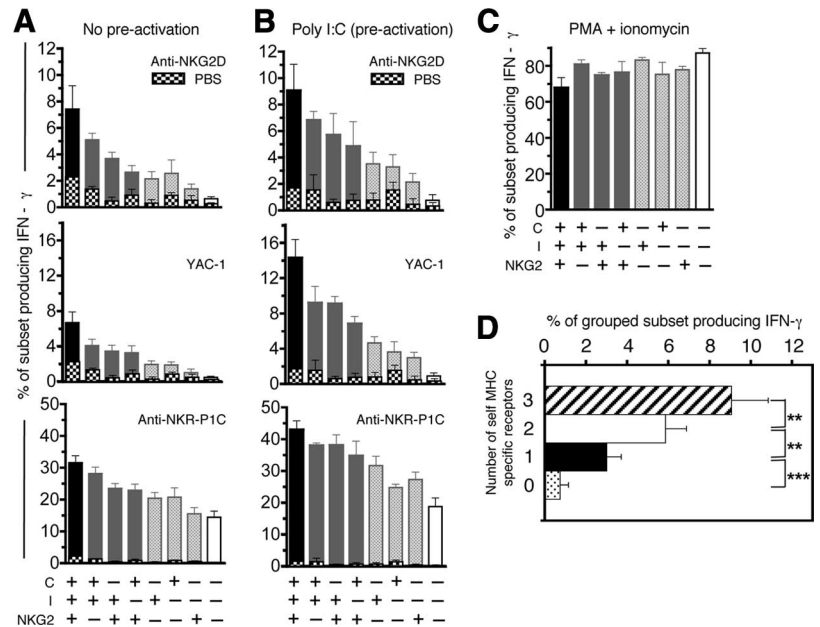
^a NK cells expressing any combinations of two or three self-MHC class I-specific inhibitory receptors in spleens from B6 mice previously treated or not with poly(I:C).

^b Observed percentages are compared to those predicted by the product rule, which predicts percentages of cells expressing a combination of receptors by multiplying the percentages of cells expressing or not each receptor. For example, the predicted percentage of C⁺I⁻NKG2⁺ cells equals (%C⁺)(%I⁻)(%NKG2⁺)/10,000.

^c Numbers represent percentages of NKp46⁺ NK cells (mean \pm SD; $n = 5$).

⁵ Abbreviations used in this paper: β_2m , β_2 -microglobulin; MFI, mean fluorescent intensity.

FIGURE 2. IFN- γ production responses vary continuously in NK cells expressing zero, one, two, or three self-specific inhibitory receptors. Splenocytes from B6 mice that were left untreated (A) or previously treated with poly(I:C) (B) were stimulated for 5 h in the presence of brefeldin A with plate-bound anti-NKR-P1C (PK136; 50 μ g/ml), anti-NKG2D (5 μ g/ml; MI6), YAC-1 tumor cells (10^6 cells), or no stimulation (PBS-coated wells). After staining, gated NKp46 $^+$ NK cells were analyzed simultaneously for intracellular IFN- γ production and expression of Ly49C, Ly49I, and NKG2. Statistical analysis for all comparisons are summarized in supplemental Table I. C, The same analysis as in A and B on spleen NK cells from poly(I:C)-treated B6 mice upon incubation with a mixture of PMA and ionomycin for 5 h. D, IFN- γ responses to plate-bound NKG2D Ab of poly(I:C)-treated B6 mice NK subsets grouped according to the number of receptors expressed. Groups differed significantly as shown (**, $p < 0.01$ and ***, $p < 0.001$) based on two-tailed paired t tests comparing subsets within the same mouse. For each panel, the data represent means \pm SD ($n = 5$).



preparation, as indicated. For analysis of IFN- γ production, round-bottom (or flat-bottom in the case of NKG2D Ab stimulation) high protein-binding plates (Costar and Fischer Scientific) were coated overnight at 4°C with the indicated concentrations of intact mAb in 100 μ l of PBS, followed by four rinses with 200 μ l of PBS, before adding NK cells. The IFN- γ production assay used the Cytotfix/Cytoperm intracellular staining kit (BD Pharmingen). Spleen cell suspensions were centrifuged in Lympholyte-Mammal (Cedarlane Laboratories) and 1×10^6 cells/well (100 μ l) were incubated for 5 h in RPMI 1640/10% FCS in the presence of 1 μ g/ml GolgiPlug (containing brefeldin A; BD Pharmingen) and 1000 U/ml recombinant human IL-2 (Hoffmann-La Roche) before transfer to V-bottom plates for staining. Where indicated, 1×10^6 YAC-1 cells or a mixture of 400 nM PMA (Sigma-Aldrich) and 3.35 μ M ionomycin (Sigma-Aldrich) were added as a source of stimulation. The degranulation assay was performed similarly, except GolgiPlug was replaced by GolgiStop (containing monensin; BD (Pharmingen) and CD107a Ab was added to the wells at the beginning of the incubation period.

CFSE-labeled bone marrow cell engraftment assay

Bone marrow graft rejection assays were performed as previously described (25). Briefly, CFSE-labeled bone marrow cells from $\beta_2m^{-/-}$ Ly5.1 and B6 Ly5.2 mice were mixed and injected (5×10^6 each) i.v. into mice that had been irradiated earlier in the day with 9.5 Gy from a ^{137}Cs source. Some mice were predepleted of NK cells or NK subsets by i.p. injection of Abs PK136 (anti-NK1.1), SW5E6 (anti-Ly49C and Ly49I), or 16a11 (anti-NKG2A). Recipient spleen cells, harvested 3 days after marrow transplantation, were analyzed for CFSE $^+$ Ly5.1 $^+$ (class I-deficient) and CFSE $^+$ Ly5.2 $^+$ (nonrejected control B6 cells) by flow cytometry. Graft acceptance in test animals was determined as the ratio of $\beta_2m^{-/-}$ (Ly5.1 $^+$) to B6 (Ly5.1 $^-$) cells among CFSE $^+$ recipient spleen cells and normalized to the mean corresponding ratio obtained when the marrow cells were injected into $\beta_2m^{-/-}$ recipients, which cannot reject class I-deficient grafts (20). Percent graft rejection was calculated as $(1 - \text{normalized acceptance}) \times 100$.

Results

Frequencies of NK cells coexpressing self-MHC-specific inhibitory receptors

In inbred B6 mice, Ly49C (specific for K b), Ly49I (specific for K d) and the CD94/NKG2A receptor (specific for a D b peptide presented by Qa-1) have been attributed with substantial reactivity against self-MHC class I molecules, with other receptors showing no or marginal reactivity (33, 40, 41). The frequencies of NK cells with each possible combination of the three receptors, or lacking all of them, were determined by multicolor flow cytometry, as shown by the representative plots in Fig. 1A. The complete anal-

ysis showed that the percentages of cells expressing each receptor combination varied between 6 and 20% (Fig. 1B). These percentages were generally in close agreement with the predictions of the “product rule” (Table I), which assumes that each receptor is expressed independently and which is calculated by multiplying together the frequencies of cells expressing, or not, each of the receptors (9). The composition of the NK cell population was similar in untreated mice and in mice pretreated with poly(I:C), which preactivates NK cells and is sometimes used to enhance NK cell reactivity (Fig. 1B). Hence, appreciable subsets of NK cells expressing zero, one, two, or three inhibitory receptors specific for self-MHC class I are represented in B6 mice.

Graded NK cell responses depending on the number of self-MHC-specific receptors expressed

To address how variations in the number of self-MHC-specific inhibitory receptors impact NK cell responsiveness, we used multiparameter flow cytometry to measure the functional responses of each of the possible NK subsets defined by expression of the three inhibitory receptors. We gated the cells based on staining with a NKp46-specific mAb, which is specific for NK cells (38). The results demonstrated a striking continuum in the responsiveness of the NK cells (Fig. 2, A and B). NK cells expressing all three receptors exhibited the greatest response. Expression of any two receptors, or any one of the receptors, was associated with successively lower responses. In general, the differences between subsets with successively fewer receptors were statistically significant (supplemental Table I 6). NK cells lacking all three receptors responded poorly in all cases. Consistent with earlier studies (25), the relative response of this subset depended somewhat on the stimulus used, with there being a higher relative response in the case of NKR-P1C stimulation than for NKG2D or YAC-1 stimulation (Fig. 2, A and B) even when lower doses of anti-NKR-P1C were used to stimulate the cells (supplemental Fig. 1). In the case of YAC-1 or NKG2D responses, the overall range of responsiveness was considerable, with the Ly49C $^+$ Ly49I $^+$ NKG2A $^+$ triple-positive subset responding up to 20-fold better than the Ly49C $^-$ Ly49I $^-$ NKG2A $^-$ triple-negative subset (Fig. 2, A and B).

⁶ The online version of this article has supplemental material.

Poly(I:C) treatment enhanced the functionality of all the NK cell subsets studied in terms of the percentage of IFN- γ -producing cells (Fig. 2B), but it did not appreciably alter the relative responsiveness of the different subsets. Consistent with previous reports (25, 28), the NK cell subsets studied responded similarly to pharmacological stimulation with PMA combined with ionomycin, indicating that all NK cell subsets are equipped to execute an activation program (Fig. 2C).

These data documented that each NK cell subset with multiple inhibitory receptors specific for self-MHC class I exhibited greater responsiveness than NK cells with only one such receptor. Fig. 2D generalizes this finding by depicting the responsiveness to plate-bound anti-NKG2D of all NK cells in B6 mice with any one, any pair, or all three of the receptors under study. The responses of each group increased significantly in each successive comparison ($p < 0.01$ for each). Hence, responsiveness of NK cells increases with the expression of additional self-MHC-specific inhibitory receptors.

The analysis also showed that NK cells expressing any one of the inhibitory receptors varied somewhat, with Ly49C⁺ or Ly49I⁺ cells exhibiting modestly but significantly higher activity than NKG2A⁺ cells in almost all cases (Fig. 2, A and B, and supplemental Table II). Furthermore, the responses of cells expressing any pair of inhibitory receptors also varied, one example being that the NK cells coexpressing Ly49C and Ly49I were in some cases significantly more responsive than the Ly49C⁺NKG2A⁺ subset (Fig. 2, A and B, and supplemental Table II). These data suggested that responsiveness varies depending on which self-MHC-specific receptor(s) is expressed, as well as on the number of different self-MHC-specific receptors expressed.

Responsiveness is determined by receptor interactions with specific MHC class I molecules

We next addressed whether the variations in NK cell responsiveness were due simply to receptor coexpression or whether they reflected the effects of receptor engagement with self-MHC class I molecules. We compared the IFN- γ response of each subset in B6 wild-type mice to that in D^b-/-, K^b-/-, K^bD^b-/-, and β_2m -/- mice after NKG2D cross-linking (Fig. 3). The absence of a ligand for the NKG2A receptor in the D^b-/- mice resulted in a lower functional response in all of the NKG2A-positive subsets when compared with B6 wild-type mice. Expression of NKG2A alone (as in Ly49C⁻Ly49I⁻NKG2A⁺ NK cells) resulted in an appreciable response in B6 mice, which express a ligand, but only a minimal response in D^b-/- mice, which do not. Furthermore, in B6 mice coexpression of NKG2A resulted in higher responsiveness of subsets that also expressed Ly49C and/or Ly49I, but in D^b-/- mice NKG2A coexpression did not enhance the responsiveness of NK cells. Therefore, NKG2A coexpression only enhanced responsiveness when a cognate ligand was available in the mice.

K^b-/- mice lack strong ligands of the Ly49C and Ly49I receptors but express the ligand for NKG2A. As predicted, NK cells in K^b-/- mice that expressed Ly49C and/or Ly49I, but not NKG2A, responded no better than NK cells lacking all three receptors. Furthermore, in K^b-/- mice but not in D^b-/- mice, NKG2A expression by itself led to a significantly higher response than observed in NK cells lacking all three receptors. NKG2A⁺ cells from K^b-/- mice that also expressed Ly49C or Ly49I responded no better than NK cells that expressed NKG2A alone. Hence, of the three receptors studied, NKG2A expression was necessary and sufficient for generating responsive NK cells in K^b-/- mice. Coexpression of additional receptors did not enhance responsiveness, because these receptors failed to engage a ligand in these mice.

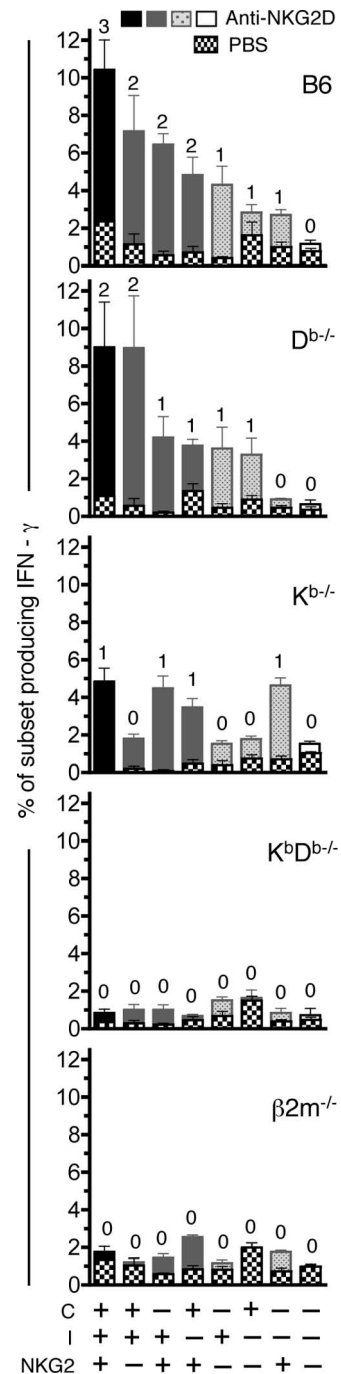


FIGURE 3. The interactions of the inhibitory receptors with self-MHC class I molecules determine the responsiveness of each subset in the NK cell population. Splenocytes from B6, D^b-, K^b-, K^bD^b-, or β_2m -deficient mice previously treated with poly(I:C) were cultured for 5 h on plates coated with anti-NKG2D (MI6) at 5 μ g/ml or with no stimulation (PBS-coated wells) in the presence of brefeldin A. IFN- γ -producing cells in each subset were analyzed as in Fig. 2. Data represent the mean \pm SD ($n = 3$ or 4). Numbers above each bar indicate the number of self-MHC class I molecules that cells in each subset are able to engage. Similar results were obtained in one replicate experiment.

The absence of both K^b and D^b, in K^bD^b-/- double knockout mice, generally resulted in a low response of all of the subsets (Fig. 3). Comparisons of the responses of subsets in these mice with those of other groups were informative in confirming the specificity of NK receptors. In the case of NKG2A⁺ NK cells, the response was much lower in K^bD^b-/- mice than in K^b-/- mice,

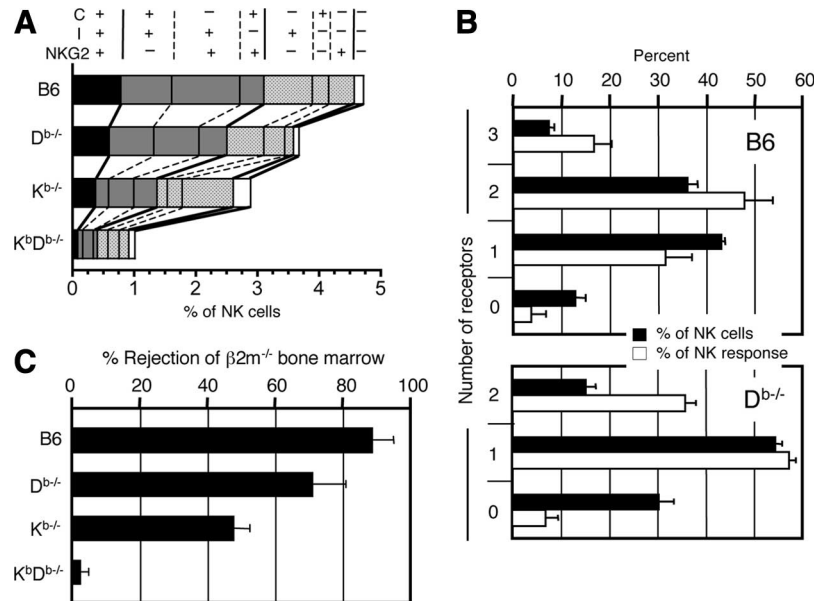


FIGURE 4. Contributions of each NK cell subsets to the overall NK cell response in vitro and in vivo. **A**, The stacked bars represent the contributions to the overall response of the different subsets expressing all three of the receptors (black bars), any two (gray bars), any one (shaded bars), or none (open bars) of them. The data describe the IFN- γ response of NK cells from poly(I:C)-pretreated mice to NKG2D Ab-coated plates (5 μ g/ml). The contributions (percentage of NK cells depicted on the x-axis) were determined by combining the data from Fig. 2 (frequency of responding cells) and Fig. 1 (frequency of the subset in the NK population) as: (frequency of a subset) \times (frequency of IFN- γ -producing cells in that subset) \times 100. Data represent the mean ($n = 3$ or 4). Symbols above the plot indicate the specific subset depicted. **B**, Comparisons of the percentages of NK cells expressing three, two, one, or zero self-specific inhibitory receptors (black bars) with the percentages of the total response attributable to that set (open bars). The percentage of the total response for each set was calculated as: $100 \times (\text{the contribution of the set to the overall response, in percent}) / (\text{percentage of total NK cells that respond})$. For example, the percentage of NK response for cells expressing two receptors = $[100 \times (\text{contribution of } C^{+}I^{+}NKG2^{-}$ cells + contribution of $C^{+}I^{-}NKG2^{+}$ cells + contribution of $C^{-}I^{+}NKG2^{+}$ cells)] / (percentage of total NK cells that respond)]. NK cells from B6 mice ($n = 4$) and $D^{b-/-}$ mice ($n = 3$) are compared. Similar results were obtained in one replicate experiment. **C**, In vivo responsiveness of NK cells was assessed by the rejection of class I-deficient bone marrow grafts. A 1:1 mixture of CFSE-labeled bone marrow cells from $\beta 2m^{-/-}$ mice (target cells) and congenic B6 normal mice (internal reference control) was injected i.v. into irradiated (9.5 Gy) B6, $D^{b-/-}$, $K^{b-/-}$, $K^{b}D^{b-/-}$, or $\beta 2m^{-/-}$ deficient hosts. Three days later, rejection was assessed in the spleens. Bars depict the percentage of target cell rejection after normalization to the “no-rejection” control ($\beta 2m^{-/-}$ deficient hosts). Data represent means \pm SD ($n = 3$ or 4 mice). Similar results were obtained in two replicate experiments.

confirming the importance of D^b in conferring high responsiveness. In the case of NK cells expressing Ly49C and/or Ly49I, a much lower response was observed with cells from $K^{b}D^{b-/-}$ mice than with cells from $D^{b-/-}$ mice, confirming the importance of K^b in conferring high responsiveness of NK cells expressing these receptors. These studies added support to the conclusion that Ly49C and Ly49I interact principally with K^b in B6 mice, whereas CD94/NKG2A interacts principally with D^b (via Qa-1 presentation). Most importantly, the NK cells in $D^{b-/-}$ mice that coexpressed Ly49C and Ly49I responded significantly better than the NK cells that expressed Ly49C but not Ly49I ($p < 0.009$) or the NK cells that expressed Ly49I but not Ly49C ($p < 0.017$). This was not the case in $K^{b-/-}$ or $K^{b}D^{b-/-}$ mice, which lack strong ligands for Ly49C and Ly49I. These data demonstrated that coexpression of multiple inhibitory receptors enhanced NK cell responsiveness only when each of the coexpressed receptors engaged MHC class I ligands.

Contribution of receptor coexpression to the overall response

The contribution of a subset to the overall response depends on both the responsiveness of the subset and its relative size, both of which varied a good deal depending on the receptor combination (Figs. 1 and 2). We combined these data to gauge the contribution of each subset to the overall response to NKG2D Ab in wild-type and K^b or D^b mutant mice, as well as the overall magnitudes of the responses in these mouse strains (Fig. 4A). The data revealed a trade-off between the size of a subset and its responsiveness. Al-

though NK cells that coexpressed all three receptors were the most responsive subset in B6 mice, they were among the least abundant. As a result, their contribution to the response was no greater or was even smaller than that of some of the other subsets, which were less responsive but more abundant (e.g., the $Ly49C^{-}Ly49I^{+}NKG2A^{+}$ and $Ly49C^{-}Ly49I^{+}NKG2A^{-}$ subsets; Fig. 4A). Similar contribution patterns were seen with other stimuli (NKR-P1C Ab and YAC-1 cells; data not shown).

In comparing NK cells from B6 mice to those from $D^{b-/-}$ mice or $K^{b-/-}$ mice, the contributions of several of the subsets contracted in a predictable manner (Fig. 4). Thus, most of the NK cell subsets that coexpressed Ly49C and/or Ly49I contributed substantially less in $K^{b-/-}$ mice than in B6 or $D^{b-/-}$ mice, consistent with the absence of a strong ligand for those receptors in $K^{b-/-}$ mice. Conversely, most of the NK cell subsets that coexpressed NKG2A contributed less in $D^{b-/-}$ mice than in B6 or $K^{b-/-}$ mice, consistent with the reactivity of CD94/NKG2A with a D^b -derived peptide.

Fig. 4B compares the percentages of NK cells in each subset to the proportion of the response that it accounts for in B6 mice and $D^{b-/-}$ mice, the only strains studied where coexpression of more than one self-MHC-specific receptors was possible. Whereas NK cells with three self-specific receptors represent only 7.5% of NK cells in B6 mice, they accounted for 17% of the response. NK cells with two self-specific receptors also accounted for a larger share of the response than their percentage in the population would predict, whereas NK cells with only one self-specific receptor accounted

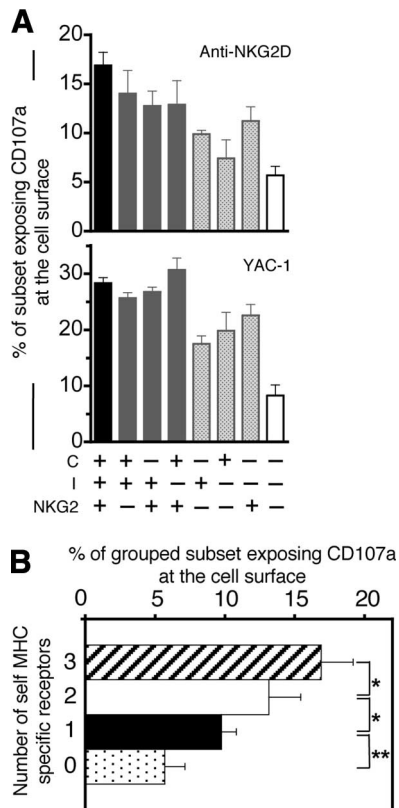


FIGURE 5. The NK cell degranulation response varies depending on the number of expressed self-specific inhibitory receptors. Splenocytes from B6 mice previously treated with poly(I:C) were stimulated with plate-bound anti-NKG2D (5 μ g/ml MI6) or with YAC-1 tumor cells (10^6 cells) for 5 h in the presence of monensin and CD107a (lysosome-associated membrane protein 1 Ab). *A*, After staining cell surface markers, gated CD3⁻NK1.1⁺ cells were analyzed by flow cytometry for CD107a exposure at the cell surface and expression of Ly49C, Ly49I, and NKG2 in all combinations. *B*, Degranulation responses to plate-bound anti-NKG2D of poly(I:C)-treated B6 mice NK subsets grouped according to the number of receptors expressed. Groups differed significantly as shown (*, $p < 0.05$ and **, $p < 0.01$) based on two-tailed paired *t* tests. For each panel, data represent means \pm SD ($n = 5$) calculated after subtracting the background activity seen in the PBS-coated wells (no stimulation control).

for a smaller share of the response than their percentage predicted. A similar pattern held in $D^b^{-/-}$ mice, where NK cells with two self-specific receptors accounted for a significantly larger share of the response than predicted based on their numbers.

In comparing the different strains, the overall *in vitro* NK response was greatest in B6 mice, which have three self-MHC-specific inhibitory receptors, than in mice with fewer self-MHC-specific receptors ($K^b^{-/-}$ and $D^b^{-/-}$ mice; Fig. 4A). The relationship between the number of available self-MHC-specific receptors in a given strain and overall NK cell responsiveness was also examined *in vivo* to gauge its biological relevance. For this purpose, the capacity of the different mouse strains to reject bone marrow grafts from class I-deficient ($\beta_2m^{-/-}$) mice was assayed by injecting the mice with a mixture of dye-labeled test cells ($\beta_2m^{-/-}$ bone marrow cells) and dye-labeled control cells that should not be rejected (B6 bone marrow cells). The percent rejection was determined as the percent loss of test cells relative to control cells, after normalizing based on the results in $\beta_2m^{-/-}$ recipients, which cannot reject class I-deficient bone marrow grafts (20).

B6, $D^b^{-/-}$, and $K^b^{-/-}$ mice all rejected the class I-deficient bone marrow grafts, whereas $K^bD^b^{-/-}$ mice accepted the grafts

(Fig. 4C). Notably, in three of three experiments (Fig. 4C and data not shown), the pattern of rejection was remarkably similar to the pattern of the *in vitro* responses, with B6 mice showing the greatest response, followed by $D^b^{-/-}$ mice and $K^b^{-/-}$ mice. The response of $K^b^{-/-}$ mice ranged from 25 to 50% of the response of B6 mice, depending on the experiment. These data suggested that the number of self-MHC-specific inhibitory receptors engaged is one determinant of the overall magnitude of the response and are consistent with previous reports that examined the overall education impact of different MHC class I molecules (34, 42). Control experiments showed that the responses were mediated by NK1.1⁺ cells, that depletion of Ly49C and I⁺ NK cells prevented rejection by $D^b^{-/-}$ mice, and that depletion of NKG2A⁺ cells resulted in reduced rejection by $K^b^{-/-}$ mice, consistent with the specificities of these receptors (supplemental Table II).

Degranulation responses by NK cells are greater in NK cells that coexpress two or more self-MHC-specific receptors

Bone marrow graft rejection is more likely to reflect NK cytotoxicity than cytokine release, which was examined in Figs. 2–4. To address directly how variations in the number of self-MHC-specific inhibitory receptors impact NK cell cytotoxic potential, we activated NK cells with plate-bound NKG2D Ab or YAC-1 tumor cells and assessed the different subsets for cell surface expression of the lysosome-associated membrane protein 1 (LAMP-1 or CD107a) (43), which is displayed on the cell surface after activation as a result of exocytosis of cytotoxic granules. Comparable results were obtained upon stimulation with plate-bound anti-NKG2D or YAC-1 tumor cells. NK cells that lacked all three receptors responded poorly, as expected, whereas NK cells with any one of the receptors responded better, and NK cells that coexpressed any two or all three of the receptors gave the greatest response (Fig. 5A). In contrast to the IFN- γ responses, there was a less reliable difference in the degranulation response between NK cells expressing any two, vs all three, of the self-MHC-specific receptors (Fig. 5B and data not shown).

Discussion

The data herein strongly support the hypothesis that the intrinsic responsiveness of NK cells is not a binary function, but rather varies over a substantial range depending on the number of inhibitory receptors expressed by the NK cells. Coexpression of three receptors for self-MHC resulted in up to a 2- to 3-fold higher response than observed with NK cells that expressed only one receptor for self-MHC and more than a 10-fold higher response than observed with NK cells that lacked all inhibitory receptors for self-MHC class I. The variations in responsiveness were not due to inhibitory interactions during the effector assays, because the stimuli used (Abs for stimulatory receptors or MHC class I-low YAC-1 cells) did not present MHC class I molecules or other ligands that could engage the inhibitory receptors at the time of assay.

The capacity for receptor coexpression also contributed to variations in responsiveness when comparing different mice. In a mouse strain capable of receptor coexpression, such as B6, the overall NK response was substantially stronger *in vitro* and *in vivo* than in a strain harboring only one self-MHC-specific receptor ($K^b^{-/-}$ mice). $D^b^{-/-}$ mice, which harbor two self-MHC-specific receptors gave an intermediate response or a response similar to B6 mice. Approximately 44% of NK cells in B6 mice express multiple inhibitory receptors specific for self-MHC. The impact of receptor coexpression is likely to be even greater in mouse strains with MHC haplotypes different from those of B6 (H-2^b), since H-2^b binds to fewer MHC-specific inhibitory receptors (approximately three) than most other MHC haplotypes (33). In strains

with a greater number of self-MHC-specific receptors, there are greater percentages of NK cells with multiple self-specific receptors and greater capacity for coexpression of higher multiples of these receptors.

A previous study by Johansson et al. (34) addressed a related issue: whether variations in the expression of MHC ligands for inhibitory receptors impacted the overall responsiveness of NK cells. They found that expression of a greater number of different MHC ligands resulted in greater overall responsiveness of NK cells, as assessed by rejection of class I-deficient bone marrow grafts by B6, K^b, and D^b mutant mice are consistent with their findings and go further by showing that the MHC ligands impact the responsiveness of specific NK subsets depending on the MHC molecules expressed.

During preparation of our manuscript, Brodin et al. (42) extended these earlier results and provided new evidence that NK cell degranulation increases as the number of expressed self-MHC-specific receptors increases. Our study demonstrates that cytokine production as well as degranulation responses increase with the number of self-MHC-specific receptors. Interestingly, in the IFN- γ response, we observed that coexpression of all three receptors led to greater responsiveness than expression of any pair (Fig. 2). This effect was less consistently observed, or even absent, in the degranulation response, as reported by Brodin et al. (42) and corroborated in our study (Fig. 5 and our unpublished data). Brodin et al. (42) suggest that in the degranulation response, the “educational benefit” of receptor coexpression is already saturating when NK cells express any pair of receptors. Our data suggest, in contrast, that the educational benefit of receptor coexpression continues to rise as the cells express more than two MHC-specific inhibitory receptors, but may be more or less in evidence depending on the response studied. The degranulation response, which may generally require a lower level of activation signals to be induced, may be saturated more readily than the IFN- γ response. Our results are consistent with recent reports examining human NK cells which demonstrate that coexpression of up to three human inhibitory MHC-specific receptors (killer Ig-like receptors and NKG2A) results in greater NK cell responsiveness (31, 44).

In some of our experiments, it appeared that compensatory mechanisms may enhance NK cell responsiveness when receptor coexpression is not possible. For example, in Fig. 4A, NK cells expressing NKG2A but not Ly49C or Ly49I responded better in K^b-/- mice, where NKG2A is the only self-MHC-specific receptor, than in B6 mice where there are two other options. However, these compensatory shifts were not observed consistently in other experiments (data not shown) or in the case of some other of the receptors studied; therefore, their significance remains uncertain.

Higher responses of NK cells coexpressing inhibitory receptors were evident when the NK cells were stimulated with NKG2D Ab, YAC-1 target cells, or NKR-P1C Ab. However, with NKR-P1C stimulation, the relative responses (i.e., compared with that of Ly49C⁻Ly49I⁻NKG2A⁻ NK cells) were smaller than with NKG2D Ab or YAC-1 tumor cells. This was presumably related to the greater overall response with NKR-P1C stimulation, which leads to a higher “background” response of the Ly49C⁻Ly49I⁻NKG2A⁻ population. A similar effect was previously observed with Ly49D stimulation (25). NKR-P1C or Ly49D Ab stimulation may provide a sufficiently strong signal to partially overcome the hyporesponsive state of Ly49C⁻Ly49I⁻NKG2A⁻ NK cells. Notably, a substantial difference in the responses of the NK subsets was observed with YAC-1 target cells, presumably a more physiological stimulus.

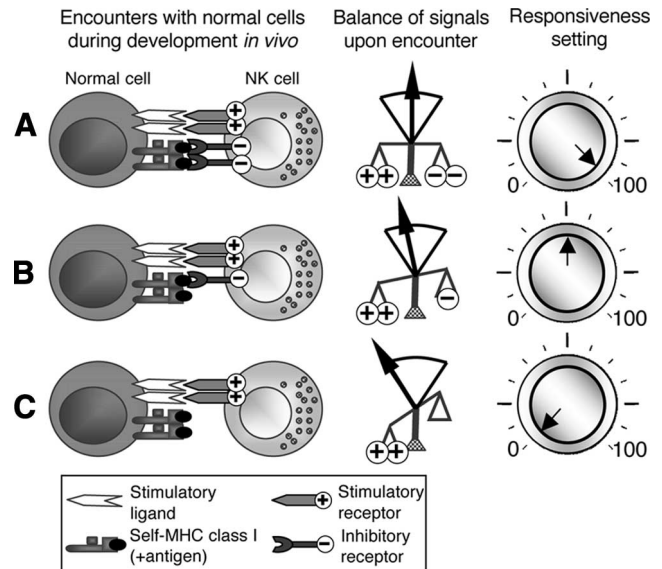


FIGURE 6. Tuning NK cell responsiveness: an educational “rheostat.” During development, NK cells are exposed to inhibitory and stimulatory interactions with normal cells (left column). The balance of signals upon encounter with neighboring normal cells (middle column) sets the responsiveness state of the NK cells (right column). *A*, Stimulatory signals from neighboring normal cells are opposed when several inhibitory receptors are coexpressed on the developing NK cell, which allows it to reach a state of high responsiveness. *B*, Expression of one receptor only partially counters stimulatory signals, such that residual persistent stimulation induces partial hyporesponsiveness. *C*, When no inhibitory receptors are expressed, strong persistent stimulation induces greater hyporesponsiveness. Thus, the responsiveness of NK cells is tuned to quantitatively different levels by the quantity of inhibitory and stimulatory interactions to which the cells are exposed during development.

We interpret our findings in terms of a rheostat model (45) in which the signaling requirements to achieve the threshold leading to a response may vary dramatically and continuously. A similar model has been proposed by Brodin et al. (42). According to this model, each NK cell’s signaling pathways undergoes a tuning process based on the number and type of inhibitory and stimulatory receptors expressed and whether normal cells in the host express corresponding ligands for these receptors in steady-state conditions (Ref. 45 and Fig. 6). A tuning process establishes a set point in each NK cell wherein the balance of inhibitory and stimulatory signaling that occurs in encounters with normal cells is marginally below the amount necessary to activate the NK cell. When NK cells lack all inhibitory receptors for self-MHC class I, the responsiveness will generally be adjusted sharply downward, below the threshold necessary to attack normal cells. In the case of NK cells expressing one inhibitory receptor for self-MHC class I, the responsiveness will generally be adjusted to a higher set point, but one that is still insufficient to support a response to normal cells. Responsiveness will be adjusted higher still when two or more inhibitory receptors for self-MHC are expressed. Each of these cells will thereby be enabled to respond optimally when confronted with self-cells that have altered expression of stimulatory or inhibitory ligands. Although NK cells lacking all inhibitory receptors for MHC class I will be insensitive to loss of MHC class I molecules on target cells, they may still give partial responses to target cells that up-regulate stimulatory ligands. This may help account for the significant cytokine responses that such NK cells yield in infection models (25).

In the present study, we generally defined responsiveness as the percentage of cells that responded upon activation by the production of cytokines or degranulation. Another relevant parameter is the average amount of cytokine produced by each cell in the subset, which can be compared in terms of the mean fluorescent intensity (MFI) of intracellular staining with IFN- γ -specific Ab. In our analysis, the abundant responding NK cells in the Ly49C⁺I⁺NKG2A⁺ subset produced virtually the same amount of cytokine as the minor fractions of responding NK cells in the Ly49C⁻I⁻NKG2A⁻ subset or the subsets expressing any pair or only one of the receptors (data not shown). In the context of a rheostat model, these observations suggest that although the threshold of signaling necessary for activation of NK cells varies in different subsets, when the threshold is exceeded, the NK cells in each subset respond to nearly the same extent. Such a model is consistent with published findings showing that as one varies the stimulus to a clonal population of T cells or a polyclonal population of NK cells, what changes is primarily the percentage of cells that respond, whereas the magnitude of cytokine production by each activated cell varies much less significantly (46–49). The notion is that as the stimulus is increased, the probability increases that a threshold concentration of signaling mediators or transcription complexes will be achieved. Once achieved, a full response ensues. These considerations do not detract from the rheostat model, however, because the data indicate that the signaling necessary to achieve the necessary threshold varies in the different subsets, even if the final response of individual cells in each subset may be largely “on” or “off” in terms of cytokine production. Notably, whereas we did not observe differences in MFI of IFN- γ staining among the subsets, we did observe modestly greater MFI of CD107a staining in the case of NK cells expressing a greater number of self-MHC-specific inhibitory receptors (data not shown) (42). Hence, once the triggering threshold has been exceeded, NK cells with a greater number of self-MHC-specific inhibitory receptors exhibit a greater degranulation response than those with fewer inhibitory receptors, but the cytokine response varies much less significantly.

The process that determines NK cell responsiveness is under debate (27, 45, 50). The disarming model proposes that high responsiveness is a default state and hyporesponsiveness is imposed when NK cells interact with excessively stimulatory self-cells (e.g., when the self-cells lack ligands for inhibitory receptors on the NK cells) (27, 51). The arming model proposes that low responsiveness is a default state and high responsiveness is imposed as a direct result of inhibitory receptor engagement (27, 28). The present data dispense with a binary model of NK cell responsiveness and therefore require modifications of both models, but do not distinguish between them. A modified disarming model posits that responsiveness is dampened commensurately with the amount of excess stimulation, which is countered by increasing the number of inhibitory receptors engaged. A modified arming model posits that responsiveness is augmented commensurately with the extent of inhibitory receptor engagement per se. Although the present findings do not distinguish these models, bone marrow chimera data support the disarming model (Refs. 51–53 and N. T. Joncker and D. H. Raulet, manuscript in preparation).

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Disclosures

The authors have no financial conflict of interest.

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