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NK Cell Responsiveness is Tuned Commensurate with the Number of Inhibitory Receptors for Self MHC Class I: the Rheostat Model

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Abstract

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. Here 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.

Introduction

Natural Killer (NK) 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 major histocompatibility complex I (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 if the NK cell becomes activated, produces inflammatory cytokines (including interferon (IFN)-γ and tumor necrosis factor (TNF)-α) and/or kills the target (7–9).

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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 molecule 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 antibodies (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 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 hyporesponsive, 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 KIR 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 hyporesponsiveness 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 an 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 (Bar Harbor, ME). Congenic B6 mice expressing Ly5.1 (B6-Ly5.2/Cr) were obtained from Charles River Laboratories (Frederick, MD). β2-microglobulin $(\beta2m)^{-/-}$, $K^{b-/-}$, $D^{b-/-}$ and $K^bD^{b-/-}$ mice on the B6 background were described previously (19,20,35, 36). B6 $\beta2m^{-/-}$ Ly-5.1 mice were derived in our facilities.

The mouse lymphoma YAC-1 cell line (TIB-160, ATCC) was cultured in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (Omega Scientific), 100 U/mL Penicillin (Gibco) and 100 μg/mL Streptomycin (Gibco) and 0.2 mg/mL L-glutamine (Sigma), 50 μM 2-mercaptoethanol (EMD biosciences), 20 mM Hepes (Fisher).

Antibodies and flow cytometric analysis

Antibodies against NK1.1 (PK136), CD3ε (145-2C11), NKG2A/C/E (20d5), Ly49I (YLI-90), CD45.1 (A20), CD45.2 (104), IFNγ (XMG1.2), CD107a (eBio1D48), NKG2D (MI6) (37) were purchased from eBiosciences and Pharmingen (San Diego, CA). Purified NKp46 (29A1.4) (38) was conjugated using the AlexaFluor647 Protein Labeling kit from Molecular Probes (Eugene, OR). The 4LO3311 hybridoma (anti-Ly49C) was a generous gift from S. Lemieux (Quebec, Canada) (39), and the mAb was purified from CELLine classic 1000 (Integra Biosciences, Chur, Switzerland) supernatant, then conjugated to biotin according to standard methods. Biotin-conjugated mAb were detected with streptavidin PE-TexasRed (Pharmingen) or streptavidin Pacific Blue (Invitrogen, Carlsbad, CA). Depleting antibodies against NK1.1 (PK136), Ly49C and Ly49I (SW5E6) and NKG2A (16a11) were purified from CELLine classic 1000 supernatants. Prior to staining, cells were pre-incubated for 20 minutes with 2.4G2 hybridoma supernatant to block FcγRII/III receptors. Flow cytometry was performed on a FC500 Dual Laser system machine (Coulter, Hialeah, FL) or a BD LSR II cytometer (BD Biosciences, San Jose, CA), and data were analyzed with the FlowJo software (Tree Star, Stanford University).

In vitro NK cell stimulation and functional assays

Mice were either not pretreated, or were injected intraperitoneally with 200 μg of poly I:C (Sigma-Aldrich, St Louis, MO) 1.5 days before harvesting spleens for NK cell preparation, as indicated. For analysis of IFN-γ production, round bottomed (or flat bottomed in the case of NKG2D antibody stimulation) high-protein binding plates (Costar, Fischer Scientfic, Pittsburg, PA) were coated overnight at 4°C with the indicated concentrations of intact mAb in 100 μL PBS, followed by four rinses with 200 μL PBS, before adding NK cells. The IFN-γ production assay employed the Cytofix/Cytoperm intracellular staining kit (Pharmingen). Spleen cell suspensions were centrifuged in Lympholyte-Mammal (Cedarlane Laboratories, Hornby, ON, Canada) and 1×10^6 cells/well (100 μL) were incubated for 5 hours in RPMI

10% FCS in the presence of 1 μg/mL GolgiPlug (containing brefeldin A) (Pharmingen, San Diego, CA) and 1000 U/mL recombinant human IL-2 (Hoffmann-La Roche, Nutley, NJ) before transfer to V-bottom plates for staining. Where indicated, 1×10^6 YAC-1 cells or a mixture of 400 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) and 3.35 μM ionomycin (Sigma) were added as source of stimulation. The degranulation assay was performed similarly, except GolgiPlug was replaced by GolgiStop (containing monensin) (Pharmingen) and CD107a antibody 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) intravenously into mice that had been irradiated earlier in the day with 9.5 Gy from a ^{137}Cs source. Some mice were pre-depleted of NK cells or NK subsets by intraperitoneal injection of antibodies PK136 (anti-NK1.1), SW5E6 (anti-Ly49C and Ly49I) or 16a11 (anti-NKG2A). Recipient spleens 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 - normalized acceptance) × 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^b) 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 Figure 1A. The complete analysis showed that the percentages of cells expressing each receptor combination varied between 6% and 20% (Figure 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 (Figure 1B). Hence, appreciable subsets of NK cells expressing 0, 1, 2 or 3 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 multi-parameter 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 an NKp46 specific monoclonal antibody, which is specific for NK cells (38). The results demonstrated a striking continuum in the responsiveness of the NK cells (Figure 2A–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 II). 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 (Figure 2A–B) even when lower doses of anti-NKR-P1C were used to stimulate the cells (Supplemental Figure 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 (Figure 2A–B). Poly I:C treatment enhanced the functionality of all the NK cell subsets studied in terms of the percentage of IFN-γ producing cells (Figure 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 (Figure 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. Figure 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 (Figure 2A–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 (Figure 2A–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 reflected the effects of receptor engagement with self-MHC class I molecules. We compared the IFN-γ response of each subset in B6 wildtype mice to that in $D^{b-/-}$, $K^{b-/-}$, $K^bD^{b-/-}$ and $\beta2m^{-/-}$ mice after NKG2D cross-linking (Figure 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 to B6 wildtype mice. Expression of NKG2A alone (as in Ly49C−Ly49I−NKG2+ 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.

The absence of both K^b and D^b, in $K^bD^{b-/-}$ double knockout mice, generally resulted in a low response of all the subsets (Figure 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, 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 $K^bD^{b-/-}$ 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 co-expressed 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 $K^bD^{b-/-}$ 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 (Figure 1 and Figure 2). We combined these data to gauge the contribution of each subset to the overall response to NKG2D antibody in wildtype and K^b or D^b mutant mice, as well as the overall magnitudes of the responses in these mouse strains (Figure 4A). The data revealed a trade off between the size of a subset and its responsiveness. 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 (eg. the Ly49C−Ly49I+NKG2A+ and Ly49C−Ly49I+NKG2A− subsets) (Figure 4A). Similar contribution patterns were seen with other stimuli (NKR-P1C antibody 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 (Figure 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.

Figure 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 >1 self MHC specific receptors was possible. Whereas NK cells with 3 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 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) (Figure 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*, in order to gauge its biological relevance. For this purpose, the capacity of the different mouse strains to reject bone marrow grafts from class I-deficient ($\beta2m^{-/-}$) mice was assayed by injecting the mice with a mixture of dye-labeled test cells ($\beta2m^{-/-}$ 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 $\beta2m^{-/-}$ 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 (Figure 4C). Notably, in three of three experiments (Figure 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%–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 I).

Degranulation responses by NK cells are greater in NK cells that co-express 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 Figures 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 antibody 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 co-expressed any two, or all three of the receptors gave the greatest response (Figure 5A). In contrast to the IFN-γ responses, there was a less reliable difference in the degranulation response between NK cells expressing any two, versus all three, of the self MHC specific receptors (Figure 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 2–3 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 (antibodies 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 that of B6 (H-2^b), because H-2^b binds to fewer MHC-specific inhibitory receptors (~3) 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. Our results comparing rejection of 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.* extended these earlier results and provided new evidence that NK cell degranulation increases as the number of expressed self MHC-specific receptors increases (42). 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 (Figure 2). This effect that was less consistently observed, or even absent, in the degranulation response, as reported by Brodin *et al.* (42) and corroborated in our study (Figure 5, unpublished data). Brodin *et al.* 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 greater 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 a less sensitive assay for this effect to be detected 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 (KIRs 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 Figure 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, so their significance remains uncertain.

Higher responses of NK cells coexpressing inhibitory receptors were evident when the NK cells were stimulated with NKG2D antibodies, YAC-1 target cells or NKR-P1C antibodies. However, with NKR-P1C stimulation, the relative responses (i.e compared to that of Ly49C−Ly49I−NKG2A− NK cells) were smaller than with NKG2D antibody 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 antibody 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.

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 states conditions (45) (Figure 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 upregulate 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 antibody. 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 while 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 differs, 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 (eg. 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 (51–53) (NJ and DR, in preparation).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annual Review of Immunology 1999;17:189–220.
2. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. European Journal of Immunology 1975;5:112–117. [PubMed: 1234049]
3. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. International Journal of Cancer 1975;16:216–229.
4. Diefenbach A, Raulet DH. The innate immune response to tumors and its role in the induction of T-cell immunity. Immunol Rev 2002;188:9–21. [PubMed: 12445277]
5. Ljunggren H-G, Karre K. Host resistance directed selectively against H-2-deficient lymphoma variants. J. Exp. Med 1985;162:1745–1759. [PubMed: 3877776]
6. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defense strategy. Nature 1986;319:675–678. [PubMed: 3951539]
7. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari M, Biassoni R, Moretta L. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annual Review of Immunology 2001;19:197–223.
8. Lanier LL. NK cell recognition. Annu Rev Immunol 2005;23:225–274. [PubMed: 15771571]
9. Raulet DH, Held W, Correa I, Dorfman J, Wu M-F, Corral L. Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. Immunol. Rev 1997;155:41–52. [PubMed: 9059881]
10. Brennan J, Mager D, Jefferies W, Takei F. Expression of different members of the Ly-49 gene family defines distinct natural killer cell subsets and cell adhesion properties. J. Exp. Med 1994;180:2287–2295. [PubMed: 7964501]
11. Hoglund P, Sundbäck J, Ollson-Alheim MY, Johansson M, Salcedo M, Öhlén C, Ljunggren HG, Sentman C, Karre K. Host MHC class I gene control of NK cell specificity in the mouse. Immunol. Rev 1997;155:11–28. [PubMed: 9059879]

12. Tanamachi DM, Hanke T, Takizawa H, Jamieson AM, Raulet DH. Expression of natural killer cell receptor alleles at different Ly49 loci occurs independently and is regulated by major histocompatibility complex class I molecules. J Exp Med 2001;193:307–315. [PubMed: 11157051]
13. Raulet DH, Vance RE, McMahon CW. Regulation of the natural killer cell receptor repertoire. Annu Rev Immunol 2001;19:291–330. [PubMed: 11244039]
14. Saleh A, Davies GE, Pascal V, Wright PW, Hodge DL, Cho EH, Lockett SJ, Abshari M, Anderson SK. Identification of probabilistic transcriptional switches in the Ly49 gene cluster: a eukaryotic mechanism for selective gene activation. Immunity 2004;21:55–66. [PubMed: 15345220]
15. Dorfman JR, Raulet DH. Major histocompatibility complex genes determine natural killer cell tolerance. Eur J Immunol 1996;26:151–155. [PubMed: 8566058]
16. Valiante N, Uhberg M, Shilling H, Lienert-Weidenbach K, Arnett K, D'Andrea A, Phillips J, Lanier L, Parham P. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. Immunity 1997;7:739–751. [PubMed: 9430220]
17. Lanier LL. NK Cell Recognition. Annu Rev Immunol. 2004
18. Moretta A, Bottino C, Vitale M, Pende D, Biassoni R, Mingari MC, Moretta L. Receptors for HLA class-I molecules in human natural killer cells. Ann. Rev. Immunol 1996;14:619–648. [PubMed: 8717527]
19. Liao NS, Bix M, Zijlstra M, Jaenisch R, Raulet D. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. Science 1991;253:199–202. [PubMed: 1853205]
20. Bix M, Liao NS, Zijlstra M, Loring J, Jaenisch R, Raulet D. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. Nature 1991;349:329–331. [PubMed: 1987491]
21. Dorfman JR, Zerrahn J, Coles MC, Raulet DH. The basis for self-tolerance of natural killer cells in beta2m^{-} and TAP-1^{-} mice. J Immunol 1997;159:5219–5225. [PubMed: 9548460]
22. Ljunggren H-G, Van Kaer L, Ploegh HL, Tonegawa S. Altered natural killer cell repertoire in *Tap-1* mutant mice. Proc. Natl. Acad. Sci. USA 1994;91:6520–6524. [PubMed: 8022815]
23. Zimmer J, Donato L, Hanau D, Cazenave JP, Tongio MM, Moretta A, de la Salle H. Activity and phenotype of natural killer cells in peptide transporter (TAP)-deficient patients (type I bare lymphocyte syndrome). J. Exp. Med 1998;187:117–122. [PubMed: 9419217]
24. Vitale M, Zimmer J, Castriconi R, Hanau D, Donato L, Bottino C, Moretta L, de la Salle H, Moretta A. Analysis of natural killer cells in TAP2-deficient patients: expression of functional triggering receptors and evidence for the existence of inhibitory receptor(s) that prevent lysis of normal autologous cells. Blood 2002;99:1723–1729. [PubMed: 11861289]
25. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. Blood 2005;105:4416–4423. [PubMed: 15728129]
26. Liao N, Bix M, Zijlstra M, Jaenisch R, Raulet D. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. Science 1991;253:199–202. [PubMed: 1853205]
27. Raulet DH, Vance RE. Self-tolerance of natural killer cells. Nat Rev Immunol 2006;6:520–531. [PubMed: 16799471]
28. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, French AR, Sunwoo JB, Lemieux S, Hansen TH, Yokoyama WM. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 2005;436:709–713. [PubMed: 16079848]
29. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA, Breso V, Frassati C, Reviron D, Middleton D, Romagne F, Ugolini S, Vivier E. Human NK cell education by inhibitory receptors for MHC class I. Immunity 2006;25:331–342. [PubMed: 16901727]
30. Hasenkamp J, Borgerding A, Uhrberg M, Falk C, Chapuy B, Wulf G, Jung W, Trumper L, Glass B. Self-tolerance of human natural killer cells lacking self-HLA-specific inhibitory receptors. Scand J Immunol 2008;67:218–229. [PubMed: 18226015]
31. Yu J, Heller G, Chewning J, Kim S, Yokoyama WM, Hsu KC. Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. J Immunol 2007;179:5977–5989. [PubMed: 17947671]
32. Hanke T, Raulet DH. Cumulative inhibition of NK cells and T cells resulting from engagement of multiple inhibitory Ly49 receptors. J Immunol 2001;166:3002–3007. [PubMed: 11207249]

33. Hanke T, Takizawa H, McMahon CW, Busch DH, Pamer EG, Miller JD, Altman JD, Liu Y, Cado D, Lemonnier FA, Bjorkman PJ, Raulet DH. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. Immunity 1999;11:67–77. [PubMed: 10435580]
34. Johansson S, Johansson M, Rosmaraki E, Vahlne G, Mehr R, Salmon-Divon M, Lemonnier F, Karre K, Hoglund P. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. J Exp Med 2005;201:1145–1155. [PubMed: 15809355]
35. Zijlstra M, Li E, Sajjadi F, Subramani S, Jaenisch R. Germ-line transmission of a disrupted β2-microglobulin gene produced by homologous recombination in embryonic stem cells. Nature 1989;342:435–438. [PubMed: 2685607]
36. Perarnau B, Saron M-F, Martin BRSM, Bervas N, Ong H, Soloski M, Smith AG, Ure JM, Gairin JE, Lemonnier FA. Single *H2K*^{b}, *H2D*^{b} and double *H2K*^{b}*D*^{b} knockout mice: peripheral CD8$^+$ T cell repertoire and anti-lymphocytic choriomeningitis virus cytolytic responses. Eur. J. Immunol 1999;29:1243–1252. [PubMed: 10229092]
37. Jamieson AM, Diefenbach A, McMahon CW, Xiong N, Carlyle JR, Raulet DH. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. Immunity 2002;17:19–29. [PubMed: 12150888]
38. Walzer T, Blery M, Chaix J, Fuseri N, Chasson L, Robbins SH, Jaeger S, Andre P, Gauthier L, Daniel L, Chemin K, Morel Y, Dalod M, Imbert J, Pierres M, Moretta A, Romagne F, Vivier E. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. Proc Natl Acad Sci U S A 2007;104:3384–3389. [PubMed: 17360655]
39. Gosselin P, Lusigan Y, Brennan J, Takei F, Lemieux S. The NK2.1 receptor is encoded by Ly-49C and its expression is regulated by MHC class I alleles. International Immunology 1997;9:533–540. [PubMed: 9138013]
40. Takei F, Brennan J, Mager DL. The Ly49 family: genes proteins and recognition of class I MHC. Immunological Reviews 1997;155:67–77. [PubMed: 9059883]
41. Yu YY, George T, Dorfman J, Roland J, Kumar V, Bennett M. The role of Ly49A and 5E6 (Ly49C) molecules in hybrid resistance mediated by murine natural killer cells against normal T cell blasts. Immunity 1996;4:67–76. [PubMed: 8574853]
42. Brodin P, Lakshmikanth T, Johansson S, Karre K, Hoglund P. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. Blood. 2008
43. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004;294:15–22. [PubMed: 15604012]
44. Yawata M, Yawata N, Draghi M, Partheniou F, Little AM, Parham P. MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. Blood 2008;112:2369–2380. [PubMed: 18583565]
45. Joncker NT, Raulet DH. Regulation of NK cell responsiveness to achieve self-tolerance and maximal responses to diseased target cells. Immunol Rev 2008;224:85–97. [PubMed: 18759922]
46. Fiering S, Northrop JP, Nolan GP, Mattila PS, Crabtree GR, Herzenberg LA. Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor. Genes Dev 1990;4:1823–1834. [PubMed: 2123468]
47. Smith KA, Popmihajlov Z. The quantal theory of immunity and the interleukin-2-dependent negative feedback regulation of the immune response. Immunol Rev 2008;224:124–140. [PubMed: 18759924]
48. Itoh Y, Germain RN. Single cell analysis reveals regulated hierarchical T cell antigen receptor signaling thresholds and intraclonal heterogeneity for individual cytokine responses of CD4+ T cells. J Exp Med 1997;186:757–766. [PubMed: 9271591]
49. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nature Medicine 1999;5:405–411.
50. Yokoyama WM, Kim S. How do natural killer cells find self to achieve tolerance? Immunity 2006;24:249–257. [PubMed: 16546094]
51. Wu M-F, Raulet DH. Class I-deficient hematopoietic cells and non-hematopoietic cells dominantly induce unresponsiveness of NK cells to class I-deficient bone marrow grafts. J Immunol 1997;158:1628–1633. [PubMed: 9029098]

52. Hoglund P, Ohlen C, Carbone E, Franksson L, Ljunggren H, Latour A, Koller B, Karre K. Recognition of β2-microglobulin-negative (β2m^{-}) T-cell blasts by natural killer cells from normal but not from β2m^{-} mice: nonresponsiveness controlled by β2m^{-} bone marrow in chimeric mice. Proc. Natl. Acad. Sci. USA 1991;88:10332–10336. [PubMed: 1946452]

53. Johansson MH, Hoglund P. Low number of H-2Dd-negative haematopoietic cells in mixed bone marrow chimeras convey in vivo tolerance to H-2Dd-negative cells but fail to prevent resistance to H-2Dd-negative leukaemia. Scand J Immunol 2004;59:71–78. [PubMed: 14723624]

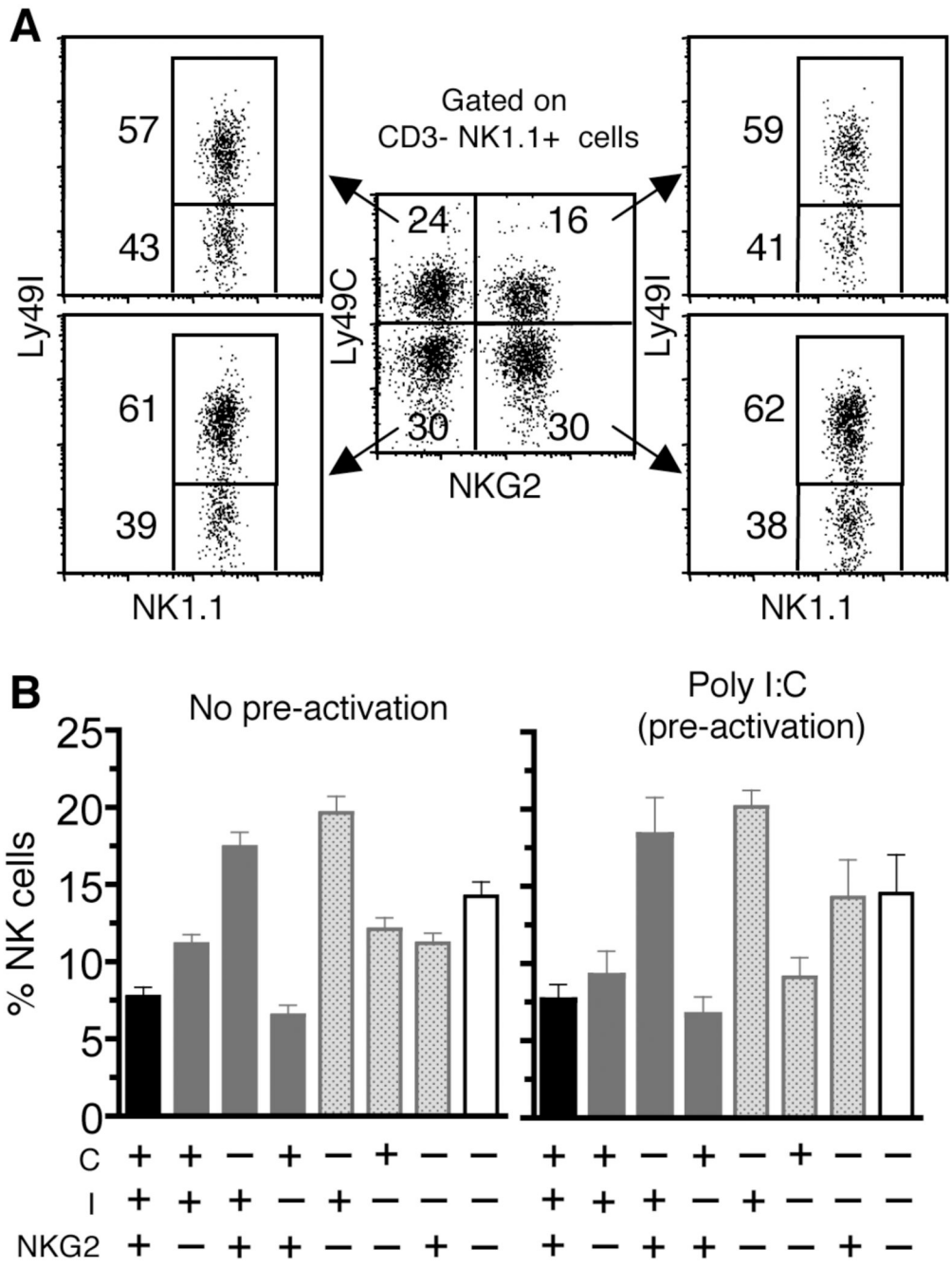

Figure 1. The self MHC class I-specific inhibitory receptor repertoire in B6 mice

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 8 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 ± 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.

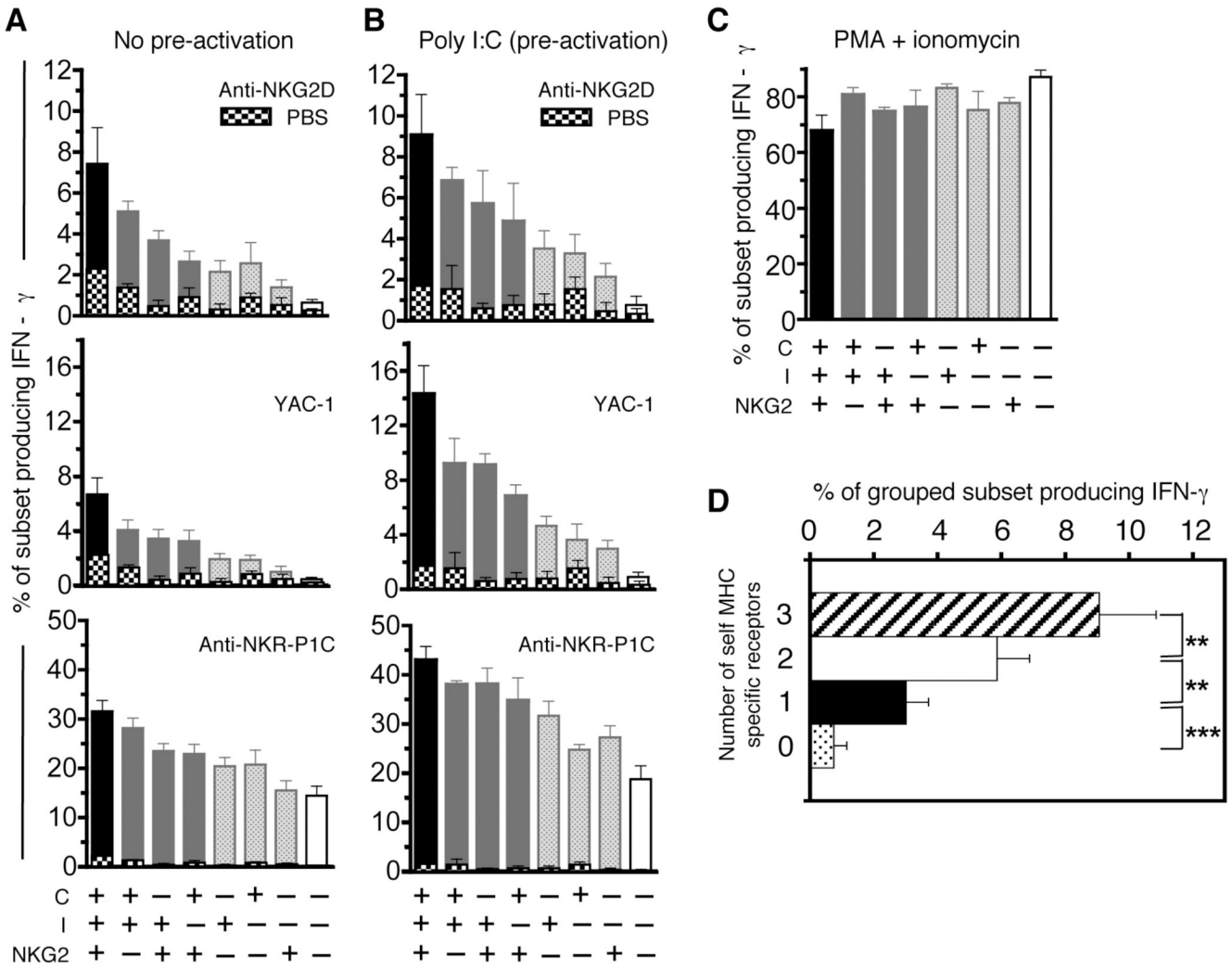

Figure 2. IFN-γ production responses vary continuously in NK cells expressing 0, 1, 2 or 3 self-specific inhibitory receptors

Splenocytes from B6 mice that were left untreated (A) or previously treated with poly I:C (B) were stimulated for 5 hours in the presence of brefeldin A with plate-bound anti-NKR-P1C (PK136, 50 μg/ml), or anti-NKG2D (5 μg/ml MI6), or with YAC-1 tumor cells (10^6 cells), or with 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 II. (C) The same analysis as in (A–B) on spleen NK cells from poly I:C treated B6 mice upon incubation with a mixture of PMA and ionomycin for 5 hours. (D) IFN-γ responses to plate-bound NKG2D antibody 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, ***p<0.001) based on 2-tailed paired t-tests, comparing subsets within the same mouse. For each panel, the data represent means ± SD (n=5).

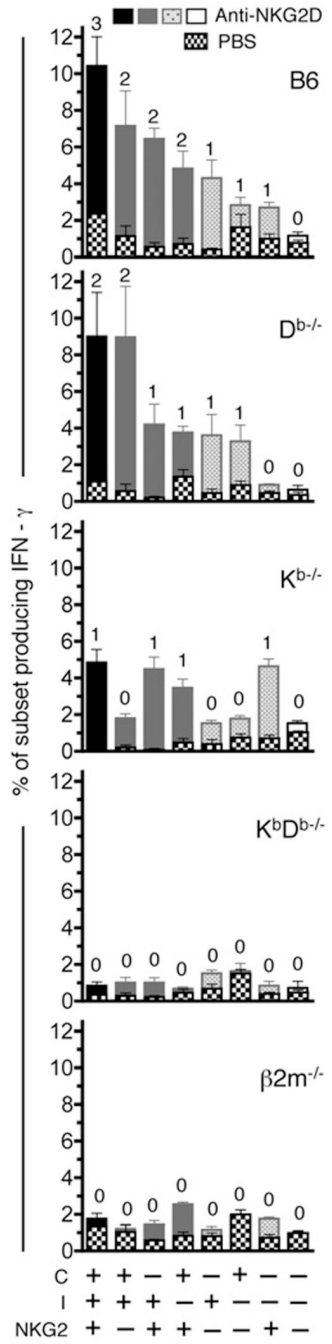

Figure 3. The interactions of the inhibitory receptors with self-MHC class I molecules determine the responsiveness of each subset in the NK cell population

Splenocytes from B6, D^b-, K^b-, K^bD^b- or β2m-deficient mice previously treated with poly I:C were cultured for 5 hours on plates coated with anti-NKG2D (MI6) at 5 μg/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 Figure 4. Data represent the mean ± 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.

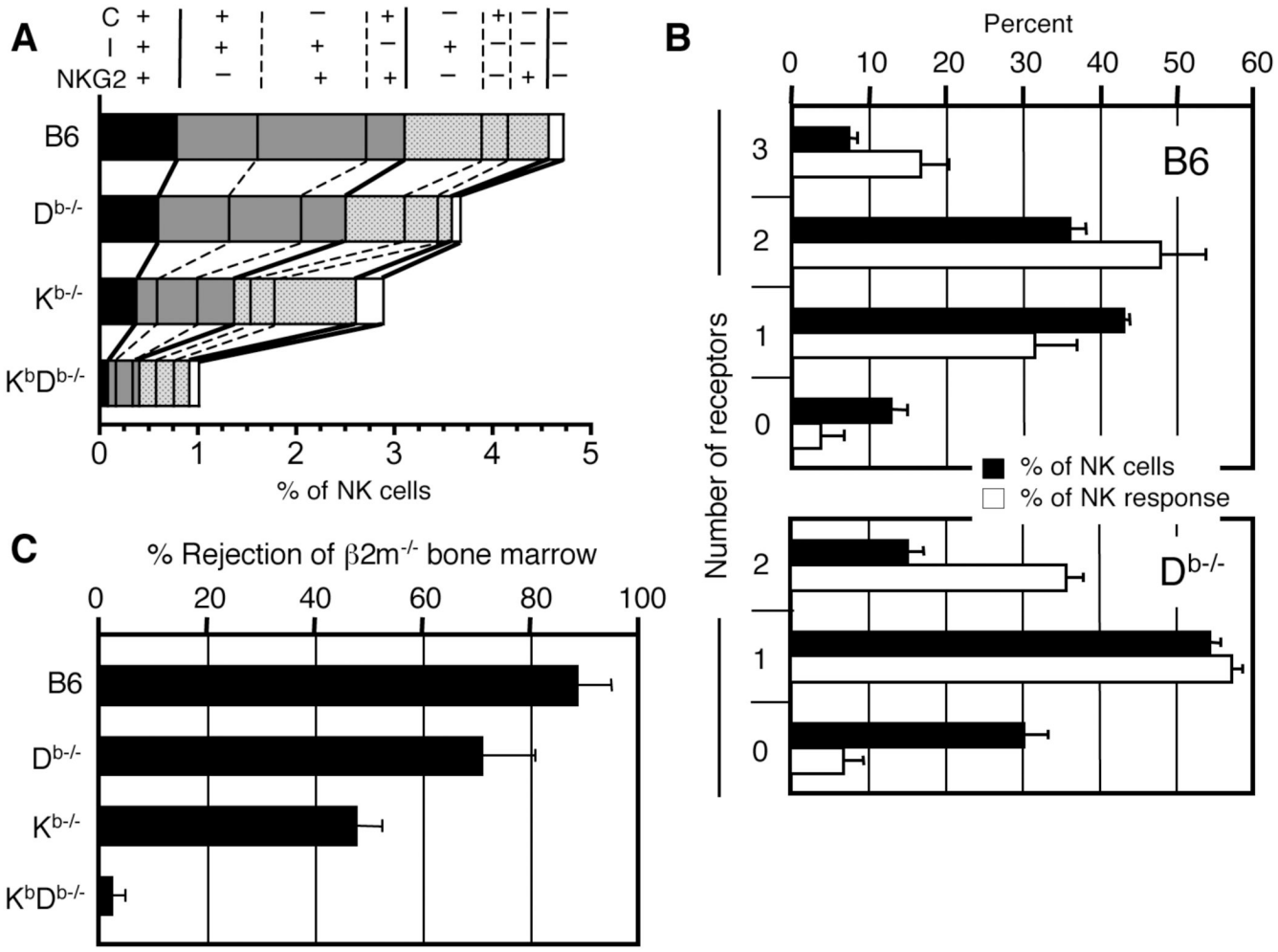

Figure 4. Contributions of each NK cell subsets to the overall NK cell response *in vitro* and *in vivo*

(A) The stacked bars represent the contributions to the overall response of the different subsets expressing all three of the receptors (black bars), any 2 (grey bars), any 1 (shaded bars) or none (clear bars) of them. The data describe the IFN-γ response of NK cells from poly I:C pretreated mice to NKG2D antibody coated plates (5 μg/ml). The contributions (percentage of NK cells depicted on the x-axis) were determined by combining the data from Figure 2 (frequency of responding cells) and Figure 1 (frequency of the subset in the NK population), as: (frequency of a subset) × (frequency of IFN-γ-producing cells in that subset) × 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 3, 2, 1 or 0 self-specific inhibitory receptors (black bars) with the percentages of the total response attributable to that set (white bars). The percentage of the total response for each set was calculated as: 100 × (the contribution of the set to the overall response, in percent) / (percent of total NK cells that respond). For example, the % of NK response for cells expressing 2 receptors= [100 × (contribution of C+I+NKG2− cells + contribution of C+I−NKG2+ cells + contribution of C−I+NKG2+ cells) / (percent of total NK cells that respond)]. NK cells from B6 mice (n=4) and D^b-deficient mice (n=3) are compared. Similar results were obtained in one replicate experiment. (C) *In vivo* responsiveness of NK cells, assessed by the rejection of class I-deficient bone marrow grafts. A 1:1 mixture of CFSE labelled bone marrow cells from $\beta2m^{-/-}$ mice (target cells) and congenic B6 normal mice (internal reference control) was injected intravenously into irradiated

(9.5 Gy) B6, D^b-, K^b-, K^bD^b- or β2m-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 (β2m-deficient hosts). Data represent means ± SD (n = 3 or 4 mice). Similar results were obtained in two replicate experiments.

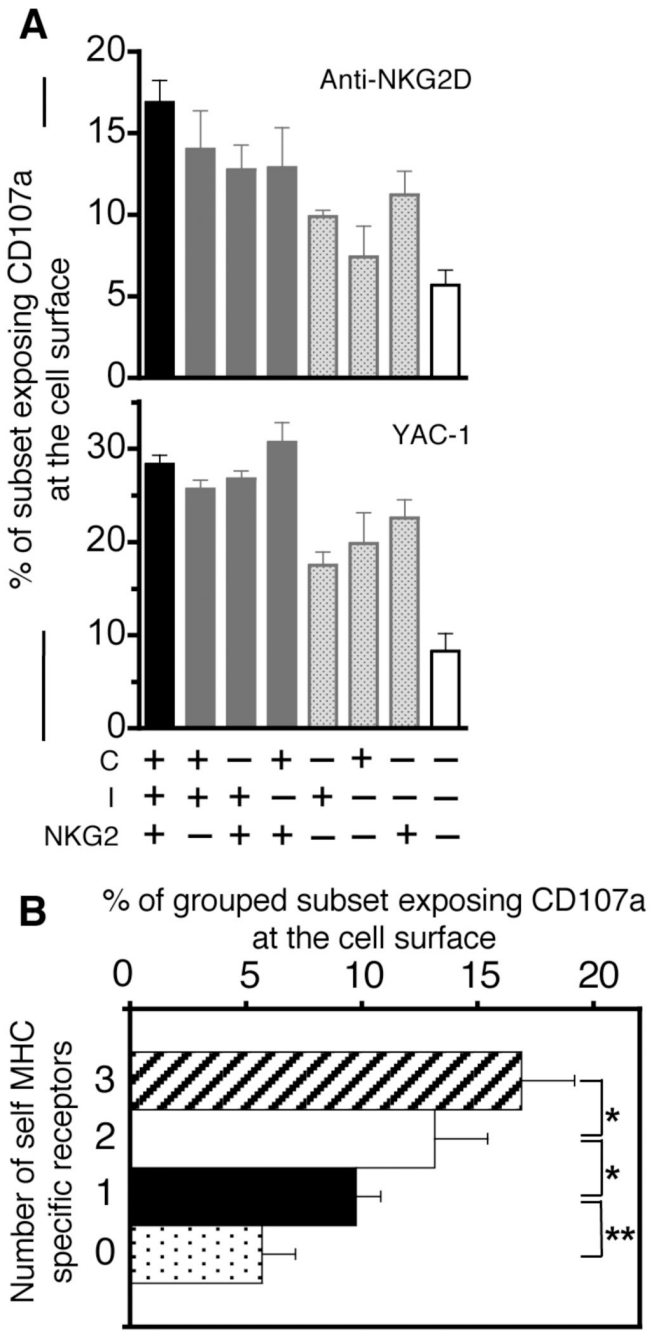

Figure 5. The NK cell degranulation response varies depending on the number of expressed self-specific inhibitory receptors

Splenocytes from B6 mice previously treated with poly I:C were stimulated with plate-bound anti-NKG2D (5 μg/ml MI6), or with YAC-1 tumor cells (10^6 cells) for 5 hours in the presence of monensin and CD107a (LAMP-1) antibody. (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 2-tailed paired t-tests. For each panel, data represent means ± SD (n=5).

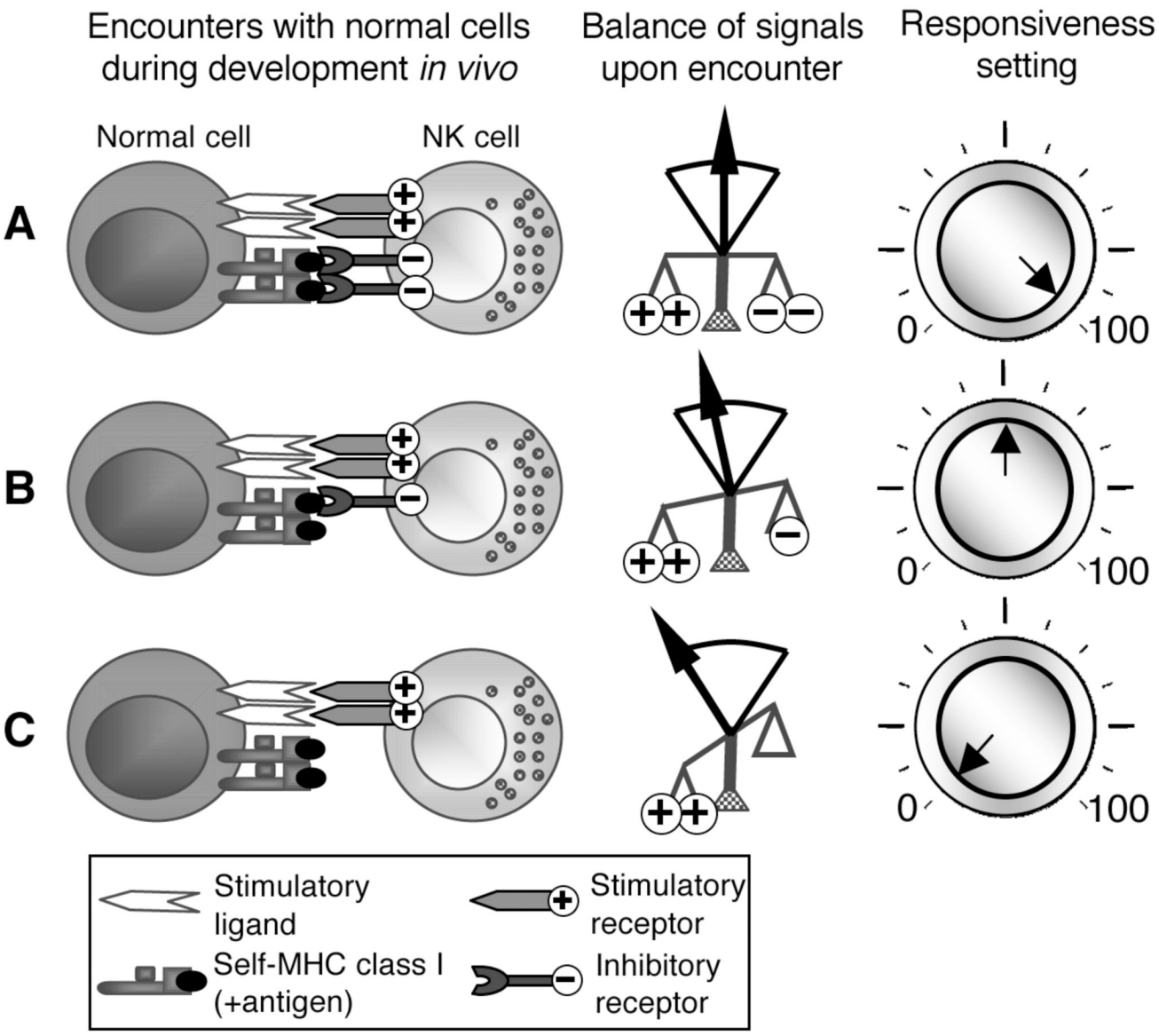

Figure 6. Tuning NK cell responsiveness: an educational "rheostat"

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.

Table I

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

	No pre-activation		Poly I:C (pre-activation)	
	observed [b]	predicted [b]	observed	predicted
C+ I+ NKG2+	7.7 ± 0.6 [c]	9.0 ± 0.5	7.6 ± 1.0	8.6 ± 1.5
C+ I+ NKG2−	11.1 ± 0.6	12.0 ± 0.7	9.2 ± 1.6	9.6 ± 1.5
C− I+ NKG2+	17.4 ± 1.0	15.0 ± 1.0	18.4 ± 2.4	17.5 ± 1.8
C+ I− NKG2+	6.5 ± 0.6	7.1 ± 0.5	6.7 ± 1.2	6.8 ± 0.8
C− I+ NKG2−	17.4 ± 1.0	20.0 ± 0.9	20.2 ± 1.1	19.7 ± 1.3
C+ I− NKG2−	12.1 ± 0.8	9.4 ± 0.8	9.1 ± 1.3	7.7 ± 1.1
C− I− NKG2+	11.2 ± 0.7	11.8 ± 0.6	14.3 ± 2.5	14.1 ± 1.8
C− I− NKG2−	15.7 ± 0.8	14.2 ± 0.9	14.5 ± 2.5	16.0 ± 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+)/10000.

[c] Numbers represent percentages of NKp46+ NK cells (mean ± SD; n=5)