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Regulation of NK cell responsiveness to achieve self tolerance and maximal responses to diseased target cells

Nathalie T. Joncker^{1,2} and David H. Raulet^{1,2}

¹Department of Molecular and Cell Biology, University of California Berkeley, Berkeley, CA, USA

²Cancer Research Laboratory, University of California Berkeley, Berkeley, CA, USA

Summary

Inhibitory receptors specific for MHC class I molecules govern the capacity of NK cells to attack class I-deficient cells (“missing-self recognition”). These receptors are expressed stochastically, such that the panel of expressed receptors varies between NK cells. This review addresses how the activity of NK cells is coordinated in the face of this variation, in order to achieve a repertoire that is self-tolerant and optimally reactive with diseased cells. Recent studies show that NK cells arise in normal animals or humans that lack any known inhibitory receptors specific for self MHC class I. These NK cells exhibit self-tolerance and exhibit functional hyporesponsiveness to stimulation through various activating receptors. Evidence suggests that hyporesponsiveness is induced because these NK cells cannot engage inhibitory MHC class I molecules and are therefore persistently over-stimulated by normal cells in the environment. Finally, we discuss evidence that hyporesponsiveness is a quantitative trait that varies depending on the balance of signals encountered by developing NK cells. Thus, a tuning process determines the functional set-point of NK cells, providing a basis for discriminating self from missing-self, and at the same time endowing each NK cell with the highest inherent responsiveness compatible with self-tolerance.

Keywords

Natural killer; MHC class I; missing-self; tolerance; inhibitory Ly49

Introduction

Natural Killer (NK) cells were originally identified by their ability to spontaneously kill certain tumor target cells *in vivo* and *in vitro* without prior sensitization (1,2). Soon after, it was found that, *in vitro*, NK cells would kill tumors cells with low levels of major histocompatibility complex class I (MHC class I) molecules at their surface (3–5). Earlier studies had shown that rejection of hematopoietic cell grafts depended on genes linked to the MHC, and it was eventually established that such rejection is mediated by NK cells (6,7). These studies established that NK cells exhibit a capacity to discriminate target cells. The finding that cells lacking self MHC class I molecules were generally more sensitive to NK cells led to the formulation of the “missing-self” hypothesis which stated that NK cells preferentially attack cells that do not express, or downregulate, some or all of the MHC class I proteins that they express (4,8). The hypothesis received strong support from the demonstration that NK cells destroy hematopoietic cells from gene-targeted MHC class I-deficient mice (9,10). The identification of MHC class I specific receptors on NK cells both

in mice (the Ly49 molecules) (11) and humans (the p58, or KIR receptors) (12–15) provided a molecular mechanism for missing-self recognition. These findings suggested that target cell lysis by NK cells occurs when the target cells fail to express MHC ligands that would otherwise engage inhibitory KIR or Ly49 inhibitory receptors expressed by NK cells.

The initial view that MHC plays the decisive role in NK-target cell interactions has been modified by studies showing that various stimulatory receptors also play an important role (16–18). In some cases alterations in the expression of ligands for stimulatory receptors are sufficient to render a target cell sensitive to NK cells, even when MHC class I levels do not vary. The current paradigm is that NK cells express several families of inhibitory receptors, most of which are 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 a given target cell determines whether the NK cell becomes activated, produces inflammatory cytokines (including interferon (IFN)- γ and tumor necrosis factor (TNF)- α), and/or kills the target cells (16,18,19).

The inhibitory, MHC specific Ly49 receptors are type II membrane glycoproteins from the C-type lectin superfamily (19), whereas KIR glycoproteins are Ig superfamily members (13,14). Each family consists of up to 10 distinct members that recognize class Ia molecules, but vary in their specificity for allelic isoforms of these highly polymorphic molecules. Ly49 receptors but not KIR are expressed by mouse NK cells whereas human NK cells show the opposite pattern. Both species express a third heterodimeric CD94/NKG2A receptor (20,21) that specifically recognizes peptides processed from class Ia molecules presented by a non-classical class Ib molecule (HLA-E in humans, Qa-1 in mice).

These inhibitory receptors carry an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic tail. Upon engagement with its ligand, the tyrosine residue in the ITIM is phosphorylated and recruits a protein tyrosine phosphatase, usually SHP-1 or SHP-2 (SH2-containing protein-tyrosine phosphatase). The phosphatases function to decrease the phosphorylation of several intracellular signaling proteins (for SHP-1 and SHP-2) (18). As a result of inhibitory receptor engagement, NK cell cytotoxicity and cytokine production are dampened, or abrogated.

In general, each inhibitory receptor exhibits a variegated expression pattern, meaning that it is expressed by only a subset of NK cells, with a good deal of overlap in receptor expression. As a result, the NK cell population consists of many subpopulations expressing various combinations of the available inhibitory receptors (22–24). The variegated expression pattern confers each NK cells with a unique set of inhibitory receptors specific for MHC class I, and consequently a distinct potential pattern of reactivity. This expression pattern ensures that some NK cells in each individual can discriminate cells lacking one MHC molecule and not another, but such a system presents a major challenge as well: if the expression of each receptor is determined by a probabilistic gene expression mechanism, as suggested by various studies (25–27) some NK cells should arise that lack inhibitory receptors specific for self MHC class I molecules. Because such cells are potentially autoreactive, developmental processes must exist that either repress the activity of such cells or prevent them from developing or maturing in the first place.

The purpose of this review is to summarize the findings of our laboratory and others regarding the mechanisms underlying the self-tolerance of NK cells. Particular emphasis will be placed on recent studies describing NK subsets that are devoid of inhibitory receptors for self-MHC, which have unleashed considerable debate in the field.

The formation of the NK cell repertoire

Inhibitory receptors specific for self MHC class I molecules play a central role in missing-self recognition by NK cells. Developing NK cells must acquire a set of inhibitory receptors that discriminate “self” (i.e. normal autologous cells) and “missing-self” (i.e. tumor cells and infected cells, which frequently downregulate MHC class I expression on their surface). Individual NK cells commonly coexpress multiple MHC class I-specific inhibitory receptors in a variegated and overlapping fashion (22,23,28). Analysis showed that the frequencies of NK cells in a given mouse strain that coexpress two or three specific Ly49 receptors is approximated by the product of the frequencies of NK cells that express each of the receptors individually (the “product rule”) (24). Along with the observation that the two alleles of a given Ly49 gene are expressed independently (25,29), these findings led to the proposal that the “repertoire” of inhibitory specificities is governed by a stochastic process. More recently, molecular mechanisms underlying stochastic expression of inhibitory receptor genes have been proposed (27,30).

The stochastic nature of receptor gene expression is predicted to yield NK cells with every possible combination of inhibitory receptors. Considering the known capacity of these receptors to discriminate among MHC class I alleles (11,12,31,32), some cells would be predicted to express, at least initially, only receptors that fail to bind the host’s MHC class I molecules. Such NK cells cannot be inhibited by host MHC molecules and are therefore potentially auto-aggressive.

Until relatively recently, a major question has been whether, in fact, such NK cells exist in a normal animal. Although theoretical considerations suggested that such cells should arise, it was possible that the theory was incorrect or that the cells arise but fail to persist or mature, and never contribute to the mature NK cell pool. In a model favored at one time by this laboratory, it was proposed that developing NK cells adapt, by initiating expression of additional inhibitory receptors until a self-specific receptor is eventually expressed (33,34). By such mechanisms, an initially random NK repertoire might be sculpted into one in which all mature NK cells express at least one, and possibly more, receptors specific for self MHC class I.

If the NK repertoire is sculpted by interactions with self MHC molecules, MHC dependent alterations should be evidenced as differences in the frequencies of NK cells expressing different inhibitory receptors. Such changes are indeed detectable, but it now appears unlikely that they are imposed primarily to ensure that all NK cells are inhibited by self MHC class I molecules. MHC-dependent differences in the repertoire are apparent as reduced numbers of NK cells expressing multiple (>1) self-specific inhibitory receptors (28,35) NK cells expressing multiple inhibitory receptors specific for self MHC may be disfavored because the different receptors on many of these cells will bind to distinct self MHC class I molecules, reducing the capacity of these NK cells to destroy diseased cells that downregulate only one of the MHC class I molecules. It must be emphasized that despite being disfavored, the expression of two or more inhibitory receptors for self MHC class I is by no means rare among NK cells. As will be discussed below, NK cell functional activity varies depending on the number of inhibitory receptors specific for self MHC class I that the NK cell expresses.

At least two models have been proposed to account for MHC-dependent changes in the NK repertoire (24,28). The sequential-cumulative model proposes that distinct Ly49 genes are induced in a stochastic but stable fashion during a phase of NK cell development, and that individual developing NK cells continue for a time to accumulate new receptors. If and when newly expressed inhibitory receptor(s) engage self MHC class I molecules with

sufficient strength, new expression of additional receptor genes is repressed (36–38). An alternative model proposes that the repertoire is fully formed at an initial stage by a stochastic process and is subsequently shaped by a selection process that favors cells that express “at least one”, but not too many, self-specific inhibitory receptors. The sequential-cumulative model is supported by a study showing that transgene-enforced expression of a self MHC-specific inhibitory receptor results in reduced expression of inhibitory receptors that do not recognize self MHC (38). However, these studies were unable to address a key issue: whether these processes ensure that each NK cell expresses at least one receptor specific for self MHC. Subsequent studies, summarized below, showed that they do not.

NK cell self-tolerance in the absence of MHC class I-specific inhibition

Although the “at least one” model was widely accepted and fits well with the notion that NK cells generally distinguish missing self from self (39), it cannot apply in the case of animals that lack proper expression of MHC class I proteins. Indeed, mice bearing mutations in the genes encoding the β 2-microglobulin (β 2m) and/or the transporter associated with antigen processing (TAP-1) contain similar numbers of NK cells as wild-type mice, and with a grossly normal repertoire (10,40,41). An identical situation pertains in TAP-deficient patients (42,43). Yet, NK cells from such hosts are self-tolerant as they do not lyse autologous class I-deficient lymphoblasts *in vitro* or class I-deficient bone marrow cells *in vivo*, nor do they attack allogeneic targets (9,10,43,44). One potential explanation for these findings was that self-tolerance in these mice depended on interactions of inhibitory receptors with the very low levels of MHC class I molecules known to remain on the surface of cells from β 2m- or TAP-1-deficient mice (45–47), but this possibility was eventually ruled out by studies showing comparable self-tolerance of NK cells from mice lacking functional MHC class I molecules altogether (40,48). These findings, taken together, established that self-tolerance does not require expression of at least one receptor specific for self MHC class I, but it remained unclear whether this conclusion applies to normal animals, as opposed to mutant animals lacking MHC class I molecules.

More recently, our laboratory reported that a subset of NK cells lacking all self MHC class I-specific inhibitory receptors develops in normal mice (49). This finding was later extended to healthy humans (50,51).

Such NK cells were partially or completely defective in killing MHC class I-deficient target cells both *in vitro* and *in vivo* and in producing IFN- γ in response to tumor cell lines or cross-linking of stimulatory receptors *in vitro* (49). Another study used mice engineered to express a single MHC class I molecule to demonstrate that NK cells that can interact with self MHC class I through the expression of a specific inhibitory receptor exhibit greater functional responses than NK cells that are devoid of such a receptor (52). Altogether, these results indicated that potentially auto-aggressive NK cells that fail to encounter cognate MHC class I ligands assume a unresponsive/hyporesponsive (depending on the type of stimulation) phenotype that contributes to self-tolerance. However, it remained unclear how NK cells that fail to interact with self MHC class I molecules become hyporesponsive. Below we summarize different models that have been proposed to explain the underlying mechanisms of NK cell self-tolerance, which have provoked considerable debate.

Role of MHC class I-independent inhibitory receptors

An obvious possible mechanism of self-tolerance of NK cells lacking inhibitory receptors for self MHC class I is that such NK cells are somehow rendered more sensitive to inhibition through receptors specific for non-MHC molecules. Several possible receptors of this type can be considered as candidates in this context.

The receptor 2B4 (CD244) and its ligand, CD48, are expressed on all NK cells both in mice and humans. A special trait of 2B4 is that depending on which of two alternative adaptor proteins it associates with, it can either activate or inhibit NK cell functions (53,54). Activated NK cells that lack 2B4 expression kill CD48⁺ allogeneic and CD48⁺ syngeneic splenocytes, suggesting that potent inhibition can be mediated by this interaction (55) and this has led to the proposal that the 2B4-CD48 interaction is responsible for self tolerance of NK cells that lack receptors for self MHC class I molecules (56). However, there is no evidence that NK cells in normal mice lacking self MHC class I specific inhibitory receptors exhibit altered 2B4 function as should be true if 2B4 inhibition is responsible for self-tolerance of these NK cells.

Other candidate non-MHC-specific inhibitory receptors are carcinoembryonic-antigen-related cell-adhesion molecule 1 (CEACAM1), an inhibitory receptor specific that engages in homophilic interactions (57), killer-cell lectin-like receptor G1 (KLRG1) (58,59), which binds E-, N-, and R-cadherins (60,61) and NKR-P1D which binds osteoclast inhibitory lectin (Ocil, also known as Clr-b) (62,63). However, none of these receptors has been shown to be expressed more frequently by NK cells that lack inhibitory receptors specific for non-MHC molecules, in normal animals. Indeed, KLRG1 expression is reduced on such cells (49,64), whereas NKR-P1D is expressed at similar levels by NK cells from wildtype and class I-deficient animals (63) and CEACAM1 is expressed very poorly by peripheral blood lymphocytes (57,65).

In summary, it is plausible that upregulation of inhibitory receptors specific for non-MHC ligands contributes to self tolerance when NK cells lack self MHC-specific inhibitory receptors, but none of the receptors studied so far has been shown to play this role. Furthermore, the available data suggest that tolerance of such NK cells occurs at least in part by a distinct mechanism, discussed below.

Alterations in activation pathways

Persistent alterations in the activation pathways of NK cells could also account for the hyporesponsive phenotype of NK cells that are not able to recognize self MHC class I molecules, therefore ensuring self-tolerance. This type of mechanism is akin to “anergy” of T and B cells, which is believed to reflect dampened stimulatory signaling capacity. Possible alterations in this category include downregulation of specific stimulatory receptors that recognize normal cells, or alterations in mediators in the stimulatory pathways downstream of these receptors resulting in dampened transmission of the stimulatory signals.

Despite the functional defects described in the previous sections, NK cells lacking self MHC class I specific inhibitory receptors in wild-type C57BL/6 and in $\beta 2m$ -deficient mice show normal cell surface expression of all the stimulatory receptors studied, including NKG2D, NKR-P1C, CD16, Ly49D (49). Because NK cell activation through any of these receptors is dysfunctional, it is likely that the defect in these cells is in a common stimulatory signaling pathway(s) necessary for cellular activation, rather than in cell surface receptor expression.

We did not observe downregulation of DAP12 or DAP10 intracellular protein levels in hyporesponsive NK cells, arguing that hyporesponsiveness cannot be explained by loss of these proximal signaling molecules (N.C. Fernandez and D.H.R unpublished observations). These data are consistent with the report that NK cells from mice deficient in DAP12/KARAP12, DAP10, Fc ϵ RI γ or CD3 ζ adaptor molecules did not display a generally hyporesponsive phenotype (52). Thus, hyporesponsiveness cannot be explained by downregulation of the receptors and associated adaptors studied to date. At this time, however, we cannot exclude that some unexamined stimulatory receptors are subject to downregulation in hyporesponsive NK cells.

Possible role of regulatory T cells

The naturally occurring CD4⁺CD25⁺ T cells (Tregs) have been shown to suppress autoreactive T cells, as well as other component of the immune system in order to prevent auto-immunity. Several recent reports show that CD4⁺CD25⁺ Tregs have the capacity to inhibit NK cell activity both *in vitro* and *in vivo* (66–68). Thus it is plausible that potentially autoreactive NK cells are controlled by Tregs.

We have performed an experiment to test whether the function of Tregs is necessary to suppress the function of NK cells that lack inhibitory receptors specific for self MHC class I. We asked whether the self-tolerance of NK cells was altered in B6-Rag1^{-/-} mice, which lack all T cells, including Tregs. The distribution of inhibitory receptors on NK cells from Rag1^{-/-} mice was grossly normal, including the presence of a subset of NK cells lacking the three self (H-2^b) MHC-specific inhibitory receptors, Ly49C, Ly49I and CD94/NKG2A. This NK subset in Rag1^{-/-} mice, like the corresponding subset in wild-type mice, was functionally hyporesponsive when stimulated with antibodies specific for the stimulatory receptor NKG2D (N.T.J and D.H.R unpublished observations). Thus, the hyporesponsiveness of NK cells lacking self MHC-specific inhibitory receptors is not dependent on the action of Tregs, though a role for other types of regulatory cells cannot be excluded.

The “Arming” and “Disarming” models

The first reports of NK cell activity in $\beta 2m$ -deficient mice demonstrated that NK cells in these mice are unresponsive against MHC class I-deficient and allogeneic targets (9,10,44). Since NK cells that arise in mice that express MHC class I are responsive whereas those in MHC class I-deficient mice are hyporesponsive, the question arose whether the presence of MHC class I-positive cells induces high responsiveness, or, alternatively, if the presence of MHC class I-deficient cells induces hyporesponsiveness. These alternatives were the basis of two models proposed to explain the adaptive tolerance of NK cells (69,70).

In what was then called the “positive model” (69) (which we later called the “arming” model (70)), functional maturation of NK cell precursors requires interactions with cognate MHC class I-positive cells. NK cells that do not encounter cognate MHC class I molecules remain in an unarmed, and therefore hyporesponsive state (Figure 1). In the “negative model” (which we later called the “disarming” model), high functional responsiveness is a default outcome. NK cells are prevented from acquiring (or retaining) full responsiveness when interactions with self cells are overly stimulatory—which can occur when self cells fail to provide sufficient inhibitory ligands to counterbalance stimulatory signals they also provide. In the extreme, failure to engage cognate MHC class I molecules results in functional anergy of the NK cell, rendering it unresponsive to cells lacking MHC class I molecules, and hyporesponsive to other stimuli. By this mechanism, the NK cell is never allowed to acquire sufficient responsiveness to attack normal self cells (Figure 1). Variants of this model differ on whether NK cells are disarmed during their development, after attaining maturity, or both.

Testing the “Arming” and “Disarming” models

These models made different predictions in the context of bone marrow chimeric mice consisting of mixtures of cells expressing MHC class I molecules or not. According to the arming model, NK cells that interact with normal cells (i.e expressing MHC class I molecules) will successfully mature and acquire the capacity to eliminate the co-developing MHC class I-deficient cells on the basis of missing-self recognition. On the contrary, the disarming model proposes that stimulatory ligands on MHC class I-deficient cells in the

chimera will provide sufficient stimulation to render all the NK cells hyporesponsive (Figure 2). An early study showed that NK cells were hyporesponsive in chimeras in which all hematopoietic cells were MHC class I-deficient and other cells were MHC class I-positive (44). Subsequently, in order to investigate the consequences when NK cells developed in a mixed hematopoietic environment, irradiated normal or $\beta 2m$ -deficient hosts were repopulated with a mixture of normal and $\beta 2m$ -deficient hematopoietic progenitors (69). In all the mixed chimeras, a mixture of MHC class I⁺ and MHC class I⁻ cells was maintained over many weeks and tolerance to MHC class I-deficient grafts was established. Tolerance also occurred when the host was MHC class I-deficient and the bone marrow cells were MHC class I-positive (69,71) (N.T.J and D.H.R unpublished observations). A separate study using H-2^b mice with mosaic expression of an H2-D^d transgene provided additional insight by demonstrating that tolerance to H2-D^d-negative cells occurred when as few as 20% of the host cells lacked H2-D^d expression. Kinetic studies showed that the percentage of H2-D^d-positive cells versus H2-D^d-negative cells did not change with time, arguing against a continuous elimination of the H2-D^d-negative cells, as might occur if NK cells were induced to high responsiveness by interactions with H2-D^d-positive cells (72). Altogether, these data support the disarming model. They suggest that either hematopoietic or nonhematopoietic cells lacking self MHC class I molecules can induce hyporesponsiveness of NK cells, as proposed by the disarming model, as distinct from the prediction of the arming model that MHC class I expressing cells in the chimeric mice should induce the differentiation of highly responsive NK cells capable of attacking MHC class I-deficient cells. Still, one cannot exclude a combined model where NK cells interacting with self MHC molecules are induced to become functionally mature, but can nevertheless be subsequently inactivated as a result of interactions with cells lacking cognate MHC molecules.

The arming and disarming models are equally pertinent for understanding how self-tolerance is imposed in the case of the recently identified NK cells in normal animals that lack inhibitory receptors for self MHC class I molecules (49,52). Although virtually all cells in normal animals express MHC class I molecules, these NK cells have no receptors that can recognize them. The arming model would propose that in the absence of signals from such receptors, these NK cells persist in a default, low responsive state, whereas the disarming model suggests, instead, that the absence of inhibition results in overstimulation of these NK cells, leading them to acquire an anergic or hyporesponsive functional state. Hence the two models predict the same outcome, albeit by different mechanisms. Given the striking similarities of these NK cells with the hyporesponsive NK cells in class I-deficient mice, and the results with bone marrow chimeras, we have argued that disarming is the likely mechanism of tolerance in both scenarios.

A study by another group, however, suggested a different interpretation. It was reported that in mice expressing D^d, a ligand for Ly49A, NK cells expressing transduced Ly49A, exhibited stronger responses than untransduced NK cells. This did not occur in H-2^b mice, which lack a relevant ligand (52,73,74). Similar to other studies, this finding indicated that expression of self MHC-specific Ly49 receptors resulted in higher functional responses. The authors went further than earlier studies by using gene transfer, and showing that the ITIM motif in Ly49A is critical for this effect. The authors coined the term “licensing” for this process, to emphasize that a developmental event endowed the cells with high functional activity only if they expressed appropriate receptors for self MHC class I molecules. The term is confusing, however, as it tends to imply that the interaction with self MHC induces high functional responsiveness (i.e the arming model), whereas the available evidence is more consistent with the disarming model.

Cellular mechanisms associated with hyporesponsiveness

Several key issues concerning these processes remain unsettled or controversial. Some studies raise the possibility that the underlying process alters the proliferation or survival of specific sets of developing NK cells, not just their activity. After reconstitution of H-2^d (but not H-2^b) mice with a mixture of bone marrow cells from normal mice and transgenic mice whose NK cells all express the H-2D^d-specific Ly49A receptor, mature NK cells showed a preponderance of transgenic NK cells, suggesting a selective advantage for the transgenic NK cells in the presence of their ligand (75). Another paper reported that immature bone marrow NK cells with inhibitory receptors specific for self MHC class I molecules incorporated more BrdU than other immature NK cells, suggesting greater proliferation of these cells (52). In evaluating these data the first point to note is that these effects are no more consistent with the arming model than with the disarming model: they can be explained either as a selective proliferation or survival of NK cells induced by engagement of receptors for self MHC class I or as a preferential death or proliferative arrest of NK cells whose receptors fail to be engaged. The second point to note is that these findings are currently difficult to reconcile with the results of other studies of NK cells in normal mice. For example, were it true that developing NK cells with a self MHC specific inhibitory receptor undergo greater proliferation, such NK cells would presumably be enriched among mature NK cells in normal mice, but the data indicate that they are either unaffected or less prevalent (24,28). Furthermore, when mice were treated continuously with BrdU, splenic NK cells expressing receptors specific for self MHC labeled no faster than other NK cells, or for that matter no faster than NK cells from class I-deficient mice (76) (M.C Coles, J.R Dorfman and D.H.R unpublished data). Taken together, these data suggest that the expression of receptors specific for self MHC has little if any impact on NK cell selection in normal mice. Additional studies will be necessary to explain these discrepancies.

A second unresolved issue concerns the signaling pathways involved in establishing NK cell self tolerance. As already mentioned, it was shown using viral transduction that the ITIM motif in Ly49A was essential in order for the receptor to impact NK cell functionality. Since the ITIM is normally associated with inhibitory activity, that data would seem at first glance to be more consistent with the disarming model, in which the NK receptor functions by preventing overstimulation of NK cells, than the arming model, in which engagement of the NK receptor induces greater functionality of the NK cells. However, it remains possible that the ITIM functions differently in immature NK cells. It was proposed that the MHC class I-specific receptor may be stimulatory during NK cell maturation/development and inhibitory during effector responses (74).

One possibility is that instead of recruiting a protein tyrosine phosphatase, as in mature NK cells, the ITIM recruits a distinct protein that promotes NK cell functionality. In an attempt to determine the role in the process of SHP-1, the major protein tyrosine phosphatase that participates in NK cell inhibition, we and others generated chimeras reconstituted with a mixture of bone marrow from wild type normal mice and SHP-1 defective motheaten-viable (*me^v*) mice. One study reported that establishment of highly responsive NK cells was preserved in SHP-1-deficient NK cells, suggesting that the Ly49 receptor may recruit a distinct signaling molecule (52). However, we found that SHP-1 deficiency is associated with hyporesponsiveness in NK cells (R.E Vance and D.H.R unpublished observations). Our findings are supported by a published study reporting impaired missing self-induced killing of targets by NK cells expressing a catalytically inactive form of SHP-1 (77). The finding that SHP-1 deficiency, like MHC class I deficiency, results in hyporesponsiveness is perhaps easier to reconcile with the disarming model than the arming model, since the phosphatase is known to curtail stimulatory signaling in mature NK cells. Clearly, though,

further studies will be necessary to establish the signaling pathways involved in the establishment of responsive versus hyporesponsive NK cells.

A third unsettled question is whether hyporesponsive NK cells are a form of immature NK cell, or alternatively a fully mature NK cell exhibiting dampened functional responsiveness. Supporting the latter possibility, hyporesponsive NK cells in normal mice as well as in $\beta 2m$ -deficient mice exhibit normal cell surface expression of all known NK cell maturation markers, including CD11b, DX5 and Ly49 receptors (49). Moreover, the fact that these NK cells are able to produce normal levels of IFN- γ upon stimulation with pharmacological agents (phorbol 12-myristate 13-acetate plus ionomycin) indicates that they are equipped to execute an activation program (49,52). More substantively, there are indications that hyporesponsive NK cells may be capable of providing important functions *in vivo* in infected animals. One indication is the finding, years ago, that NK cells in class I-deficient mice can respond and limit infections with mouse cytomegalovirus, just as NK cells do in normal mice (78). A related finding is that NK cells in normal animals that lack receptors specific for self MHC can nevertheless respond by producing IFN-gamma in mice infected with *Listeria monocytogenes* (49) or mouse cytomegalovirus (N.C Fernandez, A.M Jamieson and D.H.R unpublished data). Together, these data suggest that hyporesponsive NK cells are mature, and can even carry out *in vivo* functions under conditions of infection.

On that basis, we proposed that these NK cells exhibit a somewhat selective, or contextual, defect that is particularly evident in their capacity to mediate missing self recognition of normal cells lacking self MHC class I molecules (26,49,70). Other functions, such as responses to certain pathogens, may remain intact. This view is not incompatible with the notion that such NK cells are hyporesponsive, because the studies performed to date suggest that the responsiveness of the cells is dampened and not abrogated. When such NK cells encounter diseased cells that have upregulated stimulatory ligands, the resultant increased stimulatory signaling in the NK cell may lead to an effective response despite the reduced overall responsiveness of these NK cells. Furthermore, when encountering a diseased target cell that continues to express MHC class I molecules, the absence of self MHC-specific inhibitory receptors on these NK cells means that any stimulatory signaling will not be countered by inhibitory signaling to the same extent as would occur in the case of the highly responsive NK cells. Hence, we proposed that the hyporesponsive NK cells are particularly deficient in their capacity for missing self recognition of MHC-deficient target cells, but this defect is compatible with the notion that other NK cell functions remain partially intact.

Clearly much remains to be learned concerning the functional capabilities of NK cells lacking inhibitory receptors for self MHC class I molecules. However, their capacity to function in specific contexts suggests that they cannot be considered immature, nonfunctional cells. A fallback position is the proposal that these NK cells represent an intermediate state of NK cell maturity. At that point, the issue of the “maturity” of the cells becomes a semantic question. The fact that they exhibit reduced responsiveness compared to other NK cells cannot be considered a sufficient basis for defining the cells as immature. Given the emerging evidence, described below, that NK cell responsiveness varies over a considerable range, it appears more likely that the hyporesponsive NK cells represent mature cells that have been set, or “tuned”, to a low degree of responsiveness.

The role of stimulatory receptors in the induction of NK cell hyporesponsiveness

The disarming model implies that developing and mature NK cells are exposed to persistent stimulation delivered by self (normal) cells. This notion was originally based on solid evidence that otherwise normal cells such as lymphoblasts and bone marrow cells, when

they lack self MHC class I molecules, are attacked by normal NK cells. In all other contexts, NK cells and T cells do not attack target cells unless a ligand on the target cell engages a corresponding stimulatory receptor. We view this as *prima facie* evidence that normal cells engage stimulatory receptors on NK cells. Whereas some of these stimulatory receptors have not been defined, others have. For example, some Ly49 receptors, KIRs and CD94/NKG2 isoforms are stimulatory (18,79–81) and may recognize self MHC class I molecules, depending on the MHC haplotype. MHC-specific stimulatory receptors cannot, of course, explain lysis of MHC-deficient target cells by NK cells. Ligands for the NKG2D receptor represent another category of self-stimulatory ligands. NKG2D ligands are normally expressed poorly by normal cells and upregulated on diseased cells (82), but recent studies suggest that NKG2D ligands are upregulated on proliferating bone marrow cells in some mouse strains (83), and on proliferating lymphoblasts in some humans (84). However, NKG2D engagement is not essential for rejection of class I-deficient syngeneic bone marrow cells in B6 mice (83) (N. Guerra, N.T.J and D.H.R unpublished observations), suggesting a role for a distinct receptor/ligand pair. The responsible receptor may be as yet unidentified, or may correspond to an already identified receptor, such as 2B4, which can exhibit either stimulatory or inhibitory function depending on the adapter molecules the NK cell expresses (53,54).

According to the disarming model, when normal cell types stimulate NK cells, and at the same time fail to provide MHC class I molecules that can inhibit the NK cells, the result is over-stimulation, leading to hyporesponsiveness. The model goes further and predicts that even when an NK cell's inhibitory receptors are engaged by self MHC, if self cells also provide excessive stimulation the inhibitory signals may be overcome, resulting in NK cell hyporesponsiveness. Reports using transgenic mice that constitutively express NKG2D ligands are consistent with these predictions (85,86). In mice expressing a Rae-1 ϵ transgene either ubiquitously, or specifically in squamous epithelium (85), NK cells were refractory to stimulation with cells expressing NKG2D ligands, indicating that self-tolerance had been induced. Notably, however, these NK cells were also poorly responsive to RMA-S lymphoma cells and β 2m-deficient lymphoblasts, neither of which express NKG2D ligands. These data suggest that persistent strong stimulatory signaling in the face of normal MHC class I-mediated inhibitory signaling, may yield an equivalent outcome as does mild stimulatory signaling in the absence of MHC class I mediated inhibitory signaling, consistent with the predictions of the disarming model (Figure 3d).

Varying the number of inhibitory interactions during development tunes NK cell responsiveness: an educational “rheostat”

As already discussed, the NK cell population consists of many subpopulations expressing various combinations of the available inhibitory receptors. Each inhibitory receptor binds one or more MHC class I molecule with varying affinities and there is evidence that the extent of binding correlates with the extent of functional inhibition (31). In addition to variable expression of inhibitory receptors, NK cells vary in their expression of stimulatory receptors capable of binding normal cells, such as MHC class I-specific stimulatory receptors. These considerations lead to the prediction that developing NK cells will vary considerably one from the other in the balance of stimulatory and inhibitory receptor engagement that occurs when they encounter normal cells. In the face of these variations, the question arises whether NK cell responsiveness is binary, with high responsiveness and hyporesponsiveness being the two outcomes, or is instead continuously variable, consisting of numerous intermediate states. When an NK cell receives only a small excess of stimulation is it attenuated to the same degree as an NK cell that receives a large excess of stimulation, or is it attenuated 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?

This issue is being addressed by examining the consequences of variations in either the strength of inhibitory interactions that NK cells encounter, or the number of inhibitory receptors that are engaged by self MHC class I. To examine the effect of the strength of the interaction, mice were engineered that expressed only one relevant class I ligand (K^b , D^b , L^d , or D^d) (87). The capacity of these mice to reject class I-deficient spleen cell grafts was assessed. Mice expressing only D^d or K^b were better able to reject the challenge grafts than mice expressing only L^d or D^b (87). Because other evidence suggests that D^d and K^b mediate strong inhibition of NK cells, whereas L^d and D^d mediate weaker inhibition, the results suggest that NK cells exposed persistently to potent inhibitory signaling develop greater responsiveness than NK cells exposed to weaker inhibitory signaling.

A distinct approach was used to address how variations in the number of self MHC-specific inhibitory receptors impact NK cell responsiveness. For this approach, we took advantage of the fact that only three inhibitory receptors interact appreciably with H-2^b class I molecules in B6 mice (31). Ly49I and Ly49C are both receptors for K^b whereas the heterodimeric CD94/NKG2A receptor binds a D^b -derived signal peptide presented by the Qa-1 non-classical class I molecule. Using monoclonal antibodies specific for each of these three receptors to define NK cells expressing only one, any pair, or all three of these inhibitory receptors, we tested their responses to crosslinking antibodies specific for various stimulatory receptors. We found a *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 of the NK cells (Figure 3, a–c) (N.T.J and D.H.R in preparation). These and other data (88) suggest that the functional set-point of NK cells is tuned by the quantity of inhibitory interactions to which the cells are exposed during development.

Concluding remarks

In the case of T and B lymphocytes, reactivity is mediated through a primary antigen receptor that is unique on each cell and modulated by various costimulatory receptor interactions. In contrast, NK cells, like other components of the innate immune system, express a multitude of stimulatory and inhibitory receptors which must be considered as roughly equivalent partners in determining specificity. A given NK receptor may play a primary role in recognizing one type of diseased cell and play no role in recognition of other diseased cells. In most cases, diseased target cells display a multitude of ligands that can potentially be recognized by different stimulatory and inhibitory receptors on an NK cell, and the outcome is determined by the overall balance in signals that result from the interaction. Many of the NK receptors are expressed in a stochastic fashion, and many of the ligands (and receptors) are highly polymorphic, which means that the balance of potentially stimulatory and inhibitory ligands a given NK cell encounters even in a healthy individual must vary over a substantial range. This review has addressed the logic and the mechanisms that govern the regulation of NK cell responsiveness, so as to achieve self-tolerance on the one hand, and optimal reactivity with diseased cells on the other.

The main focus was on the role of MHC class I-mediated inhibition of NK cells, because the interactions made with MHC molecules vary considerably from one NK cell to another, or from one individual to another. This is a consequence of the random expression of inhibitory receptors on NK cells, and the great polymorphism in MHC class I molecules. We emphasized that whereas self MHC class I molecules modestly impact the repertoire of inhibitory receptors that NK cells express, some NK cells in normal animals or humans lack any known inhibitory receptors specific for self MHC class I, and yet exhibit self-tolerance. In mice or humans with mutations that prevent MHC class I expression, NK cells are similarly self-tolerant. We considered the possibilities that self-tolerance of such NK cells is due to enhanced expression or function of inhibitory receptors specific for non-MHC

molecules, or possibly, regulatory cells. We outlined our favored mechanism, which is that such NK cells are hyporesponsive to stimulation through various stimulatory receptors, presumably due to the dampening of undefined steps in the stimulatory signaling pathways. We considered two major models of the cellular interactions that induce hyporesponsiveness, which we call “arming” and “disarming”, and summarized the evidence favoring the disarming model, which proposes that persistent stimulation that normal cells provide, when unopposed by MHC specific inhibitory interactions, induces NK cells to a hyporesponsive state.

Finally, we summarized lines of evidence that suggest that the extent of hyporesponsiveness varies continuously depending on the balance of stimulation encountered by developing NK cells. NK cells with a greater number of inhibitory receptors for self MHC class I (or more reactive ones) receive less stimulation on balance and end up being less hyporesponsive than NK cells with fewer (or less reactive) inhibitory receptors. When stimulatory ligands for NK cells are overexpressed transgenically, resulting in greater stimulation on balance, NK cells end up being more hyporesponsive. Although it has yet to be shown that the mechanisms are the same, it is possible to interpret these findings as predictable outcomes of the same mechanism, which turns down the rheostat of responsiveness commensurately with the net balance of stimulatory to inhibitory signaling that NK cells encounter at steady state when interacting with cells in their natural surroundings. Thus, the functional set-point of NK cells is tuned by the quantity of inhibitory and stimulatory interactions to which the cells are exposed during development, providing a basis for discriminating self from missing-self, and at the same time endowing each NK cell with the highest inherent responsiveness compatible with self tolerance.

The consequences of this type of mechanism are broad. For one thing, NK cells have been implicated in the genesis of several autoimmune diseases that also involve T or B lymphocytes. It is possible that disturbances in the set-point mechanism described here result in NK cell autoreactivity and contribute to these diseases. Furthermore, the mechanisms described here may play a role in determining the potency of NK cell responses against pathogens or cancer cells. It has been noted that variations in MHC class I molecules between mouse strains and humans result in differences in the number of inhibitory MHC class I receptors that are engaged by self MHC, as well as the potency of these interactions (31)(34). The considerations discussed here could be expected to result in a greater or lesser overall degree of NK cell responsiveness depending on these interactions, and such differences may impact the effectiveness of NK cells in the setting of infections, cancer and autoimmunity. In addition, the mechanisms discussed here may operate post-natally when a host is persistently infected or exposed to transformed or stressed cells. Under these conditions, the NK cell rheostat may be set to a lower level on newly developing NK cells, or, in the case of already mature NK cells, reset to a lower level, resulting in less effective NK cell responses. As a result NK cell responses may be stably curtailed in various conditions of disease. In light of these considerations, work in progress to understand the signaling mechanisms that dampen the responses of hyporesponsive NK cells may reveal strategies to develop drugs that reactivate beneficial NK cell responses in conditions where NK cells are hyporesponsive, or dampen the responses in conditions where NK cells promote autoimmunity.

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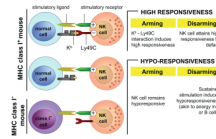


Figure 1. Functional outcomes for NK cells developing under conditions where inhibitory receptor engagement occurs, or does not occur, and interpreted according to the arming model or the disarming model

Interactions of MHC class I molecules (on normal cells) with specific inhibitory receptors (on NK cells) results in high NK cell responsiveness (top). Absence of MHC class I interactions with specific inhibitory receptors, either because the NK cell lacks a corresponding receptor (middle) or because the environment lacks MHC class I molecules (bottom) results in hyporesponsiveness. The outcome in each scenario is interpreted according to either the arming model or the disarming model (right). Note that for reasons of clarity, only one stimulatory and/or one inhibitory interaction is shown for each cell.

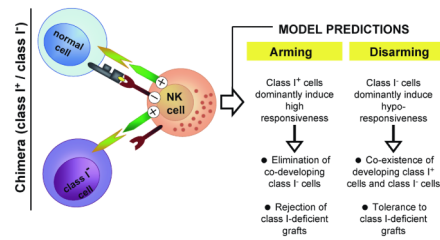


Figure 2. Predictions of the arming and disarming models in mixed MHC class I⁺/class I⁻ chimeras

NK cells developing in a chimeric environment consisting of class I⁺ and class I⁻ cells will encounter both types of cells as shown in the figure. The arming model and the disarming model make different predictions regarding the outcome in this situation. The arming model predicts that the NK cells will attain high responsiveness, which should result in elimination of co-developing class I⁻ cells and rejection of class I-deficient grafts, whereas the disarming model predicts that hypo-responsiveness will be induced, co-developing class I⁻ cells will be tolerated and there will be no rejection of class I-deficient grafts.

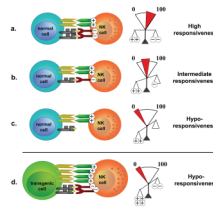


Figure 3. Tuning NK cell responsiveness: an educational “rheostat”

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. One indication of this is that NK cells that express a greater number of self MHC-specific inhibitory receptors acquire a higher degree of potential responsiveness. a) Expression of two inhibitory receptors counters stimulatory signals from neighboring normal cells, lowering the level of persistent stimulation and therefore preventing the induction of hyporesponsiveness. b) Expression of one receptor only partially counters stimulatory signals resulting in partial hyporesponsiveness. c) When no inhibitory receptors are expressed, strong persistent stimulation induces greater hyporesponsiveness. d) In instances where NK cells express inhibitory receptors for self MHC class I, but are exposed to excessive persistent stimulation (as in transgenic mice expressing stimulatory ligands at high levels), the excess stimulatory signaling induces hyporesponsiveness.