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REGULATION OF THE NATURAL KILLER CELL RECEPTOR REPERTOIRE

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■ **Abstract** Natural killer cells express inhibitory receptors specific for MHC class I proteins and stimulatory receptors with diverse specificities. The MHC-specific receptors discriminate among different MHC class I alleles and are expressed in a variegated, overlapping fashion, such that each NK cell expresses several inhibitory and stimulatory receptors. Evidence suggests that individual developing NK cells initiate expression of inhibitory receptor genes in a sequential, cumulative, and stochastic fashion. Superimposed on the receptor acquisition process are multiple education mechanisms, which act to coordinate the stimulatory and inhibitory specificities of developing NK cells. One process influences the complement of receptors expressed by individual NK cells. Other mechanisms may prevent NK cell autoaggression even when the developing NK cell fails to express self-MHC-specific inhibitory receptors. Together, these mechanisms ensure a self-tolerant and maximally discriminating NK cell population. Like NK cells, a fraction of memory phenotype $CD8^+$ T cells, as well as other T cell subsets, express inhibitory class I–specific receptors in a variegated, overlapping fashion. The characteristics of these cells suggest that inhibitory receptor expression may be a response to prior antigenic stimulation as well as to poorly defined additional signals. A unifying hypothesis is that both NK cells and certain T cell subsets initiate expression of inhibitory receptors in response to stimulation.

INTRODUCTION

Natural killer (NK) cells are bone marrow–derived lymphocytes that were originally categorized by their large, granular morphology and their ability to lyse a variety of tumor targets and infected cells (1). In addition, NK cells can secrete potent levels of cytokines (especially IFN-γ and TNF-α) and chemokines (MIP-1 family members and RANTES) (for a review, see 2). In contrast to T and B cell responses to antigen, which typically require a proliferation phase, the innate NK cell response is immediate, implying that NK cells are involved in curbing pathogens during the initial several days of infection. Indeed, there is strong evidence that

NK cells contribute to the defense against intracellular bacteria (3) and parasites (4) and that they are critical for controlling several types of viral infection (2, 5). Despite the well-described antitumor activity of NK cells in vitro (6, 7) and in certain in vivo models (8), their role in the defense against spontaneous neoplastic transformation remains less well established.

NK cells do not require the specialized gene rearrangement machinery that assembles T and B cell antigen receptor genes (9). Nevertheless, NK cells exhibit a clear capacity to discriminate target cells. In the case of tumor cell targets, sensitivity to NK cells was correlated in many instances with decreased levels of MHC expression (10). NK cells are also able to reject MHC-different bone marrow grafts, especially in scenarios where the donor graft lacks MHC molecules of the host (for example, an $MHC^{a/b}$ host often rejects $MHC^{a/a}$ grafts) (11). These observations led to the formulation of the "missing self" hypothesis by Kärre and colleagues (12), which states that NK cells ignore potential targets expressing normal levels of autologous class I molecules and attack cells that do not. This view received strong support from studies with genetically engineered mice (13–16), which demonstrated that NK cells attack otherwise normal cells that lack some or all self-MHC class I molecules. The missing self hypothesis provided a satisfying rationale for NK cell function in vivo, since transformed and infected cells often downregulate or lose class I surface expression (17, 18). A molecular mechanism for missing self-recognition was established when several MHC class I–specific receptors were discovered that inhibit NK cell function (reviewed below).

Missing self-recognition is only one of several modes of NK cell–target cell discrimination. Under some conditions, NK cells attack even class I^+ tumor cells. As reviewed below, many stimulatory receptors on NK cells have been identified, some specific for ligands that are upregulated on tumor cells and stressed cells, and others apparently specific for ligands on normal cells. These disparate recognition systems can be understood in a model in which NK cells are regulated by the balance of signaling via stimulatory receptors, specific for diverse ligands, and inhibitory receptors, specific for MHC class I molecules.

Inhibitory Receptor Overview

The first inhibitory MHC-specific receptors to be discovered were the Ly49 receptors in rodents (19, 20), which bind directly to classical MHC class I (i.e. class Ia) molecules (21–26). More than 10 different Ly49 receptors have been identified in B6 mice, though just 8 of these are of the inhibitory type (27–29). Only a single nonfunctional Ly49-like gene has been identified in humans (30, 31). The second family discovered was the killer cell immunoglobulin-like receptors (KIR) family, which appears to be functional in primates but not rodents, and which also bind directly to class Ia molecules (32–37). Most people are estimated to have on the order of 10 different KIR genes, though not all of these are inhibitory (38–41). The third family is functional in both primates and rodents, and it consists of CD94/NKG2 heterodimers (42–45). Several NKG2 isoforms can pair with the CD94 "common"

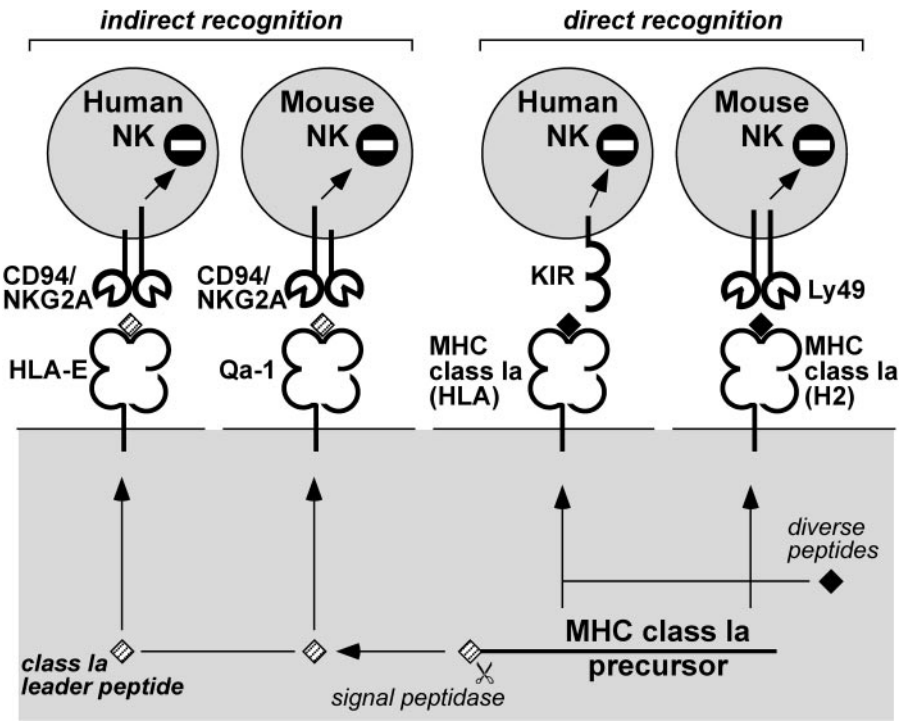

Figure 1 NK cells can detect self-MHC class Ia molecules directly or indirectly. In direct recognition (depicted on the right), MHC class I (class Ia) molecules are bound directly by NK receptors. In humans, these receptors are members of the killer cell immunoglobulin-like receptor (KIR) family, whereas Ly49 receptors fulfill a comparable role in mice. In indirect recognition (depicted on the left), NK cells recognize class Ia–derived leader peptides rather than mature MHC class Ia molecules themselves. The leader peptides are presented on the cell surface by a specialized nonclassical class I (class Ib) molecule called HLA-E (in humans) and Qa-1 (in mice), and the class Ib/leader peptide complex is bound by the heterodimeric CD94/NKG2A inhibitory receptor (present on human and murine NK cells). It should be noted that not all class Ia molecules contain a leader peptide that can be bound by HLA-E/Qa-1; moreover, not all class Ia molecules are directly recognized by KIR/Ly49 receptors.

chain; only CD94/NKG2A is known to be inhibitory. CD94/NKG2 receptors perceive class Ia molecules indirectly by specifically recognizing peptides processed from the leader sequences of class Ia molecules, bound into the groove of a nonclassical class Ib molecule, Qa-1 in mice (46) or HLA-E in humans (47–49) (see Figure 1). Direct and indirect recognition of class Ia molecules may serve complementary roles in inhibiting NK cells.

The two receptor families that recognize class Ia molecules directly, Ly49 receptors and KIR, are likely functionally analogous, even though the former are disulfide-linked homodimers bearing homology to type II C-type lectins, and the

latter are type I monomeric Ig-like receptors. Significantly, all three receptor families exhibit the capacity to discriminate between different allelic class Ia isoforms. Individual Ly49 receptors (19, 24, 50) and KIR (51, 52) exhibit varying degrees of discrimination in reactivity to different class Ia molecules. The CD94/NKG2 receptors also effectively discriminate between different class Ia isoforms because some class Ia molecules do not contain the leader peptide recognized by these receptors (53, 54).

An important feature of all the inhibitory receptors is that each is expressed on a subset of NK cells that overlaps partially with the expression of other class I–specific inhibitory receptors. Thus, multiple receptors are usually expressed on each cell, and a complex combinatorial repertoire of NK specificities is generated. The *variegated* pattern of receptor expression allows individual NK cells to discriminate among cells expressing different class I molecules. For example, a host cell that downregulates only one MHC class I molecule will elicit a strong response by the subset of NK cells whose only self-specific inhibitory receptor recognizes that particular molecule. Cells that have completely lost class I expression will be even more sensitive to attack by most NK cells. Studies of tumor cells and virus-infected cells have documented instances where both selective and complete loss of class I expression occurs (17, 55).

The variegated pattern of receptor expression suggests a stochastic mechanism in the choice of which receptor genes are expressed by each NK cell. The stochastic pattern of expression applies to Ly49 receptors in mice (56) and KIR in humans (57, 58), despite their structural dissimilarities, as well as to the NKG2 subunits of the shared CD94/NKG2 receptors (46, 58, 59). The expression, or not, of the various receptor subunits in NK cell subpopulations is evident at the level of mRNA as well (60, 61), suggesting differential transcriptional control of receptor gene expression. Receptor variegation poses interesting questions as to how expression of receptor genes is initiated and maintained in individual NK cells.

All three of the families of inhibitory receptors signal through motifs in their cytoplasmic domains, called immunoreceptor tyrosine-based inhibitory motifs (ITIM) (62). Upon receptor engagement, ITIM are tyrosine-phosphorylated and recruit protein tyrosine phosphatases such as SHP-1 and possibly SHP-2 (63–67). Since inhibition is apparent at early activation steps such as calcium mobilization (68), the phosphatases probably interfere in an early step of NK cell stimulation pathways.

Stimulatory Receptor Overview

For many years, the characterization of stimulatory receptors on NK cells lagged behind that of inhibitory NK receptors, and during this time it was widely thought that generic adhesion or costimulatory molecules, such as LFA-1, might be responsible for most NK activation (69). However, accumulating evidence reviewed in this volume of *Annual Review of Immunology* by Moretta et al implicates numerous stimulatory receptors in various NK-target interactions.

Stimulatory receptors on NK cells can be broadly divided into those that recognize MHC class I–like ligands and those that do not. Ligands for many receptors in the latter class remain unknown, but some examples of stimulatory ligands for NK cells are MICA/B, Rae1, and H60 (which bind to NKG2D) (70–72), cell bound IgG (which binds to the FcγR), and CD48 (which binds to 2B4 and CD2) (73). Interestingly, if interactions between MHC molecules and receptors on NK cells are prevented, NK cells will attack normal autologous lymphoblasts (15, 16, 74), a fact suggesting that some stimulatory receptors on NK cells react with non-MHC ligands expressed by normal cells. Candidate receptors for such reactions include NKp30, NKp44, and NKp46 receptors (75), as well as the NK1.1 antigen (NKR-P1C) (76). Where tested, most of the non-MHC-specific stimulatory receptors are expressed by most if not all NK cells (72, 75, 77–79), presumably allowing individual NK cells to respond to numerous insults.

Strikingly, MHC class I–specific stimulatory receptors are found within each of the three families of class I–specific receptors. The KIR contain the stimulatory KIR2DS and KIR3DS, the NKG2 family contains the stimulatory NKG2C and E receptors (42, 80), and the Ly49 family contains stimulatory Ly49D and Ly49H receptors (though a class I specificity for Ly49H has yet to be assigned) (81–84).

The MHC class I–specific stimulatory receptors, unlike most non-MHC-specific stimulatory receptors, are expressed in a variegated and possibly stochastic fashion on subsets of NK cells (58, 81, 84). Expression of these stimulatory receptors overlaps with expression of class I–specific inhibitory receptors such that stimulatory and inhibitory receptors of the same class I–specificity are often not expressed by the same NK cell. A beneficial consequence of this expression pattern is that a target cell that has lost an inhibitory class I allele while retaining a stimulatory class I allele would become highly sensitive to attack. In general, class I–specific stimulatory receptors tend to recognize their class I ligands with lower affinity than do their inhibitory counterparts (85, 86). Although the significance of this observation remains uncertain, an intriguing speculation is that class I–specific stimulatory receptors may function preferentially when class I levels are high, as may occur in some viral infections due to the local release of interferons. Alternatively, it is possible that the stimulatory isoforms are in some sense pathogen-specific. For example, their purpose may be (or may have been) to detect (and therefore counter) decoy class I homologs (18) that viruses produce for the purpose of inhibiting NK cells. Or, class I–specific stimulatory receptors may recognize specific complexes of self-MHC bound to viral or tumor antigen-derived epitopes.

NK stimulatory receptors generally associate with small transmembrane adapter proteins that transmit activation signals, including KARAP/DAP12 (87, 88), CD3ζ, FcRγ (89), and DAP10/KAP10 (90, 91). It is thought that each of these adapters is expressed by all NK cells but associates only with a distinct subset of the stimulatory receptors. NK cells from mice or humans with mutations in one of these genes, KARAP/DAP12, exhibit only a subtle phenotype and can still attack most NK target cells (92, 93). These data confirm the idea that NK cell activation is multifactorial and does not rely on a single signaling adapter.

NK Cell Education

Although a stochastic mechanism appears to underlie the initial expression of inhibitory receptor genes, the final functional repertoire of NK receptor expression is shaped by education processes based on the MHC class I alleles that happen to be expressed by the host. The education processes are still poorly understood, but their ultimate effects are to ensure a repertoire of NK cells that is both self-tolerant and useful. The education process must take into account both the inhibitory and stimulatory receptors that individual NK cells express.

Here we review the present understanding of the NK repertoire, including, (*a*) the mechanisms that initiate and maintain expression of inhibitory NK cell receptors, generating a diverse repertoire of specificities; (*b*) the self-tolerant state of NK cells and the evidence for the involvement of various mechanisms in imposing self-tolerance; (*c*) the evidence that education mechanisms maximize the discriminatory properties of the repertoire; and (*d*) the similarities and differences between the repertoires of NK cell inhibitory receptors expressed by NK cells versus T cells.

INITIATION AND MAINTENANCE OF RECEPTOR EXPRESSION

Stochastic Expression of Receptor Genes

All three MHC-specific NK receptor families are expressed in a variegated, overlapping fashion. The frequency of NK cells co-expressing a given combination of receptors can usually be well estimated as the product of the frequencies of NK cells expressing each receptor (the "product rule") (56, 58), suggesting that different receptor genes are expressed with a substantial degree of independence. Independent receptor gene expression is consistent with stochastic initiation of receptor gene expression in individual developing NK cells.

While the product rule provides a good estimate for most combinations of Ly49 genes or KIR genes (56, 58, 61), deviations have been noted (84), especially in the co-expression of inhibitory with stimulatory Ly49 receptors. Some combinations of inhibitory Ly49 genes also show minor deviations (T Hanke and DH Raulet, unpublished data). In addition, NK cells co-expressing CD94/NKG2A with certain KIR were underrepresented in panels of human NK clones (58). Similarly, murine NK cells co-expressing CD94/NKG2A and certain Ly49 receptors were marginally underrepresented among murine NK cells (46, 84, 94).

Deviations from the product rule are expected, since education mechanisms exist to adjust the repertoire based on the available MHC molecules, which would be expected to skew the repertoire from a random pattern (95). Moreover, certain receptor genes may exhibit commonalities in regulation, which could also result in preferential co-expression. For example, recent data indicate that the transcription factor TCF-1 may control expression of Ly49A and Ly49D but have little or no role in the expression of other Ly49 genes (96).

To the extent that it has been examined in mice and humans, it appears that the NK repertoire contains cells expressing virtually every possible set of receptors. With estimates that each NK cell can express anywhere from three to seven class I–specific receptors (58, 61), the maximum number of receptor combinations in the repertoire, or its complexity, can be approximated. Humans and mice, each with approximately 10 different inhibitory MHC-specific receptors, possess approximately 1000 different types of NK cells expressing different receptor combinations[1]. It is possible, of course, that many of these combinations would not be found in a given individual, depending on the outcome of the education processes.

Monoallelic Expression of NK Receptor Genes

Ly49 genes exhibit sequence polymorphisms that have made it possible to discriminate expression of two Ly49 alleles at the same locus in Ly49 heterozygous mice. Such analyses have revealed the striking fact that Ly49 genes are expressed in a largely monoallelic fashion (60, 97, 98). That is, most NK cells in a Ly49 heterozygote that express a given Ly49 receptor express either the maternal or the paternal allele of the gene, but not both. This applies to all Ly49 genes examined, including Ly49A, Ly49G2, and Ly49C. Where examined, a smaller, but significant, population of NK cells co-expresses both alleles. Preliminary evidence indicates that the NKG2A inhibitory NK receptor gene is also expressed in a predominantly monoallelic fashion (94). It has not yet been tested whether the KIR genes are expressed in a monoallelic fashion. Monoallelic gene expression had been previously demonstrated in the case of olfactory receptor genes (99) and was subsequently reported for several cytokine genes (100–103). In the case of the cytokine genes, co-expression of both alleles occurs in a fraction of cells, similar to the pattern observed for the Ly49 genes. It is attractive to speculate that monoallelic expression is characteristic of genes encoding molecules that must be expressed in a variegated fashion in order to generate a repertoire of specificities or functions within a given class of cells.

Why are Ly49 genes expressed in a monoallelic fashion? One possibility is that allelic versions of Ly49 receptors typically differ in specificity. Monoallelic expression would then ensure that many NK cells express only one of the specificities. Although some allelic versions of Ly49 receptors may differ in specificity, no such difference was observed for Ly49C alleles, the only case yet examined comprehensively (24). An alternative perspective is that monoallelic expression of NK receptors serves no function per se but is rather the outcome of the specialized

[1]There are 120 ways that an NK cell could express 3 different inhibitory receptors from a pool of 10 receptors. In addition, there are 210 ways that an NK cell could express four of 10 receptors. Similarly, there are 252, 210, and 120 ways that an NK cell could express 5, 6, or 7 receptors from the pool of 10. Thus, there are total of $120 + 210 + 252 + 210 + 120 = 912$ possible cell types in a repertoire of NK cells expressing 3 to 7 receptors from a pool of 10 receptors.

mechanism for selecting a random subset of NK receptor genes for expression in individual NK cells (56, 60). Indeed, data suggest that this mechanism acts locally to stably regulate *cis*-acting control sequences associated with each Ly49 allele, independent of neighboring Ly49 genes or of the Ly49 allele on the opposite chromosome. For example, the fraction of NK cells that co-expresses both Ly49 alleles of a given Ly49 gene roughly approximates (albeit in some cases exceeds) the product rule estimates derived by multiplying the frequencies of NK cells expressing each allele (97, 98) (DM Tanamachi, T Hanke, DH Raulet, unpublished results). These findings suggest that the two Ly49A alleles in a cell are expressed with some independence, presumably because they are chosen for expression by the same stochastic mechanism that selects different Ly49 loci for expression. Furthermore, the choice of a given receptor allele for expression does not influence which allele is expressed at a second Ly49 locus nearby (60). For example, in the case of NK cells that co-express Ly49A and Ly49G2, there is an approximately equal likelihood that the two expressed Ly49 genes will be on the same or different chromosomes (98) (DM Tanamachi, T Hanke, DH Raulet, unpublished results).

The mechanisms that underlie random allelic expression of Ly49 genes are not known. One possibility is that *trans*-acting factors necessary to stably initiate (or possibly repress) Ly49 gene expression are limiting during a stage or stages when the gene expression pattern is established. The factors might be limiting in the sense that only a few are available per cell. Or, they may be limiting in a kinetic sense, such that there is only a modest probability that the factors will succeed in binding the regulatory elements of a given Ly49 allele within a limited time period. One candidate for such a factor is the transcription factor TCF-1. In heterozygous TCF-$1^{+/-}$ mice, only half as many NK cells expressed Ly49A as in wild-type TCF-$1^{+/+}$ mice (96). Since halving the TCF-1 gene dosage lowered the frequency of Ly49A^{+} NK cells, it was plausibly suggested that TCF-1 represents a limiting factor for Ly49A gene expression.

Maintenance of Receptor Gene Expression

The preponderance of evidence suggests that once an NK receptor gene is successfully activated, its expression is stably maintained in the cell, even as it undergoes multiple rounds of proliferation. In the human system, long-term NK clones sustain expression of specific KIR and CD94/NKG2 receptors for many cell generations (57). In mice, sorted populations expressing specific Ly49 receptors maintain receptor expression for at least 10 days after transfer to irradiated recipient mice in vivo (104), and for at least 1–2 weeks of in vitro expansion in IL-2 (19). NK cells expressing CD94/NKG2 receptors maintained expression after transfer in vivo (105). In addition, NK cells from Ly49A heterozygous mice, sorted for expression of one of the two Ly49A alleles, maintained expression of that allele after expansion in culture (97) (DM Tanamachi, T Hanke, DH Raulet, unpublished results). These data argue for stable receptor expression, although they cannot

rule out the possibility that NK cells extinguish receptor expression under certain circumstances in vivo.

Ontogeny of Receptor Gene Expression

Little is known concerning the ontogeny of NK receptor expression in humans. Some data indicate that CD94 and KIR are already expressed by NK cells in the human fetal liver (106) (L Lanier, personal communication).

With one possible exception (107), Ly49 receptors are initially expressed after birth in mice (104, 108), and the proportion of NK cells expressing each Ly49 receptor increases gradually over the first several weeks of life. In contrast, most murine NK cells express CD94/NKG2A at birth (61, 105, 108). Since the CD94/NKG2A receptor is reactive with all known MHC haplotypes, it has been proposed that this receptor prevents most neonatal and prenatal NK cells from attacking self-cells (105, 108). After birth, the proportion of CD94/NKG2A$^+$ NK cells gradually decreases to approximately 50% in the adult. Evidence suggested that the decrease in CD94/NKG2A$^+$ NK cells is not due to loss of the receptor from cells that already expressed it (105). One possibility is that progenitor cells in the adult initiate expression of the NKG2A gene in a variegated fashion, while neonatal and prenatal progenitor cells initiate expression in all cells.

Conditions Inducing Receptor Expression

Several studies have investigated the stimuli that promote receptor expression using in vitro models of NK cell development. Human progenitor cells cultured in vitro with cytokines, particularly IL-15, developed into NK cells, many of which expressed CD94/NKG2A receptors (106, 109, 110). Little or no KIR expression was induced in these cultures, suggesting that additional signals are required for initiation of KIR expression.

Similarly, CD94/NKG2A receptors were expressed by murine NK cells differentiating in vitro under the influence of cytokines (111; see also 112). No initiation of Ly49 gene expression occurred in these cultures. The initiation of Ly49 expression required additional undefined signals from stromal cells, since efficient Ly49 receptor expression occurred when NK cells developed in cultures containing both cytokines and bone marrow stromal cells or the OP9 stromal cell line (113, 114).

Developmentally Ordered Expression of Ly49 Receptor Genes

Evidence suggests that Ly49 receptors are expressed sequentially in a cumulative fashion during development. For example, cells with the NK phenotype (NK1.1$^+$CD3$^-$), but lacking expression of most Ly49 receptors, can initiate expression of Ly49 receptors on a fraction of cells after transfer to host mice (104, 105) or after culture in vitro on bone marrow stromal cells in the presence of cytokines (114). Furthermore, a fraction of NK cells expressing Ly49A or Ly49G2 can initiate

expression of additional Ly49 receptors after transfer in vivo (104) or culture in vitro (114), while maintaining expression of Ly49A or Ly49G2, respectively.

Several studies suggest that there are restraints on the order in which different Ly49 receptors can be expressed during development. The first indication was that transferred Ly49A$^-$NK1.1$^+$ cells never initiate expression of Ly49A, either in vitro or in vivo, but do initiate expression of Ly49G2, Ly49C/I, and Ly49F on a fraction of cells (104, 114). Since Ly49A$^+$ NK cells arose in these experiments when NK1.1$^-$ lymphoid-restricted progenitor cells were employed, it was proposed that commitment to express Ly49A precedes acquisition of the NK1.1 marker, while commitment to express the other receptors can occur after NK1.1 expression. Moreover, Ly49G2$^+$NK1.1$^+$ cells were able to initiate expression of Ly49C/I genes, but not Ly49A, while Ly49A$^+$ NK cells were able to initiate expression of both Ly49G2 and Ly49C/I genes. These data are consistent with at least two models. In one, the capacity to activate Ly49 genes progresses in a sequence, e.g. Ly49A only → Ly49G2 only → Ly49C/I, etc. In the other model, all Ly49 genes are initially available for activation, and the capacity to activate some genes is lost before others, e.g. Ly49A/G2/C/I → Ly49G2/C/I → Ly49C/I. In both models, only a fraction of cells at each stage actually succeed in activating a relevant gene.

Another study was in accord that Ly49 gene expression occurs in an ordered fashion but differed as to the order (115). In this study, early NK-committed progenitors were precultured in cytokines and subsequently cloned at limiting dilution on stromal layers in the presence of cytokines. As determined by RT-PCR of RNA from clones at various days thereafter, Ly49G2 was expressed first, followed by Ly49C/I, and finally by Ly49A. The two studies are in fact not necessarily contradictory because the former study addresses the timing of commitment to express different Ly49 genes, while the latter study addresses the order in which the genes are actually expressed. The relative timing of commitment versus actual expression may vary for different Ly49 genes. Alternatively, the discrepant results may reflect differences in the experimental protocols used or the precursor cells tested.

NK CELL SELF-TOLERANCE

Tolerance to MHC-Identical Cells

That NK cells can mediate alloreactivity was first recognized in studies of bone marrow graft rejection by irradiated mice (11, 116). Many instances of bone marrow graft rejection by NK cells can be attributed to the absence on the graft of MHC molecules present in the host. For example, D8 mice, which are B6 (H-2^b) mice that transgenically express D^d as a self-antigen, reject B6 bone marrow grafts that lack D^d (13). This type of missing self recognition can also account, at least in part, for the rejection of fully MHC-different cells by NK cells. NK cells in H-2^d mice reject H-2^b bone marrow cells, H-2^d NK cells lyse H-2^b target cells in vitro,

and human peripheral blood NK cells can often lyse allogeneic PHA blasts in vitro. It should also be noted that NK cells in B6 mice reject D8 bone marrow (13), indicating that in some instances NK cells can also positively recognize the presence of allogeneic MHC molecules. The latter reaction was recently attributed to an interaction between the stimulatory Ly49D receptor and the D^d class I molecule (117).

While NK cells often attack MHC-different target cells, they exhibit tolerance to MHC-identical target cells. The self-tolerance of NK cells is clearly a property that is acquired in the host, as opposed to being directly inherited. This is implicit in the fact that there is no obvious genetic mechanism to coordinate the inheritance of receptor genes that confer MHC specificity and the MHC genes themselves, which are on a different chromosome. In fact, the alloreactivity of NK cells cannot be attributed to inheritance of specific sets of receptor genes per se, since MHC congenic mice such as B10 and B10.D2 have the same NK receptor genes (20), yet exhibit mutual NK alloreactivity in vivo (118).

NK Cell Tolerance in Class I–Deficient Mice

The control of NK cell self-tolerance by MHC molecules is also evident in studies of MHC class I–deficient mice. In perhaps the clearest example of missing-self recognition, NK cells from normal mice attack bone marrow cells or lymphoblasts from class I–deficient mice (14–16). Interestingly, however, class I–deficient $\beta 2m^{-/-}$ gene targeted mice exhibit no evidence of autoimmunity and do not reject class I–deficient cells (14). An identical phenotype is observed in other class I–deficient mouse models including $TAP^{-/-}$ (119), double mutant $TAP^{-/-}\beta 2m^{-/-}$ (120), and double mutant $K^{b-/-}D^{b-/-}$ (121, 122) mice. Similarly, NK cells from TAP-deficient humans also exhibit reduced activity against class I–deficient target cells (123). The results as a whole indicate that self-tolerance can occur in the complete absence of ligands for any of the known MHC class I–specific receptors. Class I–deficient mice contain normal (15) or even elevated (122) numbers of NK cells, indicating that self-tolerance in these mice does not result from wholesale NK cell deletion, nor does it reflect the failure of NK cells to develop.

Cell Types Mediating Self-Tolerance of NK Cells

Developing NK cells are exposed to neighboring hematopoietic cells as well as various nonhematopoietic stromal cells during development in the bone marrow. Experiments utilizing bone marrow or fetal liver chimeras suggest that encounters with both types of cells contribute to self-tolerance. The presence of class I^- hematopoietic cells in the chimeras, even when the host was class I^+, was sufficient to dominantly induce at least a partial state of tolerance as tested by subsequently challenging the mice with class I^- bone marrow grafts (16, 124). The presence of nonhematopoietic class I^- host cells had an even more substantial effect, inducing nearly complete tolerance (124). These data indicate that encounters with either

hematopoietic or nonhematopoietic class I^- cells can induce tolerance, with the latter cells playing a possibly larger role. Similar results were seen in other experimental systems (125, 126). In one set of experiments using mosaic mice that express D^d as a transgene on only a fraction of cells, as few as 10% D^d-negative H-2^b cells dominantly induced tolerance to H-2^b cells, despite the presence of neighboring D^{d+} cells (126).

The dominant activity of ligand-negative cells in inducing tolerance is significant because it rules out a simple positive selection model of NK cell education. In this model, encounters of NK cells expressing self-reactive inhibitory receptors with ligand-positive cells promote the survival or functional maturation of NK cells, whereas encounters with ligand-negative cells have no effect. The data do not rule out positive selection per se but do indicate that this cannot be the sole mechanism of tolerance induction.

Reversal of Self-Tolerance

A variety of data indicate that self-tolerance of NK cells can be broken in some instances by culturing the cells in high doses of IL-2 (125–127). In one study, NK cells from the D^d-transgenic mosaic mice described above were separated into those that expressed D^d and those that did not, and both populations were cultured in IL-2 for a short period. Within one day of separation, the D^{d+} fraction rapidly acquired the capacity to lyse B6 (H-2^b) target cells, suggesting that tolerance in this system is rapidly reversible (126). An earlier study showed that NK cells from F1 into parent bone marrow chimeras establish tolerance to grafts from the parental host, but that this tolerance was broken after culturing chimeric cells in IL-2 (125). Finally, a recent study showed that culture for four days in IL-2 reverses the self-tolerant state of NK cells from class I–deficient mice (127). The physiological significance of data obtained by culturing cells in abnormally high doses of IL-2 is open to question. The results are significant, however, because they raise a question as to the nature of a tolerant state that can be so rapidly reversed. Significantly, while IL-2 reversed tolerance in each of the experimental models discussed above, it had less (128, 129) or no (126, 130) effect in reversing self-tolerance of NK cells from normal mice. As is discussed below, one possible explanation is that culture in IL-2 reverses self-tolerance of only a fraction of NK cells in normal mice.

The "At Least One" Hypothesis

While there is general agreement that the self-tolerance of NK cells is imposed by a developmental process, the mechanisms involved are still poorly understood. One attractive hypothesis that has been discussed widely in the literature is that the formation of the NK cell repertoire is regulated such that every NK cell in a given individual expresses "at least one" inhibitory receptor specific for one or another self-MHC class I molecule. A process that equips every NK cell with a self-specific receptor would not only account for self-tolerance, it would also

maximize the number of useful NK cells, i.e. those that can attack autologous cells that have downregulated class I molecules.

Evidence in favor of the at-least-one theory came from an analysis of panels of human NK clones from two individuals. Each NK clone derived from a given donor expressed at least one known inhibitory receptor specific for one of the donor's class I MHC molecules, and each clone was inhibited from lysing self-target cells (58). One caveat of the study was that it did not conclusively demonstrate that all of the NK cells originally resident in the donors expressed self-specific receptors. If NK cells exist that do not express self-specific receptors, they may be difficult to grow continuously in culture and so cannot be cloned.

The at-least-one hypothesis suggests that MHC genes should impact the frequencies of freshly isolated NK cells expressing different inhibitory receptors. Such alterations are well documented in mice, where MHC-congenic and MHC-deficient strains can be examined (131, 132), though it has not been shown that all murine NK cells express a self-specific receptor. In contrast, MHC-dependent alterations in KIR expression by freshly isolated human NK cell populations have not been detected (133). While considerable differences in repertoire between individuals were observed, no obvious correlation with MHC allotypes was discerned. Nor was there evidence that the levels of KIR or CD94/NKG2 receptors at the cell surface are affected by the MHC allotype of the host. The failure to observe such correlations in humans is difficult to interpret, since the effects of MHC allotypic differences may be obscured by variability in non-MHC genes and possibly by environmental factors.

In the murine system, some evidence suggests that NK cells with self-MHC-specific inhibitory receptors contribute preferentially to the NK cell pool, a prediction of the at-least-one hypothesis. This evidence was obtained with a transgene that directs expression of Ly49A (specific for H-2^d) in all developing NK cells. Bone marrow cells from transgenic and wild-type mice, both H-2^d, were mixed and inoculated in irradiated H-2^d recipients. The resulting transgenic lymphocytes contained a marginally higher ratio of NK cells to B cells compared to nontransgenic lymphocytes in the same chimeras, or compared to transgenic or nontransgenic lymphocytes in similar mixed chimeras prepared in the H-2^b background (134). The authors concluded that engagement of Ly49A on NK cells enhanced the survival of the cells, their proliferation, or the pace of their development. Another report indicated that NK cells expressing a self-MHC-specific stimulatory receptor (Ly49D in H-2^d mice) usually co-expressed an H-2^d-specific inhibitory receptor (135). A caveat of this finding is that since most Ly49 receptors are H-2^d-specific, most Ly49D$^+$ NK cells co-express H-2^d-specific inhibitory receptors even in H-2^b mice.

Intriguing though these data are, other evidence in the murine system suggests that not all NK cells express self-specific inhibitory receptors. For example, the only known receptors with a clearly defined specificity for H-2^b class Ia molecules are Ly49C, Ly49I, and CD94/NKG2A. Analyses performed by single cell RT-PCR (61) or by staining NK cells with antibodies and/or tetramers against all of

these receptors (59, 94) suggest that approximately 25% of NK cells in B6 (H-2^b) mice do not express any of the known H-2^b-specific receptors. The important caveat remains that other as-yet-undiscovered H-2^b-specific inhibitory receptors may exist. There is no evidence, however, that NK cells lacking Ly49C, I, and NKG2A in H-2^b mice are inhibited by self-MHC class I molecules, suggesting that they do not express novel H-2^b-specific inhibitory receptors (RE Vance, DH Raulet, unpublished data). In conclusion, attractive as the "at least one" hypothesis is, some new data raise doubts about its validity, at least in mice.

Modulation of Cell Surface Ly49 Receptors: Role in Self-Tolerance

It is well established that the cell surface levels of Ly49 receptors are downmodulated by interactions with self-class I molecules. Ly49A, Ly49C, and Ly49G2 are all expressed at lower levels (two to tenfold) in mice that express cognate MHC ligands (130, 131,136–138). It has been proposed that the downmodulation of Ly49 receptors in the presence of the ligand is part of a process that calibrates the reactivity of NK cells with self-MHC ligands (139). The term "calibration" was initially coined to refer to receptor downmodulation but is sometimes broadened to include MHC-dependent changes in the sizes of different Ly49-expressing subsets (see below for discussion of this phenomenon). As is discussed below, however, the two phenomena are mechanistically unrelated and are probably unrelated in their functional role. Here, use of the term calibration refers solely to changes in receptor cell surface levels.

In a common representation of the calibration model, it is proposed that receptor cell surface levels vary so as to create an optimal balance between inhibition and stimulation, such that the resulting NK cell is poised at the brink of reactivity. This would have the related effects of contributing to the establishment of self-tolerance and ensuring the maximal sensitivity of each NK cell to even minor alterations in target cell MHC levels. The idea that alterations in cell surface Ly49 levels calibrate NK cell specificity assumes that such changes demonstrably alter NK cell specificity, and it implies that such changes should tend toward an optimal level. Since the KIR (133) and CD94/NKG2 receptors (RE Vance, DH Raulet, unpublished data) have not been generally observed to undergo downmodulation in the presence of MHC ligands, this form of receptor calibration presumably does not apply to these receptors.

To address whether changes in Ly49 cell surface levels alter NK cell sensitivity to class I ligands, NK cells from MHC-different mice were compared. These NK cells express different levels of Ly49 receptors. NK cells expressing high Ly49 levels were more readily inhibited by target cell class I molecules (121, 140). It is plausible that the functional differences in these studies were due to altered cell surface levels of Ly49 receptors, but other causes were not ruled out. For example, the NK cells compared are expected to differ in the number of different Ly49 receptors expressed (see below), which could also alter the sensitivity of the

cell to class I–mediated inhibition. Another possibility is that the differences in sensitivity reflect stable alterations in stimulatory receptor pathways.

While receptor levels may impact the sensitivity of NK cells to class I inhibition, some evidence suggests that Ly49 cell surface levels do not tend toward an optimum level. Using Ly49A transgenic mice, it was observed that the cell surface expression of a Ly49A transgene, like that of the endogenous Ly49A gene, was downmodulated in H-2^d mice, which (unlike H-2^b mice) contain a ligand for Ly49A (141, 142). A Ly49A transgenic mouse that expressed "normal" levels of Ly49A in the H-2^b background was compared to a Ly49A transgenic that expressed threefold lower levels of Ly49A. When the transgenes were crossed into an H-2^d background, both the high- and low-expressing lines exhibited a threefold reduction in levels of cell-surface Ly49A, due to the presence of H-2^d (141). These data suggested that Ly49 downmodulation is a generic response to ligand engagement and does not tend toward an optimal setpoint. Moreover, Ly49 receptor levels are not a stable property of mature NK cells. Thus, transfer of mature NK cells from mice lacking a Ly49A ligand to mice expressing the D^d ligand resulted in a very rapid downmodulation of Ly49A (143).

A likely possibility is that Ly49 downmodulation reflects the rapid internalization of receptor after ligand-engagement, as occurs with many other receptors. Consistent with this hypothesis, receptor downmodulation is independent of transcription (141). Moreover, MHC-dependent downmodulation of transgenically expressed Ly49A was clearly apparent in cells that normally do not express the receptor, such as B cells (DM Tanamachi, DH Raulet, unpublished data). These findings suggest that modulation of receptor levels can occur independently of NK cell development and is not a consequence of it. Of course, tolerance-inducing mechanisms would need to take into account any alteration in the sensitivity of NK cells to MHC ligands that resulted from receptor modulation.

Self-Tolerance of NK Cells Lacking Self-MHC-Specific Inhibitory Receptors

As already discussed, class I–deficient mice contain normal numbers of NK cells that are not autoaggressive, arguing that NK cells need not express self-MHC-specific receptors to achieve self-tolerance (15). As mentioned, evidence suggests that normal mice may also contain a class of NK cells that do not express self-MHC-specific receptors. An important finding is that several effector functions are impaired in NK cells from class I–deficient mice, compared to those of normal mice. Equally significant is that the impairment is not absolute. For example, NK cells from class I–deficient mice exhibited reduced but not absent antibody-dependent cellular cytotoxicity (ADCC) (120) and reverse-ADCC (redirected lysis) (M-F Wu, DH Raulet, unpublished data) activities. In addition, NK cells from class I–deficient mice usually exhibited a reduced capacity to attack YAC-1 tumor target cells, though this phenotype is variable and was not observed in all experiments (15, 16, 121). The picture that emerges is that NK

cells from class I–deficient mice are essentially devoid of activity against class I–deficient normal cells, and they exhibit reduced functional activity in several other assays. NK cells with defects in components of the inhibitory signaling pathway, such as the SHP-1 tyrosine phosphatase, may also acquire a hyporesponsive phenotype (144). Thus, hyporesponsiveness may result either from the absence of class I ligands or from impaired signaling by class I–specific inhibitory receptors.

As discussed above, NK cells from class I–deficient mice cultured in high doses of IL-2 attain the capacity to lyse autologous lymphoblasts, indicating that these conditions reverse the hyporesponsive state (127). In contrast, similar cultures of unseparated NK cells from class I^+ mice are less affected (126, 128–130), and cultures of sorted NK cells expressing known self-specific inhibitory receptors retain the self-tolerant state (129, 130, 145). These findings are consistent with the notion that NK cells in normal mice are a mixture of two types of cells, those that express self-MHC-specific inhibitory receptors and those that lack self-MHC-specific receptors and are hyporesponsive. Presumably, culture in IL-2 reverses self-tolerance of only the latter set.

At least three general models can be considered for the self-tolerance of NK cells from class I–deficient mice. One model proposes that stimulatory signal transduction pathways, or even the stimulatory receptors themselves, may be downregulated or dampened in NK cells that have never encountered cognate class I ligands (120, 121). While at least some stimulatory receptors such as NKR-P1C (NK1.1 antigen, (15)) and Ly49D (T Hanke, DH Raulet, unpublished data) are expressed at normal levels on NK cells in class I–deficient mice, this may not be true for other stimulatory receptors or for relevant signaling molecules. A second related model is that the inhibitory signal transduction pathway may exhibit a basal level of activity even in the absence of ligands; stable elevation of this basal activity in the NK cells that develop in class I–deficient mice could swing the balance in favor of nonresponsiveness. A third model is that NK cells can express undiscovered inhibitory receptors specific for unidentified non-MHC ligands (120). Such receptors may be preferentially expressed by NK cells that have never encountered class I ligands, accounting for the failure of these cells to attack normal class I–deficient target cells.

MECHANISMS TO MINIMIZE RECEPTOR CO-EXPRESSION MAXIMIZE NK CELL FUNCTIONALITY

Several lines of evidence indicate that the receptor repertoire of NK cells, in terms of the frequencies of NK cells expressing different receptors, is impacted by MHC class I molecules expressed by the host. For example, in $\beta 2m^{-/-}$, $TAP^{-/-}$, or $\beta 2m^{-/-}TAP^{-/-}$ mice, the frequencies of NK cells expressing each of several Ly49 receptors were marginally higher, and the frequencies of NK cells co-expressing various receptor pairs or trios were substantially higher (120, 131, 132)

as compared to class I^+ mice. Thus, the most pronounced effect of MHC class I expression is in limiting the extent of Ly49 receptor overlap.

It is important to emphasize that the alterations in frequencies of Ly49-defined NK cell subsets discussed here are accomplished by a completely different mechanism than the previously discussed alterations in cell surface Ly49 levels. Receptor cell surface levels adapt very rapidly to the presence of cognate ligands and are controlled by a posttranscriptional process. In contrast, receptor expression per se is controlled at the level of mRNA (97, 98, 141), presumably transcription, and appears to be a stable developmentally regulated property of NK cells.

Studies with Ly49 transgenic mice suggested that the effect of MHC molecules on the Ly49 repertoire is to limit the number of NK cells co-expressing multiple Ly49 receptors specific for self-MHC. A transgene encoding the Ly49A receptor was expressed early in the developmental pathway in all NK cells. The transgene had no effect on the endogenous repertoire in mice that lacks ligands for Ly49A. In H-2^d mice, which express the D^d Ly49A ligand, the transgene caused a substantial reduction in the frequency of NK cells expressing Ly49G2, endogenous Ly49A (141), and some other Ly49 receptors (T Hanke, DH Raulet, unpublished data). Recent experiments demonstrate that a Ly49G2 transgene exerts a similar effect on the expression of endogenous Ly49 receptors (T Hanke, DH Raulet, unpublished data). Thus, expression of one receptor specific for self-MHC reduces the probability that other self-specific receptors will be expressed.

Similar conclusions were obtained in in vitro experiments using a clonogenic system for NK cell development. When Ly49A transgenic NK precursors differentiated on class I–deficient bone marrow stromal cell layers, most of the resulting clones contained $Ly49G2^+$ NK cells. When the same precursors differentiated on H-2^d stromal cells (displaying a Ly49A ligand), many fewer clones contained $Ly49G2^+$ NK cells (114). These data suggest that during NK cell development, Ly49 interactions with MHC molecules on stromal cells act to minimize co-expression of self-specific Ly49 receptors.

Why should the co-expression of multiple self-MHC-specific receptors by NK cells be disfavored? An attractive possibility is based on the finding that MHC class I loss events are often selective, in that cells may lose one class Ia molecule and not another. For example, HIV-infected cells reportedly downregulate HLA-A and HLA-B molecules, but not HLA-C molecules (55). Rather than downregulating all class I expression, tumor cells often extinguish expression of just one or another class Ia gene, or even one or another class Ia allele in the case of MHC heterozygotes (17). NK cells that co-express receptors specific for different self–class I molecules may be unable to attack self-cells that have lost just one. Minimizing co-expression of self-specific receptors could also maximize the sensitivity of NK cells to small changes in the cell surface levels of host cell class I molecules.

At least two mechanistic models can account for the restricted co-expression of self-MHC-specific receptors by NK cells. In a selection model, NK cells that express “too many” self-MHC-specific receptors would die or fail to proliferate.

An alternative, adaptive model was suggested by the finding that NK cells acquire expression of Ly49 receptors in a sequential and cumulative fashion. It was proposed that this process is regulated by Ly49–class I interactions (56). If an NK cell expresses only nonself class I–specific receptors, it is free to activate additional receptor genes. If it expresses a sufficient number of self-MHC-specific receptors, however, subsequent rounds of receptor gene initiation would be inhibited. For example, in the Ly49A transgenic mice, engagement of the transgenic receptor inhibits subsequent initiation of other NK receptor genes, such as Ly49G2. This "regulated-sequential" model predicts that early engagement of Ly49 receptors, as in the Ly49A transgenic H-2^d mice, will inhibit expression of both self-specific and nonself-specific Ly49 receptors. The selection model predicts that only cells expressing self-specific endogenous receptors will be affected (95). Consistent with the regulated-sequential model, data indicate that utilization of both self-specific and nonself-specific receptors is diminished in the Ly49A transgenic mice (T Hanke and DH Raulet, unpublished data).

INTEGRATION OF STIMULATORY AND INHIBITORY SIGNALING IN NK CELL DEVELOPMENT

Since class I–deficient but otherwise normal cells are sensitive to NK cells, some stimulatory ligands must be constitutively expressed by at least some normal cells. Furthermore, certain class I–specific stimulatory receptors (e.g. KIR2DS, KIR3DS, Ly49D, CD94/NKG2C) are expressed in a variegated fashion by different NK cell subsets. Thus, depending on which stimulatory receptors are expressed, and whether the host animal happens to express ligands for MHC-specific receptors, the summed autostimulation received by individual NK cells is expected to vary. These considerations lead to the question: How are the stimulatory and inhibitory signals that emanate from self-cells integrated to ensure self-tolerance of individual NK cells?

Coordination of stimulatory and inhibitory signals can be easily accounted for in a selection model of NK cell tolerance, assuming that selection is delayed until after the cell has initiated expression of all relevant receptors. When the NK cell eventually interacts with self "selecting cells," the outcome of selection (i.e. cell death, survival, and/or proliferation) would be determined by the balance of stimulatory and inhibitory signaling that the NK cell receives. However, as discussed above, several lines of evidence suggest that NK cell tolerance is, at least in part, an adaptive process.

How could stimulatory and inhibitory signaling be balanced in an adaptive process? Several mechanisms can be envisaged: (*a*) One possibility is that the first receptors expressed by developing NK cells are stimulatory receptors. At this stage, the cells would presumably be immature and nonlytic. The engagement of self-ligands by these stimulatory receptors would then transmit signals that induce the de novo expression of inhibitory receptors, which are expressed in

a sequential and cumulative fashion. When the cell eventually expresses one or more self-MHC-specific inhibitory receptors of sufficient strength to overcome the stimulatory signal, induction of additional receptors would be inhibited. At this point, the NK cell could mature fully by upregulating its sensitivity to stimulatory ligands. In encounters with self-cells, however, inhibition would just counteract stimulation, preventing autoaggression. (*b*) A converse mechanism is that NK cells express inhibitory receptors before they express stimulatory receptors. NK cells would then be permitted to initiate expression of stimulatory receptor genes to match the level of inhibition provided by the inhibitory receptors. (*c*) In a third model, these mechanisms may be combined. NK cells would ratchet up their stimulatory and inhibitory receptors in concert, such that the expression of an inhibitory receptor stimulates the expression of a stimulatory receptor, which in turn stimulates the expression of another inhibitory receptor. (*d*) A fourth model balances not the number or strength of each type of expressed receptor, but the efficiency of the relevant signal transduction pathway. Depending on the number and strength of inhibitory and stimulatory receptors expressed by each NK cell, the efficiency of the stimulatory and/or inhibitory signal transducing pathways could be stably adjusted to come into balance. Only then would the NK cell mature.

The available data does not allow these possibilities to be readily discriminated. It is unclear, for example, whether stimulatory or inhibitory receptors are generally expressed in a specific order relative to each other in NK cell development. A recent ontogenic analysis shows that the stimulatory Ly49D and Ly49H receptors are expressed on NK cells at about the same time after birth, or just slightly delayed, compared to the inhibitory receptors (84). Since new precursors are continuously differentiating, however, the ontogenic pattern does not necessarily reflect the order in which receptors are expressed on individual developing NK cells. It also remains possible that some stimulatory receptors, for example those specific for non-MHC ligands, are expressed early in the differentiation process, whereas the MHC-specific stimulatory receptors are expressed later. Consistent with the idea that some stimulatory receptors are expressed early in ontogeny, if not in development, fetal NK cells exhibit lytic activity against tumor targets (107, 146). Moreover, Fc receptors and the NKR-P1C stimulatory receptor are both expressed early in NK cell ontogeny (147, 148).

Some evidence supports the conclusion that stimulatory signals induce the expression of inhibitory receptors on developing NK cells. Mice deficient in the *src*-family tyrosine kinase *fyn* tend to express fewer inhibitory Ly49 receptors than do *fyn*-positive mice (W Held, personal communication). Since the *fyn* kinase is involved in lymphocyte activation, as opposed to inhibition, one interpretation of the data is that stimulatory signals promote de novo expression of inhibitory receptors. The data are also consistent with a selection model, in which developing NK cells that receive excess stimulation are selected against. If stimulatory signaling is partly impaired, more NK cells with fewer inhibitory receptors would survive the selection process.

THE INHIBITORY RECEPTOR REPERTOIRE EXPRESSED BY T CELLS

Although the inhibitory MHC class I-specific receptors are often described as NK receptors, each type is also expressed by fractions of various T cell subsets. Unlike NK cells, relatively few human and mouse T cells express these receptors. The role of the inhibitory receptors in the immune response of T cells has not been clearly established. A comparison of NK cells and T cells in terms of their repertoire of inhibitory receptors is nevertheless worthwhile, as it may hint at the underlying shared mechanisms in repertoire formation and function.

Phenotype of T Cells Expressing Inhibitory Class I–Specific Receptors

Individual KIR and CD94 receptors are normally expressed by up to 5% of peripheral blood T cells (149–154). Although CD4 T cells with KIR could be detected and cloned (150), the vast majority of KIR^+ T cells are $CD8^+CD4^-$ cytotoxic T lymphocytes (CTL). A corresponding population has been described in mice. Approximately 10% of murine $CD8^+TCR\alpha\beta^+$ T cells express at least one of the inhibitory Ly49 receptors, while expression of these receptors on conventional CD4 T cells is very rare (155, 156). A significant proportion of the $CD8^+$ $Ly49^+$ T cells also express other NK markers, including CD94/NKG2A, NKR-P1C, and the DX5 antigen (155, 156) (CW McMahon, RE Vance, DH Raulet, unpublished data).

NK receptor expression is also prominent in several nonconventional T cell populations: $TCR\gamma\delta$ T cells, intraepithelial lymphocytes, and the CD1d-restricted T cells often called NK T cells. In adult humans, the majority of peripheral $\gamma\delta$ T cells bear $V\gamma9V\delta2$ TCR that recognize nonpeptide phosphoantigens in an MHC-independent manner. The various KIR are expressed by 1%–20% of circulating $V\gamma9V\delta2$ T cells, and the CD94/NKG2A receptor is expressed by about 80% of $V\gamma9V\delta2$ cells (157–161). CD1d-restricted T cells are a unique population of $CD4^-CD8^-$ or $CD4^+CD8^-$ $TCR\alpha\beta^+$ T cells in mice and humans that recognize lipid antigens presented by the nonclassical class I molecule CD1d (162). In mice, these cells usually express NKR-P1C and often express Ly49 family members. Notably, however, KIR was rarely expressed in a panel of human CD1d-restricted T cell lines, although NKR-P1A was expressed on essentially all such cells (163). It remains to be established whether human CD1d-restricted T cells in vivo express KIR.

In general, T cells that express inhibitory class I–specific receptors exhibit the phenotypic markers of memory T cells. In humans, KIR^+ CTL bear surface markers indicative of previous activation (CD45RO, CD29, CD44, CD18, CD57), and they lack markers for naïve T cells (CD45RA) (152). Likewise, murine $Ly49^+$ CTL express Ly6C and CD122 (IL-2Rβ) and are $CD44^{hi}$ (156). Many of these memory markers are also expressed on KIR^+ $\gamma\delta$ T cells, CD1d-restricted T cells, and NK cells, suggesting that prior and/or chronic activation may underlie inhibitory receptor expression by these cells.

Specificity of T Cells Expressing Inhibitory Receptors

Inhibitory receptors cannot be detected on the majority of human CTL clones, but KIR^+ and $CD94/NKG2A^+$ CTL specific for HIV or melanoma antigens have been documented (164–169). Interestingly, many of the KIR^+ T cells in humans are present as expanded oligoclonal populations (152). Oligoclonal $CD8^+$ T cell populations have been proposed to represent T cells responsive to persistent antigens or self-antigens (170). In mice, by contrast, the $CD8^+Ly49^+$ population is apparently not oligoclonal, since it exhibits a near normal TCR $V\beta$ distribution and is fairly consistent in size in individual animals of a given strain (156, 171). The mice studied, however, were somewhat younger than the age when oligoclonal $CD8^+$ T cell populations typically become detectable in mice (172). Whether oligoclonal murine $CD8^+$ T cell expansions express Ly49 receptors has not been reported, and it remains unclear whether the $CD8^+Ly49^+$ and $CD8^+KIR^+$ populations are strictly analogous.

It is perhaps significant that the nonconventional T cell subsets that express inhibitory receptors are thought to be specific for self-antigens. These include the $V\gamma9V\delta2$ population, specific for self-phospho-antigens, and the $TCR\alpha\beta^+$ CD1d-restricted T cells, specific for lipid antigens presented by CD1d. Notably, only some $V\gamma9V\delta2$ T cell clones were found to be overtly self-reactive, as defined by the capacity to lyse class I–deficient Daudi cells; the Daudi-reactive clones were much more likely to express CD94/NKG2A receptors than were clones that didn't lyse Daudi targets (158). These findings raise the possibility that inhibitory receptors on T cells may function in some cases to prevent uncontrolled autoreactivity, which is consistent with the proposal that inhibitory receptor expression by T cells is generally a response to persistent antigenic stimulation.

Function of Inhibitory Receptors on T Cells

Several methods have been used to demonstrate that class I–specific inhibitory receptors can functionally inhibit both cytolysis and cytokine secretion by human $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ CTL clones (149, 150, 158, 159, 165, 166, 168, 173–177). For more detailed reviews, see (178, 179). All of these studies utilized CTL clones (or in some cases, cultured lines), and whether freshly isolated cells behave in a similar manner remains to be determined.

In mice, studies with Ly49-transgenic mice indicate that these NK receptors can inhibit alloantigen-induced and antiviral responses by conventional T cells (24, 180, 181). However, CTL that naturally express Ly49 receptors appear to be less sensitive to inhibition, as Ly49 expressed on CTL isolated from normal mice was found to inhibit only early activation events, but not target cell killing or cytokine release (156). Likewise, TCR-mediated stimulation of Jurkat T cells transfected with Ly49G2 (182) and primed CTL from mice expressing a KIR transgene (183) did not appear to be attenuated by inhibitory receptor cross-linking. It is not clear whether the failure to observe strong inhibition in the latter experiments reflects the relatively low cell surface level of Ly49 receptors on T cells,

the use of nonphysiological T cell triggering assays, or a difference in the relevant signal transduction pathways between naïve T cells and memory T cells. There is evidence that murine CD1d-restricted T cells can be inhibited via Ly49 engagement, as antigen-independent target lysis by NK1.1$^+$ Ly49$^+$ T cells cultured from the liver is reduced by Ly49 cross-linking (155).

Stage at Which Inhibitory Receptors Are Expressed by T Cells

Much evidence suggests that expression of inhibitory receptors by T cells typically occurs during or after activation of fully mature T cells. One indication, as previously noted, is that T cells that express inhibitory class I–specific receptors bear surface markers indicative of previous activation. In addition, recently matured conventional CD4$^+$ and CD8$^+$ T cells in the thymus do not express inhibitory receptors, nor do cord blood T lymphocytes from newborn humans (152, 153) or T cells with a naïve phenotype in the periphery of adult mice or humans (156). Additional evidence with fetal liver chimeric mice demonstrated that Ly49 receptor expression by CD8$^+$ T cells is dependent on expression of class I molecules by hematopoietic cells, suggesting that inhibitory receptor expression requires class I antigen presentation (156). Furthermore, in TCR transgenic mice raised in the absence of antigen, T cells expressing the TCR transgene were notably devoid of Ly49 receptors (156). It is more difficult to establish the stage at which inhibitory receptor expression occurs on CD1d-restricted T cells or Vγ9Vδ2 T cells, but it is perhaps telling that both types of cells are responsive to self-antigens and exhibit a memory or activated phenotype. Together, the data suggest that upregulation of inhibitory receptors occurs only after encounter with antigen, at least with respect to CD8$^+$ T cells, and possibly more generally.

In the case of the CD94/NKG2A receptor, upregulation on mature T cells has been demonstrated directly. Both IL-15 and TGF-β induce CD94/NKG2A expression on a large fraction of human CD8 T cells undergoing antigenic stimulation in vitro (109, 184). Interestingly, KIR are upregulated very poorly under the same conditions. In mice the CD94/NKG2A receptor is expressed on essentially all in vitro–stimulated CD8$^+$ T cells, T cell clones, and LCMV-specific CTL isolated from mice at the peak of infection (AJ Zajac, CW McMahon, R Ahmed, DH Raulet, unpublished data). Little Ly49 expression is observed under any of these conditions. These data suggest that CD94/NKG2A receptors can be expressed rapidly by mature CD8$^+$ T cells of any specificity, whereas expression of Ly49 and KIR is limited to special conditions or antigen specificities.

Stability and Overlap of Inhibitory Receptor Expression by T Cells

Little direct analysis has been done of the stability of KIR or Ly49 expression by T cells, though KIR$^+$ clones and sorted cells reportedly maintain receptor expression in culture (57, 185). In one study, it was observed that fewer

TABLE 1 Overlapping expression of Ly49 family members on murine $CD8^+$ CTL[a]

	Ly49G2 [7.2, 61.0][b]		Ly49C/I [3.2, 27.1]		Ly49F [8.7, 73.7]	
	% of $CD8^+$	% of $Ly49^+CD8^+$	% of $CD8^+$	% of $Ly49^+CD8^+$	% of $CD8^+$	% of $Ly49^+CD8^+$
LY49A [3.7, 31.3]	2.4[c] (0.27)[d]	20.3 (19.1)	0.7 (0.12)	5.9 (8.5)	2.6[c] (0.32)[d]	22.0 (23.1)
LY49G2 [7.2, 61.0]			1.3 (0.23)	11.0 (16.5)	4.9 (0.63)	41.5 (45.0)
LY49C,I [3.2, 27.1]					1.8 (0.28)	15.3 (20.1)

[a]Analysis by three color staining of nylon wool passed splenocytes pooled from three B6 mice, using JR9-318 (Ly49A), 4D11 (Ly49G2), SW5E6 (Ly49C and I), and HBF-719 (Ly49F) mAbs. Unpublished data of CW McMahon and DH Raulet.

[b]The numbers in brackets refer to cells expressing the indicated Ly49 as a percentage of $CD8^+$ T cells (first number) or of $Ly49^+$ $CD8^+$ T cells (second number). $Ly49^+$ $CD8^+$ cells are defined as the $CD8^+$ population that stains with a mixture of all the Ly49 mAbs listed (and therefore expresses one or more Ly49 receptors).

[c]Percentage of cells that co-express the two indicated Ly49 family members.

[d]Percentage of cells predicted by the product rule to co-express the two indicated Ly49 receptors.

CD1d-restricted $NK1.1^+$ T cells in the liver express Ly49 receptors compared to those in the thymus (186). The authors proposed that Ly49 expression is extinguished on many of the thymic CD1d-restricted T cells before or shortly after they migrate to the liver. It has not been ruled out, however, that the liver is populated by a selected set of thymic CD1d-restricted T cells, which is relatively deficient in $Ly49^+$ cells. The stability of CD94/NKG2A expression was investigated in mice that had cleared an LCMV infection months earlier. Although most of the cells still expressed detectable CD94/NKG2, the cell surface levels were substantially lower than at the peak of the response (AJ Zajac, CW McMahon, R Ahmed, DH Raulet, unpublished data). Whether activated $CD8^+$ T cells ever completely extinguish CD94/NKG2A expression is uncertain.

T cells, like NK cells, express inhibitory receptors in a variegated fashion, with extensive overlap in the expression of different receptors. The frequencies of human T cells expressing each KIR varies, and individual KIR^+ T cells often co-express additional KIR, ILT2/LIR-1, or CD94/NKG2 receptors (151–153, 169). Similar variegated overlapping expression of Ly49 receptors can also be observed on murine $CD8^+$ T cells (Table 1). On the other hand, some intriguing differences were noted between the pattern of receptor expression in NK cells and T cells. First, some receptors are expressed at different relative frequencies in the two populations. For example, Ly49F is one of the least frequently expressed inhibitory receptors among murine NK cells, but it is the most commonly expressed receptor among $CD8^+$ T cells (156) (Table 1). Furthermore, in mice, the stimulatory Ly49 isoforms (Ly49D and Ly49H) cannot be detected on CTL (84, 155, 156); however, human T cells reportedly do express stimulatory KIR (149, 154, 187). Second, as a fraction of all $CD8^+$ T cells, or even of memory ($CD44^{hi}$) $CD8^+$ T cells,

the frequencies of cells co-expressing pairs of Ly49 receptors are higher than predicted by the product rule (Table 1). The product rule assumes that all cells in the studied population have the potential to express each receptor. A higher than expected overlap can be explained if stochastic Ly49 expression is allowed in only a subset of the population examined. Indeed, if one assumes that this subset can be defined as the population that expresses at least one of the five receptors tested in Table 1, the product rule predicts the overlap of different receptors with reasonable accuracy (Table 1).

Influence of MHC on the Receptor Repertoire

A key issue regarding the expression of inhibitory receptors by T cells is whether MHC molecules shape the repertoire, as has been observed for NK cells. Thus far, it has been difficult to observe alterations in the inhibitory receptor repertoire among T cells in normal mice that can be attributed to MHC molecules. The data on this point are sparse, however, and further investigation is warranted. Interestingly, it is reported that mice that express transgenes for both a human inhibitory KIR and its HLA ligand accumulate an abnormally large number of memory phenotype CD8 T cells (S Ugolini, E Vivier, personal communication). This observation has led to the hypothesis that class I binding by inhibitory NK receptors on CTL may encourage the formation or maintenance of memory cells. If so, it might be predicted that memory $CD8^+$ T cells would be selectively enriched for expression of self-MHC-specific inhibitory receptors. This possibility has not been rigorously tested. Also untested is whether mechanisms operate in T cells to minimize co-expression of self-MHC-specific inhibitory receptors, as observed in the case of NK cells.

SUMMARY: How to Generate a Functional and Self-Tolerant NK Repertoire

Although many fundamental questions remain, many features of the NK repertoire are beginning to emerge. In principle, the mechanisms that regulate the NK repertoire should ensure that NK cells are self-tolerant (nonresponsive to normal self cells) and maximally useful (responsive to abnormal or missing self). The following is a summary of some of the key findings from the disparate material discussed in the body of the review. At the same time, it is useful to put the results in the context of a model. Therefore, while there is currently little consensus on the broad underlying mechanisms, one speculative view of some of the key processes is presented below.

In adult animals, NK precursors are believed to arise in the bone marrow. Once committing to the NK lineage, these early precursors undergo a process of NK receptor acquisition, which is promoted by cytokines such as IL-15 and undefined signals from stromal cells. It appears that many inhibitory receptors are specific for MHC class I, whereas stimulatory receptors recognize a broader range of ligands

including MHC molecules, non-MHC ligands that are upregulated on transformed or stressed cells, and constitutively expressed non-MHC self-ligands.

Importantly, both inhibitory and stimulatory receptors specific for MHC class I are distributed among NK cells by a stochastic mechanism that operates independently on each receptor gene allele. Consequently, the genes are expressed in variegated fashion and are predominantly monoallelically expressed, at least in the case of Ly49 and NKG2A genes. The pattern of inhibitory receptor expression by T cells is also consistent with a stochastic process, though clear qualitative differences have been noted between the inhibitory receptor repertoires of T cells and NK cells. In contrast to the MHC-specific receptors, several of the non-MHC-specific stimulatory receptors (e.g. NKRP1C, NKG2D, NKp46) appear to be expressed by all or nearly all NK cells. Some data suggest that expression of non-MHC-specific stimulatory receptors is an early event in NK cell development. On the other hand, MHC-specific stimulatory receptors in mice, such as Ly49D, appear at a similar or even delayed time in ontogeny as inhibitory Ly49 receptors.

Repertoire Formation as a Zero-Sum Game

An interesting possibility is that acquisition of inhibitory receptors occurs in NK cells that are receiving a net stimulatory signal from interactions with self-cells (Figure 2). Studies of mice harboring a mutation in a stimulatory signaling pathway ($fyn^{-/-}$) are consistent with this possibility. In addition, it would parallel the situation for $CD8^+$ T cells, where several lines of evidence suggest that stimulation through the T cell receptor promotes the acquisition of inhibitory receptors. In the case of NK cells, stimulation at the early stages of development might be mediated by receptors specific for non-MHC self-ligands. Subsequent expression of other stimulatory receptors reactive with self-ligands, such as self-MHC-specific stimulatory receptors, might provoke additional rounds of inhibitory receptor expression (see Figure 2). This mechanism would tend to counteract the expression of stimulatory receptors, pushing the cell toward a "zero-sum" balance between stimulation and inhibition (depicted as the diagonal line in Figure 2). Conversely, it is also possible that an excess of self-specific inhibitory receptors promotes expression of stimulatory receptors, but there are no data on this possibility.

Evidence indicates that acquisition of inhibitory MHC-specific receptors occurs in a sequential fashion. Acquisition of the CD94/NKG2A inhibitory receptor may be an early event, but only approximately 50% of NK cells in the adult express this receptor. In mice, Ly49 receptor expression is apparently a relatively late event, and there appears to be an order in which different Ly49 family members have the potential to be expressed. Expression of a given receptor gene allele is quite stable once initiated. Over time, these processes establish the complex combinatorial repertoire of NK cells, with each cell expressing multiple inhibitory (and stimulatory) specificities.

Evidence indicates that co-expression of multiple self-MHC-specific inhibitory receptors by NK cells is minimized. This finding is readily explained by the proposal that initiation of inhibitory receptor gene expression occurs only when NK

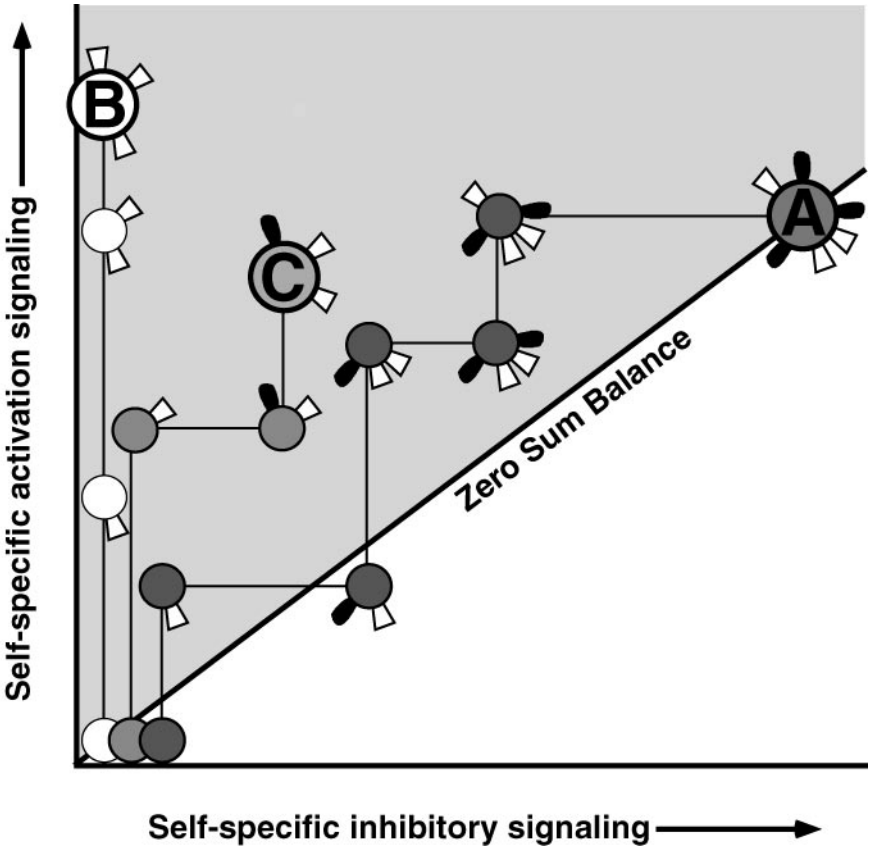

Figure 2 Model for how stimulatory and inhibitory interactions are integrated during the receptor acquisition phase of NK cell development. In this model, the expression and subsequent signaling by stimulatory receptors (white) drives the expression of inhibitory receptors (black). The shaded section indicates a net stimulatory signal, which provokes de novo expression of inhibitory receptors. Receptors are acquired stochastically until the process is terminated by unknown factors. Cell "A" has achieved balance in the amplitudes of positive and negative signals (influenced by the number of receptors, ligand affinities, and other factors) and is therefore self-tolerant and maximally sensitive to changes in host cell class I MHC expression. Cell "B" lacks self-specific inhibitory receptors (it may express non-self-specific inhibitory receptors); these cells are rendered hyporesponsive by an unknown mechanism. Cell "C" has both stimulatory and inhibitory self-specific receptors but has not attained a zero sum balance of signaling; subsequent fine tuning mechanisms may conceivably bring the cell into balance. Not depicted in the Figure is the possibility that some cells may express inhibitory receptors before stimulatory receptors.

cells receive a net stimulatory signal (Figure 2). Expression of one self-MHC-specific inhibitory receptor would at least partially counteract stimulatory signals, decreasing the likelihood of subsequent rounds of de novo expression of inhibitory receptors. A benefit of this proposed mechanism is that it imparts to NK cells a greater capacity to attack self-cells that have extinguished expression of only one of the several class Ia molecules normally expressed in an individual. In addition, since the process would tend toward an approximate balance of stimulation vs inhibition in many NK cells, the population as a whole would be more sensitive to changes in MHC class I levels.

Importantly, there appears to be an upper limit to the average number of inhibitory receptors that NK cells can express. Even in class I–deficient mice, where Ly49 and CD94/NKG2A receptors cannot engage ligands and counteract stimulatory signals, only a subset of all the receptor genes are expressed in each NK cell (albeit a greater average number than in normal mice). The basis for this limit remains uncertain. One possibility is that NK cells are only permitted to acquire receptors for a limited period of time during their development. Alternatively, the factors required to initiate expression of these receptor genes may be present in limiting quantities within the developing NK cell.

NK cells are clearly alloreactive but self-tolerant, and a major current challenge is to understand how self-tolerance is achieved. The zero-sum model proposed above, if allowed to continue to completion, would ultimately result in each NK cell expressing at least one self-MHC-specific inhibitory receptor to counteract stimulatory signals. The resulting NK cells would presumably be self-tolerant because stimulation and inhibition by self-ligands would be balanced. However, although the data are not definitive, some evidence suggests that a significant number of NK cells in normal mice do not express self-MHC-specific receptors. Furthermore, in the complete absence of MHC class I–mediated inhibition, as in class I–deficient mice, NK cells clearly achieve self-tolerance. It can be proposed that some NK cells in normal mice never succeed in expressing self–class I specific receptors, possibly because of the stochastic nature of the receptor acquisition process and the upper limit in the number of receptors that individual NK cells can express. These uninhibited cells may achieve self-tolerance by downregulating either the stimulatory receptors specific for self-ligands or components of the corresponding stimulatory signal transduction pathways. The adoption of this hyporesponsive state may occur either during the receptor acquisition process itself or, alternatively, during a later phase of NK development. In fact, it is plausible that the developing NK cells start out in a hyporesponsive state, only acquiring a heightened level of stimulatory sensitivity when inhibitory receptors are subsequently expressed and engaged. Regardless of the specific mechanism, it is important to emphasize that these cells are not predicted to be useless, as the NK cells in class I–deficient mice retain some capacity to attack tumor cells and certain other NK target cells. Perhaps the hyporesponsive state applies preferentially to stimulatory receptors that are self specific as opposed to tumor cell specific.

Together, the receptor acquisition and education processes would establish different sets of useful NK cells. Figure 3 summarizes the discriminatory properties

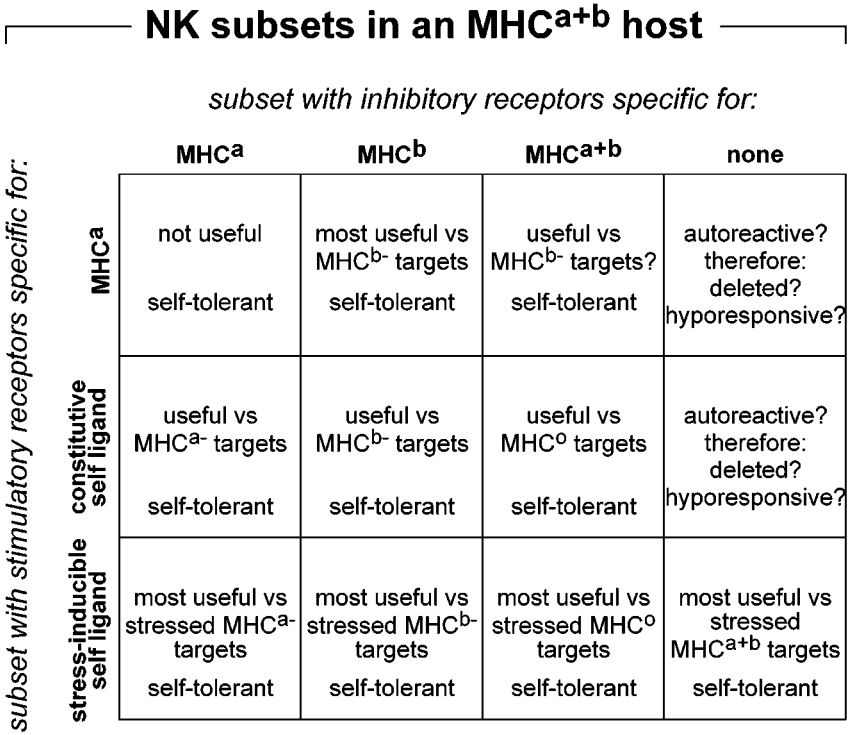

Figure 3 The mechanisms that distribute stimulatory and inhibitory receptors to NK cells have the potential to generate NK subsets with distinct functional characteristics. The figure depicts 12 potential subsets in a host expressing MHC^{a} and MHC^{b} molecules. The NK cells in this host could potentially express inhibitory receptors that are: (*a*) specific for MHC^{a} only (but not MHC^{b}); (*b*) specific for MHC^{b} only (but not MHC^{a}); (*c*) specific for MHC^{a} and MHC^{b}. A fourth category of NK cells is also considered: that is, those failing to express any self-specific MHC receptors ("none"). Three categories of stimulatory receptors are considered: (*a*) stimulatory receptors specific for MHC^{a}; (*b*) stimulatory receptors specific for constitutively expressed self-ligands; and (*c*) stimulatory receptors specific for self-ligands that are only induced on stressed or infected cells. The expected functional specificities of each NK subset is indicated within the relevant box. The fate of NK cells failing to express self-specific inhibitory receptors remains uncertain. Some of these cells may be deleted or rendered hyporesponsive by an as-yet-undetermined mechanism. It should be emphasized, however, that these cells are not necessarily useless, particularly if they express stimulatory receptors specific for inducible self-ligands (lower right box).

of different types of NK cells that would be expected to arise in the mature NK repertoire. This picture is consistent with recent research suggesting that NK cells employ multiple recognition systems so as to be responsive to diverse abnormalities arising from various infections and tumorigenesis.

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