Self-tolerance of natural killer cells

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Abstract | Natural killer (NK) cells, similar to other lymphocytes, acquire tolerance to self. This means that NK cells have the potential to attack normal self cells but that there are mechanisms to ensure that this does not usually occur. Self-tolerance is acquired by NK cells during their development, but the underlying molecular and cellular mechanisms remain poorly understood. Recent studies have produced important new information about NK-cell self-tolerance. Here, we review the evidence for and against possible mechanisms of NK-cell self-tolerance, with an emphasis on the role of MHC-specific receptors.

Adaptor molecules
Proteins, often without intrinsic biochemical activity, that function mainly to bind one signalling protein and bring it into proximity to another.

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Natural killer (NK) cells are lymphocytes that develop from bone-marrow precursors. Although they resemble T and B cells in many respects, NK cells do not express antigen receptors encoded by genes that undergo recombination-activating gene (RAG)-dependent recombination. So NK cells are considered to be cells of the innate immune system[1]. Since their discovery[2,3], NK cells have been found to have an important role in mediating innate immune responses to viruses[4,5], tumours[6] and MHC-mismatched bone-marrow grafts[7]. NK cells can directly lyse target cells[8] and can also have regulatory roles: for example, they are significant early producers of interferon-γ (IFNγ), which can influence T-cell responses[9]. Unlike T and B cells, the specificity of NK cells for target cells is not dominated by a single antigen receptor but, instead, is determined by a range of stimulatory and inhibitory receptors expressed at the surface of each NK cell[10–12]. So NK-cell activation depends on the set of stimulatory and inhibitory ligands that the target cell presents (TABLE 1), as well as on whether the NK cell expresses receptors specific for these ligands and, ultimately, on the balance of signalling through the stimulatory versus inhibitory receptors.

This Review evaluates several models of the cellular and molecular interactions that lead to NK-cell self-tolerance. A key focus of the Review is on recent studies from two laboratories that show that some NK cells are tolerant to self despite lacking inhibitory receptors specific for self MHC class I molecules[13,14]. How NK cells become self-tolerant is an important unresolved question in immunology, with implications for our understanding of the role of NK cells in autoimmunity, tumour surveillance, bone-marrow transplantation and antiviral responses.

Self-recognition by NK cells

An important feature of NK cells is their ability to distinguish target cells that differ only in their expression of MHC class I molecules. This ability is due to the expression of several families of MHC-class-I-binding receptors at the surface of NK cells (TABLE 1). Many MHC-class-I-binding receptors are inhibitory, but others are stimulatory. The inhibitory receptors recruit SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatases such as SHP1 and SHP2, whereas the stimulatory receptors associate with the adaptor molecule DAP12 (DNAX activation protein 12; also known as KARAP), which recruits protein tyrosine kinases. Each individual organism encodes a set of ten or more such receptors, which belong to more than one protein family, and each receptor generally has specificity for distinct polymorphic variants of MHC class I molecules. Importantly, each NK cell usually expresses only a selection (for example, three to five) of these receptors[15–17], so the NK-cell population has a diverse repertoire of different MHC class I specificities[11].

It is becoming increasingly clear that the recognition of MHC class I molecules is just one (important) facet of recognition by NK cells. In addition to the recognition of MHC class I molecules, NK cells express stimulatory and inhibitory receptors that are specific for various other ligands at the surface of target cells, including DNA-damage- and/or stress-induced ligands, virus-encoded ligands and certain constitutively expressed ligands (TABLE 1; FIG. 1).

Missing-self recognition by NK cells. NK cells that express inhibitory receptors for self MHC class I molecules are released from this inhibition when they encounter self cells that have downregulated expression of MHC class I

molecules, as occurs frequently in virus-infected cells or tumour cells (FIG. 1). The recognition and lysis of MHC-class-I^{low} target cells by NK cells is aptly referred to as missing-self recognition[18]. It is thought that the downregulation of MHC class I molecules by virus-infected cells is a strategy to evade cytolysis by CD8$^+$ T cells and that missing-self recognition by NK cells is a counter-response by the immune system.

Importantly, viral infection or transformation of target cells does not seem to be a prerequisite for missing-self recognition. Even untransformed, uninfected target cells can be lysed by NK cells if the target cells

Table 1 | **Receptors on natural killer cells**

NK-cell receptor	Ligands	Comments	References
MHC-class-I-specific receptors (inhibitory and stimulatory receptors are present in each family)			
CD94–NKG2 heterodimer	Qa-1 (mouse) HLA-E (human)	NKG2A is inhibitory; NKG2C and NKG2E are stimulatory; Qa-1 and HLA-E bind peptides cleaved from the signal peptides of many classical MHC class I molecules	70–72
Ly49-family members	Various MHC class I molecules	Several Ly49 genes are present in mice; not functional in humans	73
Ly49A	H2-D^d	No functional ligand in H2^b mice	74
Ly49C	H2-D^d H2-K^d H2-K^b Other H2 molecules	–	75
KIR-family members	Various MHC class I molecules	Several KIR genes are present in humans, but not present in mice	76
LIR1 (ILT2, LILRB1)	Various HLA class I molecules HCMV-encoded UL18	LIR1 is present in humans, but not present in mice; LIR1 is expressed by several cell lineages	77,78
Stimulatory receptors (specific for non-MHC class I molecules)			
NKG2D	RAE1-family members (mice) H60 (mice) MULT1 (mice) ULBP (humans) MICA and MICB (humans)	NKG2D has low homology to NKG2A, NKG2C and NKG2E; NKG2D binds diverse ligands in mice and humans; ligands are induced by stress and/or DNA damage	79–84
Ly49H	MCMV-encoded m157	–	85–89
Ly49D	H2-D^d	–	90–92
FcγRIII	Antibody-coated target cells	–	93
NKR-P1C	ND	NKR-P1C is a pan NK-cell marker in C57BL/6 mice	49
NKR-P1F	CLR-G	–	49
NKp30	ND	HCMV-encoded pp65 might be an antagonist; *Nkp30* is a pseudogene in most mouse strains	94,95
NKp44	ND; possibly viral haemagglutinin	–	96,97
NKp46	ND; possibly viral haemagglutinin	NKp46 is present in humans and mice	97,98
2B4 (CD244)	CD48	2B4 functions to inhibit or stimulate, depending on the associated signalling molecules	99
DNAM (CD226)	CD112 CD155	–	100
Inhibitory receptors (specific for non-MHC class I molecules)			
2B4 (CD244)	CD48	2B4 functions to inhibit or stimulate, depending on the associated signalling molecules	99
KLRG1 (MAFA)	Cadherins	–	51,101–103
NKR-P1B	CLR-B	–	49,50
CEACAM1	CEACAM1	–	34

This is not a comprehensive list; selected receptors particularly relevant to this Review were chosen. Common alternative abbreviations are shown in parentheses. Only key references are indicated. For more details, see several recent reviews[10,11]. CEACAM1, carcinoembryonic-antigen-related cell-adhesion molecule 1; CLR, C-type-lectin-related; DNAM, DNAX accessory molecule; FcγRIII, low-affinity Fc receptor for IgG; H60, histocompatibility 60; HCMV, human cytomegalovirus; ILT2, immunoglobulin-like transcript 2; KIR, killer-cell immunoglobulin-like receptor; KLRG1, killer-cell lectin-like receptor G1; LILRB1, leukocyte immunoglobulin-like receptor, subfamily B, member 1; LIR1, leukocyte immunoglobulin-like receptor 1; MAFA, mast-cell function-associated antigen; MCMV, mouse cytomegalovirus; MIC, MHC-class-I-polypeptide-related sequence; MULT1, murine ULBP-like transcript 1; ND, not determined; NK, natural killer; NKG2, NK group 2; NKp, NK-cell protein; NKR-P, NK-cell receptor protein; RAE1, retinoic acid early transcript 1; ULBP, cytomegalovirus UL16-binding protein.

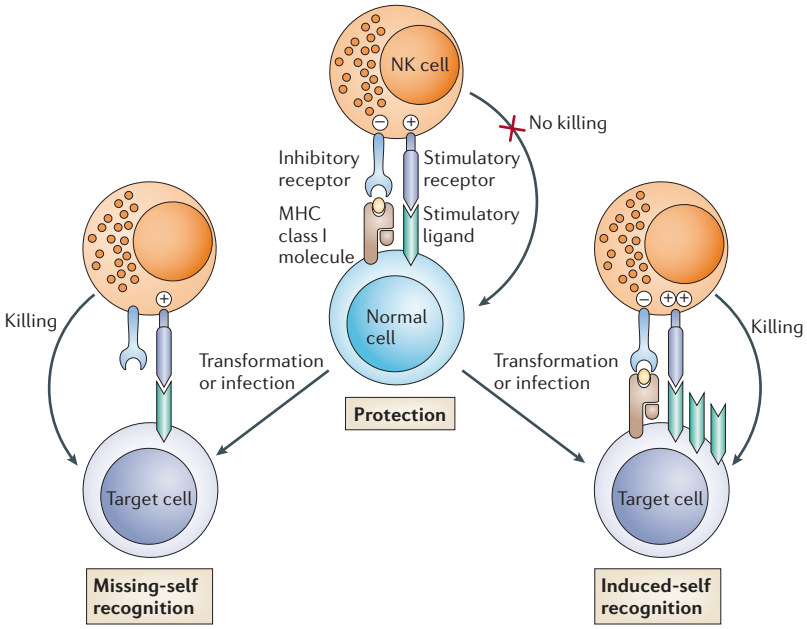

Figure1 | **The balance of inhibitory and stimulatory signals received by a natural killer cell determines the outcome of interactions with target cells.** Normal target cells are protected from killing by natural killer (NK) cells when signals delivered by stimulatory ligands are balanced by inhibitory signals delivered by self MHC class I molecules. If, however, a target cell loses expression of self MHC class I molecules (as a result of transformation or infection), then the stimulatory signals delivered by the target cell are unopposed, resulting in NK-cell activation and target-cell lysis (known as missing-self recognition). Transformation or infection might also induce expression of stimulatory ligands such that constitutive inhibition delivered by inhibitory receptors is overcome (known as induced-self recognition). In many contexts, it is probable that both missing-self and induced-self recognition operate simultaneously to provide NK cells with the maximal ability to discriminate between normal cells and transformed or infected target cells.

fail to express a full complement of normal self MHC class I proteins. This was first shown in studies of bone-marrow transplantation in which donor bone-marrow cells lacking an MHC class I allele of the recipient were often rejected by NK cells[19]. Perhaps the clearest demonstration of missing-self rejection is the potent elimination of bone-marrow cells or lymphoblasts that do not express MHC class I molecules (such as those from mice that are deficient in β_2-microglobulin (β_2m), transporter associated with antigen processing 1 (TAP1) or both H2-K^b and H2-D^b) by NK cells from an otherwise genetically identical MHC-class-I-expressing host[20–25]. It is important to emphasize that missing-self recognition does not operate independently of stimulatory recognition. To lyse target cells or to produce effector cytokines, NK cells, similar to T cells, must be triggered by stimulatory receptors. In the case of missing-self recognition of bone-marrow cells or lymphocytes, the target cells are not diseased, indicating that even normal cells express ligands that engage stimulatory receptors at the surface of NK cells. A variety of stimulatory ligands are present at the surface of normal cells from mice of different genetic backgrounds. For example, the MHC molecule H2-D^d can be a stimulatory ligand in H2^d mice[26], and a ligand for the stimulatory receptor NKG2D (NK group 2, member D) is stimulatory in mice of the BALB/c background[27]. Bone-marrow cells from C57BL/6 mice seem to express yet another, unidentified, stimulatory ligand, because grafts from β_2m-deficient C57BL/6 mice are rejected by NKG2D-deficient C57BL/6 hosts (N. Guerra and D.H.R., unpublished observations). So missing-self recognition of normal (non-diseased) cells by NK cells depends on both stimulation and the absence of inhibition.

The need for self-tolerance. That normal cells express stimulatory ligands for NK cells highlights the potential autoreactivity of NK cells. If such stimulation were unopposed, NK cells would presumably show autoimmune activity and/or reject self bone-marrow cells or lymphoid cells. That this does not usually occur is partly because normal animals express MHC class I molecules and other molecules that can engage inhibitory receptors and counter the stimulatory signals. However, MHC class I molecules show enormous allelic variation, and individual inhibitory NK-cell receptors bind certain MHC class I variants but not others. MHC class I genes are inherited independently of NK-cell receptor genes[28], so genetic mechanisms cannot ensure that the NK-cell receptors of an individual show specificity for the appropriate self MHC class I molecules. Furthermore, a given MHC-class-I-specific receptor is usually only expressed by a subset of NK cells, and the set of receptors that each NK cell expresses seems to be determined by a process that is largely random[11]. Therefore, although many NK cells are self-tolerant as a result of the expression of inhibitory receptors for self MHC class I molecules, some NK cells might arise that do not express such receptors. So a key issue to be addressed is whether such NK cells arise and, if they do, how these cells are prevented from attacking their host.

Possible mechanisms of self-tolerance

Several mechanisms that lead to self-tolerance of NK cells have been proposed (FIG. 2).

The 'at-least-one' model. An early model to explain NK-cell self-tolerance proposed that a developmental process is superimposed on the initially random expression of MHC-class-I-specific receptors such that every mature NK cell is somehow endowed with at least one inhibitory receptor specific for a self MHC class I molecule[15,17,29] (FIG. 2b). Two processes have been considered. First, a cellular selection process could ensure the selective expansion or survival of only those immature NK cells that happened to express inhibitory receptors specific for self MHC class I molecules, or a selection process could involve the specific deletion of NK cells that lack inhibitory receptors for self MHC class I molecules. Second, it is possible that individual developing NK cells could sequentially express or 'audition' each receptor encoded in its genome until a self-MHC-class-I-specific receptor is eventually expressed[29–31].

Transporter associated with antigen processing 1 (TAP1). A molecule that transports short peptides from the cytosol to the endoplasmic reticulum and is required for normal cell-surface expression of MHC class I molecules.

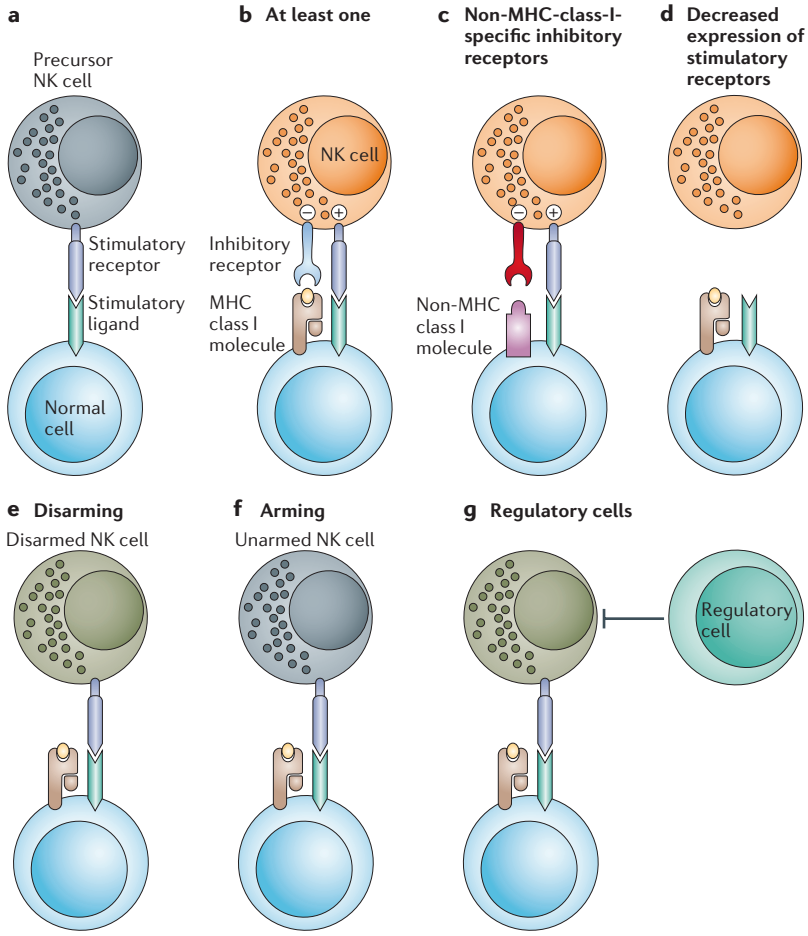

Figure 2 | **Possible mechanisms leading to natural-killer-cell self-tolerance.** It is thought that, during natural killer (NK)-cell development, interactions between precursor NK cells and normal self cells educate NK cells such that they attain tolerance to normal self cells (**a**). Six possible models that could account for self-tolerance of NK cells (**b–g**) are depicted. Some of these models are not mutually exclusive. First, there might be mechanisms that would ensure that mature NK cells express at least one inhibitory receptor specific for a self MHC class I molecule (**b**). Second, mature NK cells might express at least one inhibitory receptor for non-MHC class I molecules present at the surface of target cells (**c**). Third, NK cells that fail to express an inhibitory receptor for a self MHC class I molecule might reduce their expression of stimulatory receptors (**d**). Fourth, NK cells that fail to express an inhibitory receptor for a self MHC class I molecule might be actively rendered hyporesponsive (that is, 'disarmed') by downregulation of stimulatory signalling pathways (**e**). Fifth, NK cells that fail to express an inhibitory receptor for a self MHC class I molecule might never be 'armed': that is, their stimulatory signalling pathways might not be turned on (**f**). And sixth, potentially autoreactive NK cells might be prevented from attacking self cells by the action of putative regulatory cells (**g**).

Expression of non-MHC-class-I-specific inhibitory receptors. Self-tolerance of cells that lack self-MHC-class-I-specific inhibitory receptors could be achieved through the expression of inhibitory receptors specific for other broadly expressed ligands, providing an alternative source of NK-cell inhibition[32–34] (FIG. 2c). Such an inhibitory receptor could explain self-tolerance if the receptor is expressed in greater amounts or provides a stronger signal specifically in the subset of NK cells that happened to lack inhibitory receptors for self MHC class I molecules.

Decreased expression of stimulatory receptors. NK cells that lack self-MHC-class-I-specific inhibitory receptors could achieve self-tolerance by expressing lower amounts of relevant stimulatory receptors than do NK cells that express self-MHC-class-I-specific inhibitory receptors (FIG. 2d). Lower expression of stimulatory receptors could result from specific downregulation of the receptors in such NK cells or from a failure of these cells to upregulate such receptors during development.

Disarming. NK cells that lack self-MHC-class-I-specific inhibitory receptors could be 'disarmed': that is, they could be induced to enter a state of hyporesponsiveness by chronic stimulatory encounters with self cells[1,13,35] (FIG. 2e). In this model, chronic stimulation could induce a loss of responsiveness in mature NK cells, akin to anergy in T or B cells. Alternatively, encounters of immature NK cells that lack self-MHC-class-I-specific inhibitory receptors with self cells could block the final steps of maturation of these NK cells, thereby 'freezing' the cells in an immature, hyporesponsive state. In either variant of the model, stimulatory-receptor signalling is dampened, resulting in self-tolerance.

Arming. In this model (FIG. 2f), self-MHC-class-I-specific inhibitory receptors induce functional maturation (that is, 'arming') of precursor NK cells. NK cells that lack self-MHC-class-I-specific inhibitory receptors persist as immature, hyporesponsive (self-tolerant) cells. This model resembles a version of the at-least-one model, in which maturation is induced by expression of at least one self-MHC-class-I-specific inhibitory receptor, and the functionally immature NK cells persist in the animal.

Regulatory cells. This model supposes that the function of potentially autoreactive NK cells is specifically inhibited by regulatory cells (FIG. 2g), akin to the inhibition of autoreactive T cells by $CD4^+CD25^+$ regulatory T (T_{Reg}) cells. It is conceivable that cells of a non-T-cell lineage could also suppress the activity of autoreactive NK cells.

Responsiveness of NK-cell subsets in normal mice

Self-tolerance of NK cells might reflect several mechanisms working in tandem. Numerous studies, including new research[13,14], demonstrate the existence of NK-cell subsets that differ in functional responsiveness depending on the MHC-class-I-specific inhibitory receptors that they express and/or the MHC class I genes that are expressed by the host. Before reviewing how these new findings might influence the models of NK-cell self-tolerance, we summarize the key results.

Hyporesponsive NK cells in MHC-class-I-deficient animals. Hyporesponsive NK cells were first observed in a special case: mice that are deficient in cell-surface expression of MHC class I molecules as a result of a mutation in the gene that encodes β_2m[20,21]. Such mice contain normal or even increased numbers of NK cells, but these NK cells are self-tolerant, as they do not attack autologous lymphoblasts or bone-marrow cells *in vivo* or *in vitro*[20,22]. These NK cells showed dampened, but still appreciable,

Anergy
A state of unresponsiveness that is sometimes observed in T and B cells that are chronically stimulated or are stimulated through the antigen receptor in the absence of co-stimulatory signals.

CD4⁺CD25⁺ regulatory T cells
A population of T cells that has been shown to regulate the function of other T cells.

Hyporesponsive NK cells
A subset of natural killer (NK) cells in normal animals and all of the NK cells in MHC-class-I-deficient animals show dampened (but not necessarily absent) responses to stimulatory-receptor engagement compared with typical NK cells in normal animals. The term does not imply any mechanism for how these NK cells arise.

Redirected lysis
Lysis that occurs when an Fc-receptor-expressing target cell is coated with antibodies specific for a stimulatory receptor on a cytolytic cell. The antibodies, bound to the target cell through their Fc region, bind the antigen receptor, thereby mimicking antigenic stimulation and triggering lysis of the target cell.

Concanavalin A
(conA). A lectin that activates T cells.

functional activities, including Fcγ-receptor-mediated cytolysis of antibody-coated target cells[23], redirected lysis mediated by antibodies specific for the stimulatory receptor NKR-P1C (NK-cell receptor protein 1C) (M. F. Wu and D.H.R., unpublished observations), and reduced lysis of certain tumour target cells[21] (although the latter defect was not always observed[22]). More recently, it was shown that NK cells from β_2m-deficient mice have reduced responses to antibody-mediated crosslinking of stimulatory receptors such as NKR-P1C, NKG2D or Ly49D[13,14]. Reduced functional activity of NK cells was also observed in mutant mice with MHC class I molecule deficiencies, such as mice with mutated *Tap1* (REF. 36) or classical MHC class I (*H2-K*^{b} and *H2-D*^{b}) genes[24], as well as in TAP-deficient humans[37,38]. The reduced functional responsiveness of these NK cells could explain self-tolerance of NK cells *in vivo*, based on the idea that weak stimulation provided by normal self cells is insufficient to trigger these NK cells.

We have termed the lower-functioning NK cells hyporesponsive to highlight that they have lower (but not absent) functional activity than do most NK cells in normal animals. The term does not imply any particular mechanism with respect to how these cells arise or persist, and it can therefore be applied regardless of the mechanism under discussion. Another research group has used the terms licensed and unlicensed to refer to higher-functioning and hyporesponsive NK cells, respectively[14]. Although this group has recently avowed that their use of these terms is intended to be mechanism neutral[39], the term licensing usually denotes an active mechanism in which functionality is conferred to a subject: for example, in the expression 'licensing of dendritic cells', which refers to induced maturation of dendritic cells[40]; or in the familiar expression 'licensed to drive'. Therefore, we and others[41] have understood licensing to imply the arming model, in which recognition of MHC class I molecules induces high functionality in developing NK cells. Because the mechanism underlying NK-cell tolerance might involve induction of hyporesponsiveness, rather than induction of functionality, we avoid the licensing terminology here.

Hyporesponsive NK cells in normal animals. It was not initially clear whether the existence of hyporesponsive NK cells in MHC-class-I-deficient mice (in which MHC class I molecules are absent from the surface of all cells) had any bearing on NK-cell functionality in normal animals. The presence of hyporesponsive NK cells in normal mice was recently established in a study that found that 10–15% of NK cells in C57BL/6 mice lack expression of the three self-MHC-class-I-specific receptors (that is, Ly49C, Ly49I and CD94–NKG2A) that are present in this strain[13]. Functional assays revealed that, despite the absence of inhibitory receptors for self MHC class I molecules, these NK cells did not lyse concanavalin A (conA)-stimulated blasts from MHC-class-I-expressing C57BL/6 mice, so these cells are self-tolerant. Importantly, these NK cells showed a hyporesponsive functional phenotype similar to the NK cells in MHC-class-I-deficient mice[13]: they were impaired in their capacity to lyse various target cells that are normally sensitive to NK cells, including conA-stimulated blasts from MHC-class-I-deficient mice, the prototypical NK-cell tumour-cell target YAC1, and cell lines transfected with NKG2D ligands. Furthermore, the NK cells that lack inhibitory receptors specific for self MHC class I molecules produced only small amounts of cytokines such as IFNγ in response to crosslinking of the stimulatory receptors NKR-P1C, NKG2D and Ly49D[13]. Importantly, NK cells lacking these three receptors were hyporesponsive in C57BL/6 mice, but these cells were not hyporesponsive in mice of a distinct MHC class I genotype in which MHC class I molecules do bind one or more of the three receptors. So hyporesponsiveness occurs specifically in NK cells that fail to bind self MHC class I molecules.

The existence of responsive and hyporesponsive NK-cell subsets in MHC-class-I-expressing mice was also reported in another study[14]. The authors examined mice that were engineered to express a single peptide–MHC-class-I complex, recognized by a single inhibitory receptor on NK cells (Ly49C)[14]. In these mice, Ly49C⁺ NK cells showed considerably more functionality than Ly49C⁻ NK cells when stimulated *ex vivo* with tumour cells or with antibodies specific for stimulatory receptors. In addition to this compelling data, the authors also reported that NK cells expressing the inhibitory receptor Ly49A, which is specific for H2^d MHC class I molecules, showed high functionality if obtained from H2^d mice but were functionally defective if obtained from H2^b mice, which lack a cognate MHC class I molecule as a ligand for Ly49A[14]. The latter finding is somewhat surprising considering that a large proportion of Ly49A⁺ NK cells in H2^b mice express one or more self-H2^b-specific inhibitory receptors: for example, 33% or more express Ly49C and/or Ly49I[17,29,42], and ~36% express NKG2A[43]). So, many of the Ly49A⁺ NK cells in H2^b mice do express a self-MHC-class-I-specific inhibitory receptor, and we see only a very small difference in their functional activity compared with Ly49A⁺ NK cells from H2^d mice (E. Treiner and D.H.R., unpublished observations). Despite this discrepancy, both of these recent studies[13,14] provide evidence that NK cells that lack self-MHC-class-I-specific inhibitory receptors are hyporesponsive compared with NK cells that do express such receptors.

Consistent with the mouse data, recent studies have also identified a proportion of primary human NK cells that lack receptors for self HLA class I molecules and have shown that these NK cells respond poorly to stimulatory-receptor engagement (E. Vivier, personal communication). These findings are in contrast to an earlier study reporting that a large number of NK-cell clones isolated from two donors with different HLA haplotypes expressed at least one inhibitory receptor specific for a self HLA class I molecule[15]. A possible explanation for the discrepancy is that the procedure used in the earlier study to isolate long-lived NK-cell clones selected against the hyporesponsive NK-cell subset.

A key issue is whether the hyporesponsiveness observed in cell culture is relevant *in vivo*. One of the recent studies showed that NK cells lacking expression of self-MHC-class-I-specific inhibitory receptors were

unable to reject β_2m-deficient bone-marrow grafts *in vivo*[13]. These data indicate that the dampened capacity of these NK cells to attack MHC-class-I-deficient cell types *in vitro* also applies *in vivo*. These findings support the proposal that hyporesponsiveness *in vitro* is relevant to self-tolerance of NK cells *in vivo*, because bone-marrow grafts from MHC-class-I-deficient mice, which are rejected by responsive NK cells, presumably express many of the same stimulatory ligands for NK cells that are expressed by normal cells in the body.

Maturation state of hyporesponsive NK cells. There remains some disagreement about whether hyporesponsive NK cells are just immature cells or whether they are mature NK cells in which functional responsiveness has been somehow dampened. The idea that the NK cells are mature has precedents in the case of other mature lymphocytes, which can be rendered anergic under various circumstances. The data show that the hyporesponsive NK-cell population in normal mice and in β_2m-deficient mice has a fully mature cell-surface phenotype, including expression of CD11b and various Ly49 molecules (that is, Ly49-family members other than the three self-specific receptors)[13]. Furthermore, although their functional activity is dampened, these NK cells are not entirely devoid of function in most assays. So hyporesponsive NK cells seem to be mature, although the possibility that they are immature in some key respects cannot be excluded.

Explanation of dampened functional activity of hyporesponsive NK cells. The molecular basis of the dampened functional activity of hyporesponsive NK cells remains unknown. Hyporesponsive NK cells express normal amounts of the stimulatory receptors that have been tested, including those used in antibody-mediated crosslinking studies to show the hyporesponsiveness of these cells. The possibility that there is a global defect in effector functions is contradicted by the finding that these cells produce normal amounts of intracellular IFNγ in response to pharmacological activators of protein kinase C (such as phorbol esters) plus calcium ionophores (such as ionomycin)[13,14]. These data indicate that the signalling defect in these cells is downstream of cell-surface stimulatory receptors but still relatively proximal in the signalling pathways. It is unlikely that the dampened stimulatory responses reflect increased activity of SHP1, the main mediator of inhibitory-receptor signalling, because neither SHP1-deficient NK cells (R.E.V. and D.H.R., unpublished observations) nor NK cells that express a dominant-negative SHP1 molecule (REF. 44) show autoreactivity and, conversely, are somewhat hyporesponsive. An interesting but unexplained finding is that culture of hyporesponsive NK cells with high doses of interleukin-2 usually reverses the hyporesponsive state[13,14,45].

Status of models of NK-cell self-tolerance

As described in this section, new data on hyporesponsive NK cells, as well as numerous other findings, refine our understanding of NK-cell self-tolerance.

Status of the at-least-one model. The previously mentioned finding that normal animals contain a subset of NK cells that lacks inhibitory receptors for self MHC class I molecules seems to be a clear refutation of the at-least-one model. There are, however, at least two caveats. First, if it is subsequently proven that the hyporesponsive NK cells that lack inhibitory receptors for self MHC class I molecules constitute an immature NK-cell subset, it could be argued that the at-least-one model does hold true in the case of mature NK cells. However, as already discussed, hyporesponsive NK cells seem to show a mature cell-surface phenotype, as defined using available markers. Second, although NK cells that lack inhibitory receptors for self MHC class I molecules exist, it remains possible that the frequency of such cells is minimized by developmental mechanisms that influence the NK-cell repertoire[29,46,47]. Studies in mice show that inhibitory receptors specific for MHC class I molecules are expressed in a sequential and cumulative manner during NK-cell development and that the average number of inhibitory receptors that is expressed tends to increase when the host lacks MHC class I molecules[30,31,48]. Taken together, these reports indicate that developing NK cells that initially fail to engage an MHC class I molecule with an inhibitory receptor can go on to express additional inhibitory receptors for MHC class I molecules. This process of 'auditioning' new receptors is clearly limited, however, as is shown by the existence of NK cells that lack self-MHC-class-I-specific inhibitory receptors in normal mice[13,14]. The process of initiating the expression of new MHC-class-I-specific inhibitory receptors might only occur in a limited developmental period such that each developing NK cell cannot audition all possible inhibitory receptors[11].

Collectively, the studies described above argue against the at-least-one model but indicate that there are at least two routes that a developing NK cell can take to become self-tolerant. First, if an NK cell ultimately expresses inhibitory receptors for self MHC class I molecules, then the consequent inhibition can prevent the cell from mediating autoreactive responses. Or second, if an NK cell fails to express inhibitory receptors for self MHC class I molecules, then the cell has a dampened stimulatory response that prevents it from mediating autoreactive responses.

Role of non-MHC-class-I-specific inhibitory receptors. Several families of inhibitory receptors specific for ligands that are not MHC class I molecules have been described (TABLE 1). Among these receptors are NKR-P1B and NKR-P1D[28,49,50], killer-cell lectin-like receptor G1 (KLRG1)[51], carcinoembryonic-antigen-related cell-adhesion molecule 1 (CEACAM1) in humans[34], and possibly some of the Ly49-family members in mice. A special case is the receptor 2B4 (also known as CD244), which can use different adaptor molecules to impart either inhibitory or stimulatory signals[52–54]. 2B4, NKR-P1B, NKR-P1D and CEACAM1 can inhibit NK-cell function, raising the possibility that these receptors might have a role in NK-cell self-tolerance[34,55]. For 2B4 and CEACAM1, blocking the receptor can lead to increased reactivity of NK cells that lack self-MHC-class-I-specific

Dominant-negative SHP1
A catalytically inactive version of SRC-homology-2-domain-containing protein tyrosine phosphatase 1 (SHP1; which is encoded by the gene *Ptpn6*) that still binds the cytoplasmic tail of receptors, thereby blocking the function of wild-type SHP1.

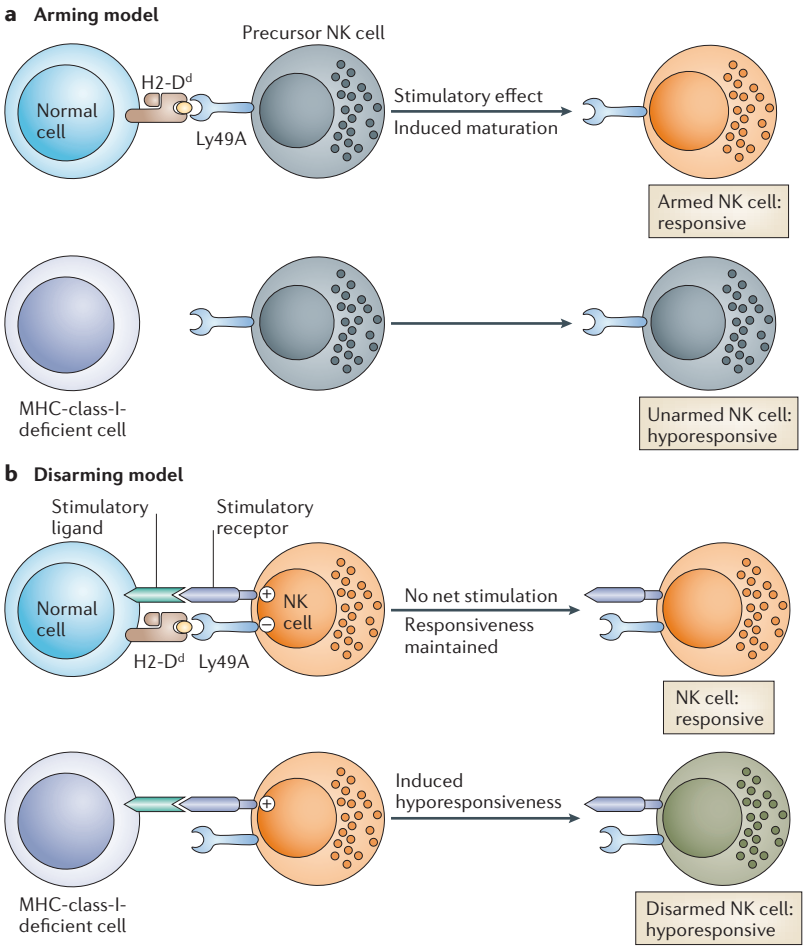

Figure 3 | **Comparison of arming and disarming as mechanisms of natural-killer-cell self-tolerance. a** | In the arming model, positive signals received by a precursor (immature) natural killer (NK) cell through interactions with MHC-class-I-expressing target cells are required to induce functional maturation (that is, 'arming') of the cell. For simplicity, the receptor Ly49A (which recognizes H2-D^d at the surface of target cells) is depicted, although other inhibitory receptors could fulfil a similar role. It should be noted that, in mature NK cells, Ly49A delivers an inhibitory signal, but in the arming model, Ly49A is proposed to function positively, resulting in NK-cell maturation. NK cells that fail to interact with MHC-class-I-expressing target cells (for example, in mice that do not express cell-surface MHC class I molecules) remain unarmed and unresponsive, and therefore do not attack MHC-class-I-deficient target cells. **b** | In the disarming model, NK cells express a variety of stimulatory and inhibitory receptors, and therefore interact with self cells. However, only those NK cells in which inhibitory and stimulatory signals are balanced are allowed to retain (or acquire) responsiveness. NK cells that receive unopposed positive signals (for example, in an MHC-class-I-deficient host) are 'disarmed' and thereby rendered unresponsive. There are two variants of this model: NK cells could be disarmed during their development or after attaining maturity, in response to sustained (chronic) positive signalling.

receptors[33,34]. It is important to note, however, that the existence of inhibitory receptors specific for non-MHC class I molecules does not necessarily account for the self-tolerance of NK cells that lack expression of self-MHC-class-I-specific inhibitory receptors. Indeed, at present, there have been no genetically controlled studies showing that inhibitory receptors specific for non-MHC class I molecules are increased in amount or functional activity specifically on NK cells that lack self-MHC-class-I-specific inhibitory receptors.

Nevertheless, it is unlikely that the action of any of these receptors can account for the hyporesponsiveness of NK cells described here, because such NK cells were hyporesponsive even when stimulated in the absence of target cells that might express inhibitory ligands: that is, when stimulated with immobilized antibodies specific for stimulatory receptors[13,14]. Because tolerance might be imposed by several mechanisms working in tandem, it remains possible, however, that self-tolerance is accomplished by specifically increasing the amount or signalling capacity of inhibitory receptors specific for non-MHC class I molecules.

Role of decreased expression of stimulatory receptors. A study of the expression of stimulatory receptors by hyporesponsive NK cells showed normal cell-surface amounts for all of the receptors tested, including NKG2D, NKR-P1C and Ly49D[13,14]. The steady-state amounts of perforin and signalling adaptor molecules such as DAP10 and DAP12 were also normal[13]. So hyporesponsiveness cannot be due to decreased expression of these stimulatory receptors or signalling adaptor molecules. It cannot be excluded, however, that specific reductions in the cell-surface expression of unexamined stimulatory receptors has a role in self-tolerance.

Disarming and arming: distinct features. On the basis of these considerations, the disarming and arming models, which are outlined in FIG. 3, are the models that are favoured at present to explain the existence of hyporesponsive NK cells that lack inhibitory receptors for self MHC class I molecules. Both models are consistent with the existence of a hyporesponsive NK-cell subset but have important (and opposing) distinguishing features (FIG. 3).

One distinguishing feature of the arming model is that only functionally immature NK cells undergo arming, whereas disarming could, in principle, apply to mature or immature NK cells. Another distinguishing feature is that, in the arming model, engagement of an MHC class I molecule by an inhibitory Ly49 molecule leads to a positive signal that promotes the functional maturation of NK cells. It should be noted that this is somewhat paradoxical, because engagement of such receptors clearly inhibits NK-cell functions of mature NK cells.

The disarming model, by contrast, predicts that inhibitory receptors provide an inhibitory signal during NK-cell biogenesis, just as they do during the effector stage. The model also assumes (as discussed earlier) that a distinct set of stimulatory receptors is engaged by self ligands on many, if not all, developing NK cells. In the disarming model, unlike the arming model, the role of the inhibitory receptors is to counter these stimulatory signals. If the inhibitory receptors are not engaged, the stimulatory signal prevails, and the NK cell is subject to chronic stimulation. In the disarming model, such unopposed chronic stimulation induces a state of hyporesponsiveness in which stimulatory signals are strongly dampened. A strength of the model is that the acquisition of the hyporesponsive phenotype is similar

to the outcome for other developing lymphocytes that receive persistent stimulation through their antigen receptors: that is, anergy[56,57]. Another strength of the model is that it stipulates that the inhibitory receptor functions as it normally does, by providing an inhibitory signal.

Status of the arming model. Observations in the literature, although not necessarily interpreted as such, are consistent with the arming model of NK-cell self-tolerance. As one example, Kim *et al.*[14] reported that the development of highly responsive NK cells resulted from Ly49-derived signals that were distinct from the normal inhibitory signals delivered by Ly49 molecules at the surface of mature NK cells. Members of the Ly49 family of receptors contain a short amino-acid motif known as an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tail. In mature NK cells, this motif recruits SHP1 or SHP2 (REFS 58,59). Through its phosphatase activity, SHP1 terminates the signals that emanate from other cell-surface stimulatory receptors at the surface of mature NK cells. Interestingly, however, Kim *et al.*[14] presented data showing that normal amounts of SHP1 were not required for the functional maturation of NK cells. This result was especially surprising because Kim *et al.*[14] also showed that the ITIM in the receptor Ly49A was nevertheless required for the appearance of responsive NK cells, indicating that unidentified signalling molecules interact with ITIMs and promote NK-cell maturation. Other known ITIM-binding phosphatases did not seem to be substituting for SHP1. Because SHP1 and other phosphatases normally provide inhibitory signals, the evidence that they do not participate in the generation of responsive NK cells is consistent with the arming model, in which the receptor imparts a signal that stimulates NK-cell maturation.

For reasons that we cannot explain, the findings of Kim *et al.*[14] are at odds with our preliminary studies (R.E.V. and D.H.R., unpublished observations) in which we generated bone-marrow chimeras using mixed SHP1-mutant and wild-type bone marrow. Our results indicated that SHP1-deficient NK cells (but not wild-type NK cells developing in the same bone-marrow chimera) do acquire a hyporesponsive phenotype similar to that of NK cells from MHC-class-I-deficient mice. In addition, a published study showed that NK-cell function was partially impaired in mice transgenic for a dominant-negative SHP1 molecule[44]. Although there is clearly much work to be done, these results could indicate that, during NK-cell development, a lack of inhibitory-receptor signalling (rather than lack of a novel Ly49-derived arming signal) is the factor that leads to NK-cell hyporesponsiveness, a finding that would be more consistent with the disarming model. Indeed, in a recent review, Yokoyama and Kim[39] acknowledge that SHP1-derived signals might be relevant to the development of NK-cell functionality; therefore, the maturation of functional NK cells might not depend on a novel signalling process after all.

Another result that has been viewed as being consistent with the arming model[41] is the finding that NK cells that express inhibitory receptors for a self MHC class I molecule preferentially proliferate during a specific developmental phase[14]. This observation seems to support the counterintuitive idea that encounters with an MHC class I molecule stimulate developing NK cells that express cognate 'inhibitory' receptors, as occurs in the arming model[41]. However, an alternative interpretation of the data is that successful maturation (that is, avoidance of chronic stimulation by self cells) is followed by a proliferative phase. So the selective proliferation of NK-cell subsets that express self-MHC-class-I-specific inhibitory receptors is not necessarily evidence that favours the arming model above other models.

Interestingly, previous studies had failed to find similar evidence of selective proliferation of NK-cell subsets. For example, studies using bromodeoxyuridine labelling did not find that NK cells expressing $H2^b$-specific receptors isolated from $H2^b$ mice were labelled significantly more than NK cells from MHC-class-I-deficient mice (either mice deficient in β_2m or both H2-K^b and H2-D^b)[60], as would be predicted if NK cells that express self-MHC-class-I-specific Ly49 molecules proliferate selectively during NK-cell development. Analyses of other receptors did not show more labelling in mouse strains that express a cognate MHC class I molecule than in strains that do not (P. Isnard and D.H.R., unpublished observations). So if selective proliferation of NK-cell subsets occurs, it does not seem to exact much impact on the NK-cell repertoire.

Status of the disarming model. The disarming model makes predictions that are clearly distinct from the arming model with respect to the consequences of encounters between developing NK cells and host cells that lack cognate MHC class I molecules specific for inhibitory receptors. The disarming model proposes that hyporesponsiveness is induced by encounters with host cells that lack MHC class I molecules (but which presumably express stimulatory ligands for NK cells). So tolerance should be dominantly induced, even if the cells of the host include a mixture of cells that do and do not express MHC class I molecules (as occurs in a chimera). By contrast, the arming model implies that interactions with cells that lack ligands for inhibitory receptors are irrelevant. Instead, encounters with cells that express cognate MHC class I molecules for these receptors should dominantly 'arm' the NK cells.

With respect to these predictions, two independent reports published several years ago seem to support the disarming model. In one study, bone-marrow chimeras were prepared with bone marrow from wild-type ($H2^b$) and MHC-class-I-deficient (β_2m–deficient) mice[35]. It was shown that tolerance of NK cells to MHC-class-I-deficient bone-marrow cells was dominantly induced by MHC-class-I-deficient cells in the host. Tolerance occurred despite the maintenance of normal numbers of NK cells in the host, and it did not matter whether the NK cells were themselves deficient in MHC class I molecules.

The second study investigated tolerance to allogeneic bone-marrow cells in mice that expressed an *H2-D*^{d} transgene[45,61]. As sometimes occurs in transgenic mice,

Immunoreceptor tyrosine-based inhibitory motif
(ITIM). A short amino-acid sequence (the consensus sequence of which is V/IXYXXV/L, where X denotes any amino acid) that is found in the cytoplasmic tail of inhibitory receptors. ITIMs are thought to mediate inhibitory signalling by recruiting phosphatases such as SHP1 (SRC-homology-2-domain-containing protein tyrosine phosphatase 1).

Bromodeoxyuridine labelling
(BrdU labelling). A technique in which dividing cells exposed to BrdU incorporate it into their DNA. These cells can be identified by intracellular staining with antibodies specific for BrdU. Non-dividing cells do not incorporate BrdU.

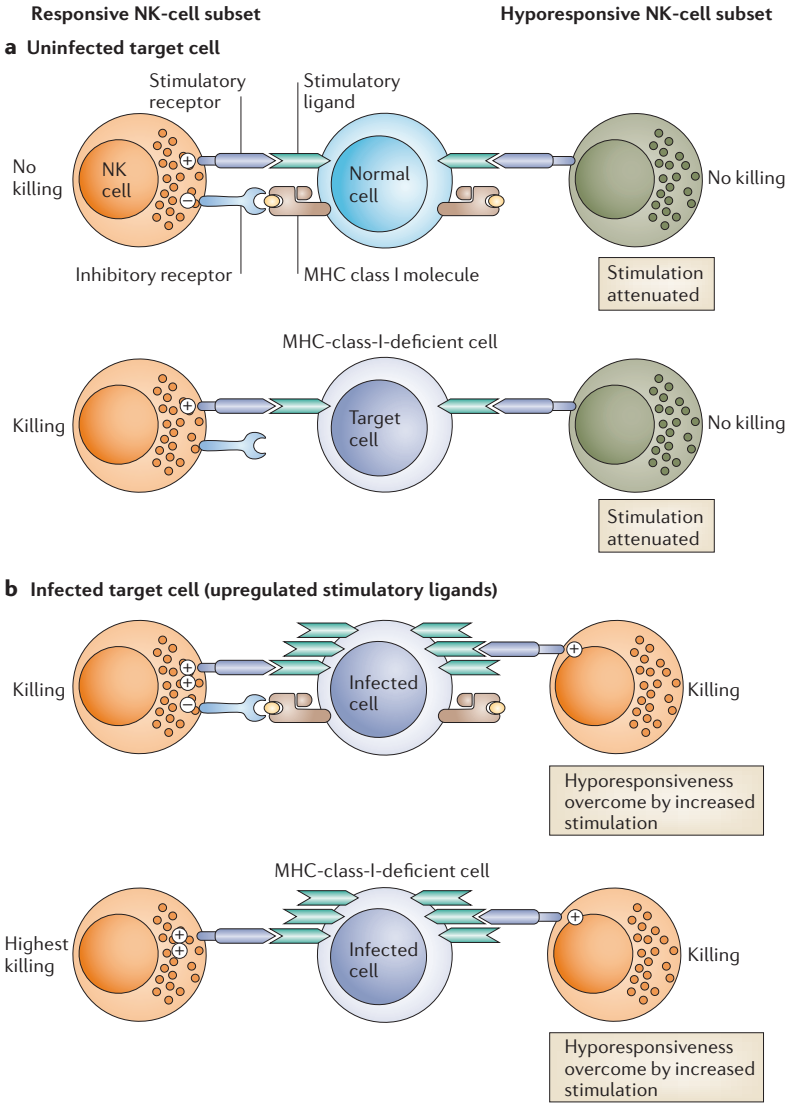

Figure 4 | **Contextual hyporesponsiveness.** The terms hyporesponsive, unlicensed, unarmed, disarmed and anergic might mainly apply to one aspect of natural killer (NK)-cell recognition: the preferential recognition of cells with reduced expression of MHC class I molecules (known as missing-self recognition). Depicted are the outcomes of interactions between uninfected target cells (**a**) or infected target cells (**b**) and responsive (left) or hyporesponsive (right) NK-cell subsets. The responsive NK cells express a self-MHC-class-I-specific inhibitory receptor, whereas the hyporesponsive NK cells do not. The hyporesponsive cells do not respond to target cells with decreased expression of MHC class I molecules but can respond to cells with increased expression of stimulatory ligands.

one of the transgenic lines showed a mosaic transgene-expression pattern: that is, only a proportion of cells expressed the transgene. As a result of the mosaic expression pattern, the mice developed tolerance to allogeneic cells lacking H2-D^d. By contrast, another transgenic line, in which all of the host cells expressed H2-D^d, did not develop tolerance to allogeneic cells lacking H2-D^d. So the host cells lacking H2-D^d dominantly induced tolerance of NK cells. Tolerance was induced even if as few as 20% of host cells did not express H2-D^d, indicating that even intermittent contact with such cells is sufficient to induce tolerance.

These two studies therefore support the disarming model. By contrast, the simplest version of the arming model would predict that tolerance should not occur in MHC-class-I-chimeric or -mosaic mice, because reactive NK cells should be armed as a result of interactions with cells expressing MHC class I molecules, which are present in the hosts.

An issue that arises in these studies is that the nature of the cell type that educates NK cells is unknown. It is possible that a specific cell in the bone-marrow microenvironment is responsible for arming or disarming NK cells. However, the study using mixed bone-marrow chimeras seems to indicate that either MHC-class-I-deficient haematopoietic or non-haematopoietic cells could dominantly induce NK-cell hyporesponsiveness, although non-haematopoietic cells seemed to have a greater effectiveness than haematopoietic cells[35]. Because the type of cell that is responsible for NK-cell education is unknown, studies using mice with a mosaic transgene-expression pattern could not establish the extent of mosaicism on all potentially relevant cells. The authors did report, however, that even fibroblasts from these mice showed mosaic expression of MHC class I molecules, leaving open the possibility that either haematopoietic or non-haematopoietic cells could induce tolerance[45].

Status of regulatory cells in NK-cell self-tolerance. Two intriguing recent reports have provided evidence that T_{Reg} cells can inhibit the function of NK cells[62,63]. On the basis of these findings, the role of T_{Reg} cells in self-tolerance should be investigated further, but T_{Reg} cells probably cannot account for the hyporesponsive NK cells discussed in this Review, which have been detected in studies of NK cells from RAG-deficient mice (which lack all T cells) (REF. 14, and E. Treiner and D.H.R., unpublished observations). It remains plausible that T_{reg} cells, or regulatory cells of other lineages, have a role in NK-cell self-tolerance.

Contextual hyporesponsiveness

It is important to emphasize that hyporesponsive NK cells might be impaired in some contexts and not others (FIG. 4). In most studies, such NK cells respond at least partially to some target cells, such as tumour cells[13,21,23,64,65]. Furthermore, in response to infection with *Listeria monocytogenes* or mouse cytomegalovirus (MCMV), NK cells that lack self-MHC-class-I-specific receptors produced as much IFNγ *in vivo* as did NK cells that express such receptors[13]. Most importantly, although all NK cells in MHC-class-I-deficient (β_2m-deficient) mice have the hyporesponsive phenotype, such NK cells were as capable as wild-type NK cells of controlling MCMV infections *in vivo*[66]. These findings indicate that hyporesponsiveness is contextual. A possible explanation[11] is that hyporesponsive NK cells are not only less sensitive to stimulatory ligands expressed by normal self cells but also unable to receive self-MHC-class-I-induced inhibitory signals; therefore, they might respond well to diseased self cells that upregulate stimulatory ligands.

This line of reasoning indicates that such NK cells are specifically impaired in their ability to amplify their activity towards cells with decreased expression of MHC class I molecules (that is, they are impaired in missing-self recognition), but they might carry out many other NK-cell recognition functions effectively. In light of this, the terminology that is used at present (that is, hyporesponsive, disarmed, unarmed, and unlicensed) might be misleading if such impairment is mainly manifested in the case of only one type of NK-cell recognition (missing-self recognition).

Concluding remarks

The available data support the conclusion that tolerance of NK cells can be accomplished by at least two mechanisms: NK cells can express inhibitory receptors for self MHC class I molecules, or NK cells that lack inhibitory receptors for self MHC class I molecules can show diminished sensitivity to stimulatory ligands. The possibility that hyporesponsiveness of these cells also reflects other mechanisms, such as increased signalling by inhibitory receptors specific for non-MHC class I molecules, remains to be addressed. A key issue is whether the hyporesponsive phenotype is conferred actively (as occurs in the disarming model), or whether, conversely, the responsive phenotype is conferred actively (as occurs in the arming model). The available data are most parsimoniously interpreted as supporting the disarming model, but further studies are necessary to resolve the issue fully. Indeed, the two models are not mutually exclusive. In addition, the responsiveness of NK cells might not be binary (that is, either responsive or hyporesponsive) but might show a more continuous range that sets a stable set point for each NK cell depending on the receptors that it happens to express and the environment in which it matures or resides[11,67].

Several key questions remain. What molecular alterations in signalling pathways account for NK-cell hyporesponsiveness? What functions, if any, might NK cells that are hyporesponsive to normal self cells have in responses to tumour cells or infected cells? Is hyporesponsiveness reversible, and as a consequence, do NK cells participate in the genesis of autoimmune diseases[68]? And how do NK-cell tolerance mechanisms relate to recent findings showing that the inclusion of donor NK cells in human bone-marrow transplants given to HLA-mismatched individuals increases responses to host leukaemic cells and reduces the incidence of T-cell-mediated graft-versus-host disease[69], but the donor NK cells do not damage the host? The remaining questions should yield to a combination of genetic, biochemical, cellular and pharmacological approaches. Elucidating NK-cell self-tolerance should provide a clearer understanding of how various NK-cell recognition strategies and functional activities cooperate to fight disease.

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Competing interests statement
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