

Development and tolerance of natural killer cells

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A natural killer (NK)/T-cell-restricted progenitor cell in the fetal blood, with a phenotype of NK1.1⁺ CD117 (c-kit)⁺, can give rise to NK cells. IL-15 is probably required for NK cell differentiation and, in addition, stimulates expression of at least one of the MHC-specific inhibitory receptor families expressed by NK cells. Evidence supports the role of multiple mechanisms in rendering NK cells self tolerant – including selection for expression of self-specific inhibitory receptors, anergy and modulation of the cell surface levels of MHC-specific inhibitory receptors.

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Abbreviations

ADCC	antibody-dependent cell cytotoxicity
β2m	β2-microglobulin
IRF	interferon-regulatory factor
KIR	killer-cell immunoglobulin-like receptor
NK	natural killer
R	receptor
TAP	transporter associated with antigen processing

Introduction

Natural killer (NK) cells play a significant role in controlling viral and possibly other infections and may play a role in controlling malignancies [1]. They employ cytotoxicity and cytokine production as major effector mechanisms. While the mechanisms used by NK cells to discriminate susceptible from nonsusceptible target cells are still incompletely understood, great progress has been made recently in uncovering several families of NK cell receptors that confer specificity for class I MHC molecules [2,3]. Many of these receptors are inhibitory and consequently prevent NK cells from attacking normal self cells that express high levels of autologous class I MHC molecules. Thus, cells that extinguish expression of class I MHC molecules as a consequence of infection, transformation or mutation are rendered sensitive to cytotoxicity by NK cells [4]. Some tumor cells expressing high levels of autologous class I molecules are nevertheless sensitive to cytotoxicity by NK cells, indicating that class-I-deficiency is not the only mechanism of target cell discrimination by NK cells.

Clearly, the study of NK cell development is in its infancy and only the broad outlines of the process can be presently discerned. This review will address recent advances in the field — starting with precommitted progenitor cells and the role of cytokines and stromal cells in early differentiation events, followed by an overview of the

mechanisms underlying expression of MHC-specific receptors and formation of the NK cell repertoire.

Early stages of NK cell development

Evidence for an NK/T-cell-restricted progenitor cell

NK cells, like other hematopoietic cells, are derived from pluripotent hematopoietic stem cells. Evidence for a restricted NK/T cell progenitor came initially from analysis of early fetal thymocytes, many of which — like NK cells — express the Fcγ receptor (R) III [5]. This FcγRIII⁺ fetal thymocyte population gave rise to TCRαβ⁺ T cells after intrathymic transfer or to NK cells after intravenous transfer but was incapable of giving rise to myeloid cells or B cells.

More recent studies in mice have indicated that the FcγRIII⁺ fetal thymic population is heterogeneous [6,7]. A fraction of the cells expresses the NK cell markers NK1.1 and DX5 but fails to express CD117 (c-kit) — NK1.1⁺ DX5⁺ CD117[−] cells — and another fraction exhibits the NK1.1⁺ DX5[−] CD117⁺ phenotype. The CD117[−] subset exhibits *ex vivo* cytotoxic activity against YAC-1 target cells, suggesting that it contains functionally mature NK cells. The CD117⁺ subset was capable of reconstituting αβ T cell development in fetal thymic organ culture and NK cell development when cultured with the OP-9 stromal cell line but failed to give rise to B cells or myeloid cells. The CD117[−] population failed to give rise to αβ T cells [6]. Possibly related is the finding that human precursor thymocytes express NKR-P1A, a human isoform of the murine NK1.1 antigen [8]. It was concluded that the CD117⁺ population represents restricted progenitor cells for the T and NK lineages (while the CD117[−] population represents mature NK cells). While these studies suggest the existence of a restricted NK/T cell progenitor, it will be important in future experiments to determine whether individual NK1.1⁺ DX5[−] CD117⁺ cells can give rise to descendants in both the T and NK lineages.

NK cell development normally occurs extrathymically, posing the question of whether restricted NK cell progenitors exist in the periphery. Analysis of fetal mouse blood cells revealed a CD90⁺/CD117^{lo} population that was capable of giving rise to αβ T cells after intrathymic transfer. It was concluded that this population represents a restricted αβ T cell progenitor [9]. Subsequent studies have shown that most of these cells express NK1.1 and exhibit a similar overall phenotype to the NK/T-cell-restricted progenitors in the fetal thymus [10[•]]. Most significantly, cells in this population can differentiate into either T or NK cells, but not myeloid or B cells. Collectively these studies suggest that restriction of a progenitor cell to the NK/T cell lineages may occur prethymically. NK cell development — while it may occur briefly in the fetal thymus — occurs primarily extrathymically, with critical steps occurring in the bone marrow [11].

Role of IL-15

Mature NK cells express the IL-15R α chain, the IL-2/15R β chain and the common-cytokine-receptor chain (γ_c), but do not express the IL-2R α chain that is necessary for formation of the high affinity IL-2R. While high doses of IL-2 can stimulate NK cell proliferation, NK cells develop normally in IL-2 $^{-/-}$ mice [12]. Significantly, however, NK cell development was impaired in mice with homozygous mutations in either IL-2/15R β or γ_c genes [13,14*]. These observations raised the possibility that NK cell development requires IL-15, the receptor for which employs a unique IL-15R α chain, but shares the IL-2/15R β chain (and γ_c) with the IL-2R. Indeed, bone marrow stromal cells secrete IL-15 and exogenously added IL-15 supported NK cell development *in vitro* from human CD34 $^{+}$ progenitor cells (CD34 is expressed on cells at several stages of differentiation, including pluripotent hematopoietic stem cells) in the absence of bone marrow stromal cells [15,16]. In the murine system, IL-15 was necessary for differentiation of functional NK cells *in vitro* from a Sca2 $^{+}$ CD117 $^{+}$ lymphoid-cell-restricted bone marrow progenitor-cell population. Preculturing these cells in a cocktail of cytokines (IL-6, IL-7, stem cell factor [SCF] and flt3-ligand) was necessary to render the cells responsive to IL-15 [17**]. Mice with a homozygous mutation in the interferon-regulatory factor (IRF)-1 gene had diminished NK cell functional activity [18,19*,20*], which correlated with impaired production of IL-15 by bone marrow stromal cells and could be restored *in vitro* by IL-15 [21**]. Evidence was provided that IL-15 gene expression depends on the IRF-1 transcription factor [21**]. Collectively, these studies suggest an important role for IL-15 in NK cell development.

A requirement for IL-15 may account for the phenotype of NK cells in marrow-disrupted mice, such as those treated with bone-seeking isotope ^{90}Sr or the osteopetrosis-inducing agent β -estradiol. While most hematopoiesis shifts to the spleen in these animals, NK cell development is severely curtailed. NK1.1 $^{+}$ cells appear in significant numbers in the spleen, but these cells lack NK lytic activity [22]. Culture of these cells with a low dose of IL-15 or a high dose of IL-2 can, however, induce functional NK cell activity [23]. Since IL-15 is produced by bone marrow stromal cells, it is plausible that limited access to IL-15 in the spleen may account for the failure of NK cells to fully differentiate in the marrow-ablated animals.

Expression of MHC-specific receptors by developing NK cells

An issue of considerable recent interest concerns the acquisition by developing NK cells of class-I-specific receptors. How is receptor expression tied to functional maturation and what signals are necessary for inducing expression of these receptors? The emerging picture is that the different receptor families are to some extent independently regulated.

Expression of CD94–NKG2 inhibitory receptors

The CD94–NKG2 receptors constitute the only (known) inhibitory class-I-specific receptor family that is shared

by humans and rodents [24,25]. Interestingly, CD94 is expressed on an NK-cell-like population in the human fetal liver — suggesting that these receptors are expressed early in NK cell ontogeny [16]; however, it was not established whether the partner NKG2 proteins are also expressed at this early stage nor whether the receptors are functional. The ontogeny of CD94/NKG2 expression has not yet been investigated in mice; however it is intriguing that NK cells cultured from fetal mouse livers or from the spleens of new-born mice exhibit class-I-dependent inhibition of cytolytic function despite their failure to express Ly49 molecules, the other known family of murine MHC-specific inhibitory receptors [17**,26,27]. These results raise the possibility that functional CD94–NKG2 receptors are expressed early in NK cell development.

Several reports suggest that the expression of CD94–NKG2 receptors is induced by cytokines, particularly IL-15. Culturing immature human thymocytes with IL-15 resulted in NK cell maturation and expression of CD94–NKG2A receptors but not of killer-cell immunoglobulin-like receptors (KIRs) [28**]. Culture of CD34 $^{+}$ fetal-liver-derived progenitor cells with a mixture of cytokines, including IL-15, also led to CD94 expression [16]. Interestingly, CD94–NKG2A receptors can be expressed by activated T cells and — as in NK cells — are induced by culturing the cells with IL-15 [29].

Expression of Ly49 inhibitory receptors

In mice, expression of the Ly49 family of class-I-specific receptors is a relatively late event in NK cell development. At birth, the spleen and bone marrow contain significant numbers of NK1.1 $^{+}$ CD3 $^{-}$ cells but these cells do not express inhibitory Ly49 receptors. Receptor expression occurs gradually over the first few weeks of life, reaching a plateau by 1 month of age [26,30*].

Unlike CD94–NKG2A expression in the human system, Ly49 receptor expression was not induced by culturing bone marrow progenitor cells or splenic NK1.1 $^{+}$ CD3 $^{-}$ Ly49 $^{-}$ cells in cytokines — including IL-15 and flt-3-ligand ([17**]; C Roth, DH Raullet, unpublished data); however, splenic NK1.1 $^{+}$ CD3 $^{-}$ cells that did not express certain Ly49 receptors were induced to express them on a subset of cells after intravenous transfer *in vivo* [30*]; therefore, the failure of the tested cytokines, by themselves, to induce Ly49 expression does not reflect an incapacity of the cells to express the receptors. Presumably, an untested cytokine or signal(s) produced by stromal cells is necessary to induce expression of Ly49 receptors during NK cell development.

Expression of KIRs

The signals that induce expression of KIRs by developing NK cells, like those inducing Ly49 receptors, have not been defined. IL-15 and several other tested cytokines failed to induce KIR expression on human KIR $^{-}$ NK or T cell populations [28**].

In summary, it appears likely that CD94–NKG2 expression is induced by IL-15 and may be an early event in NK cell ontogeny, at least in humans. Expression of Ly49 receptors or KIRs by murine and human NK cells, respectively, appears to require signals other than IL-15. Expression of Ly49 receptors by murine NK cells occurs relatively late in NK cell ontogeny, while the ontogeny of KIR expression has not been reported.

Formation of the NK receptor repertoire

Features of the repertoire

Aside from the general signals that induce expression of a given class of inhibitory receptors, a series of important questions surround the establishment of the repertoire of inhibitory receptors. The receptor families each have several members, which often react with different class I molecules or allomorphs. Class-I-specific receptors are clonally distributed on NK cells, but unlike T and B cell antigen-receptors, coexpression of two or more receptors by each NK cell is the rule rather than the exception. A clonal analysis led to the estimate that each human NK cell clone expresses an average of 4–5 MHC-specific inhibitory receptors (KIRs and/or CD94–NKG2) [31**]. In the murine system, individual NK cells commonly coexpress two or three Ly49 receptors [32–34]; furthermore, evidence suggests that many murine NK cells coexpress Ly49 and CD94–NKG2 receptors [25]. The available data suggest that once a receptor is expressed by an NK cell, subsequent expression of the receptor is quite stable [30*,35]. In the murine system, expression of Ly49 receptors was shown to be a cumulative process — NK cells expressing one receptor could go on to express others after *in vivo* transfer, while maintaining expression of the initially expressed receptor [30*].

Models of NK cell self tolerance

It has long been clear that NK cells are self tolerant — they fail to attack normal autologous cells yet frequently attack normal allogeneic, semiallogeneic or class-I-deficient cells such as lymphoblasts or hematopoietic stem cells. Three general, nonexclusive mechanisms of NK cell tolerance have been considered. In one, an education process promotes the development of NK cells that express at least one self-MHC-specific inhibitory receptor, sufficient to prevent lysis of normal autologous cells (the ‘at least one model’ [34]). A second model emphasizes the quantitative nature of the inhibitory signal and proposes that the cell surface levels of self-MHC-reactive inhibitory receptors are calibrated to ensure that self MHC molecules will mediate an adequate inhibitory signal (the ‘receptor calibration model’ [36]). A third model proposes that NK cells that lack self-MHC-specific inhibitory receptors can exist in an anergic state, in which they are unable to attack normal cells though they may retain some functional activities (the ‘anergy model’ [37*,38*]).

The ‘at least one’ model

The best evidence, to date, that education mechanisms select for expression of self-specific NK receptors comes

from an analysis of a large panel of human NK clones from two individuals. The results established that each clone expressed at least one KIR or inhibitory CD94–NKG2 receptor that was specific for one or another of the appropriate self class I molecules [31**]. Although the data clearly demonstrate the expression of self-specific NK receptors by clones, it remains possible that a fraction of peripheral NK cells are anergic and consequently are refractory to cloning.

The analysis of Ly49A⁺ NK cells from H-2^b mice also bears on the ‘at least one’ model. Ly49A is not a self-MHC-specific inhibitory receptor in H-2^b mice, yet is expressed by 20% of the NK cells in these mice. Are these cells self tolerant and, if so, by what mechanism? Analysis showed that these cells are self tolerant, in that they lyse H-2^b lymphoblasts poorly if at all [39,40]. Significantly, however, they retain the capacity to lyse class-I-deficient lymphoblasts, suggesting that they are subject to inhibition by H-2^b-encoded class I molecules and are not anergic. Yet inhibition was not mediated by Ly49A, since anti-Ly49A antibodies failed to block inhibition mediated by H-2^b target cells [40]. The results suggest that the Ly49A⁺ NK effector cells in H-2^b mice must also express non-Ly49A, H-2^b-specific inhibitory receptors, though these receptors were not identified. While these results are consistent with the ‘at least one’ model, they by no means rule out the existence of anergic cells since the Ly49A⁺ population in H-2^b mice could include a mixture of anergic cells and cells with H-2^b-specific inhibitory receptors.

Although evidence is lacking that each NK cell in normal mice expresses a receptor that is specific for self class I, there is evidence that MHC class I molecules influence the repertoire of receptors expressed by murine NK cells. Most striking was the effect of class I deficiency on the frequencies of NK cells coexpressing different Ly49 receptors [41,42]. Invariably, the frequencies of NK cells coexpressing two or more of the receptors tested were higher in class-I-deficient mice than in normal mice. These observations suggest that recognition of class I molecules by developing NK cells limits the extent of coexpression of Ly49 receptors.

A sequential expression model of NK receptor expression was proposed to account for these findings as well as for the cumulative nature of Ly49 receptor expression [30*,34]. The model has two key propositions: first, that developing NK cells initiate Ly49 receptor expression gradually and cumulatively; second, that inhibitory-receptor engagement of host class I molecules ultimately prevents expression of new receptors. In such a model, NK cells will acquire new receptors until a sufficient number of self-specific receptors are expressed to exceed a signaling threshold that prevents expression of yet other receptors. Data obtained with Ly49-transgenic mice support the model, in that expression of a transgenically encoded Ly49 receptor specific for self class I molecules early in development inhibited expression of endogenously encoded Ly49 receptors [43**].

The sequential expression process would be expected to promote expression of self-MHC-specific receptors by NK cells; however if the receptor accumulation process occurs for only a limited time-period during NK cell development, not all NK cells would necessarily succeed in initiating expression of a self-MHC-specific receptor. Such a limitation is suggested by the finding that each NK cell in class-I-deficient mice does not express all Ly49 receptors, though there is a clear increase in overall receptor usage [37*,41,42]. This consideration raises the possibility that other mechanisms may be necessary to establish tolerance of NK cells that fail to express a self-MHC-specific inhibitory receptor.

The anergy model

The most compelling evidence for NK cell anergy is that hyporesponsive NK cells develop in class-I-deficient mice [44–46]. These NK cells are unable to attack class-I-deficient lymphoblasts or bone marrow cells. Because this phenomenon was discovered in β_2 -microglobulin (β_2m)^{-/-} mice, the possibility was considered that the NK cells were not in fact anergic, but were inhibited by the presence of low levels of classical class I molecules expressed on cells in the absence of β_2m . This possibility has been rendered highly unlikely by the demonstration that NK cells from β_2m ^{-/-} mice fail to attack target cells expressing lower levels of class I molecules (i.e. target cells from β_2m ^{-/-} and transporter associated with antigen processing [TAP]^{-/-} double-mutant mice; [37*]) or no classical class I molecules (i.e. target cells from K^b ^{-/-} D^b ^{-/-} double-mutant mice; [38*]); furthermore, the NK cells in β_2m ^{-/-} TAP^{-/-} double-mutant mice and K^b ^{-/-} D^b ^{-/-} double-mutant mice also exhibited self tolerance [37*,38*].

The anergy of NK cells in class-I-deficient mice is conditional on the type of target cell employed. Lymphoblasts or bone marrow cells are insensitive to attack by these NK cells, but some capacity to lyse tumor target cells and mediate antibody-dependent cell cytotoxicity (ADCC) is retained [37*,38*,45,46] and these activities are even subject to some degree of class-I-mediated inhibition. Significantly, tumor-cell lysis and ADCC by NK cells from class-I-deficient mice are usually quantitatively impaired in comparison to normal NK cells [37*,45,46]. Thus, 'anergy' here may reflect a dampening of the sensitivity of NK cell activating pathways in response to target stimulation, sufficient to prevent lysis of nontransformed somatic cells. The existence of anergic NK cells in class-I-deficient mice does not guarantee that such cells exist in normal mice but it is plausible that some NK cells, having failed to express self-MHC-specific inhibitory receptors, would acquire the anergic phenotype. Thus, it is possible that NK cells in normal mice are a mixture of anergic cells and cells that are self tolerant by virtue of expressing self-MHC-specific inhibitory receptors.

The receptor calibration model

The receptor calibration model was prompted by the finding that the cell surface levels of Ly49 inhibitory receptors are higher in the absence of a strongly binding MHC ligand for the receptor [47]. Receptor upregulation might in some cases

sufficiently enhance reactivity with weak ligands to engender NK cell self tolerance. It was recently shown that higher cell surface levels of Ly49A correlated with greater sensitivity of the cells to inhibition by a strong class I ligand [48*]. Thus, the regulation of the cell surface levels of Ly49 receptors can modulate the sensitivity of NK cells to self MHC ligands. It is unlikely, however, to account for tolerance of NK cells that fail to express any self-MHC-specific inhibitory receptors.

NK cell self tolerance in mosaic mice

As a means to explore the mechanisms of NK cell self tolerance, mosaic mice have been constructed in which cells expressing different MHC molecules coexist. One model system employed fetal liver chimeras between normal and class-I-deficient mice. When a mixture of class-I⁺ and class-I-deficient fetal liver cells was allowed to develop in an irradiated normal host or a class-I-deficient host, the resulting hematopoietic compartment was mosaic — consisting of a mixture of class-I⁺ and class-I-deficient cells. Compared to NK cells from wild-type mice, the NK cells from these mosaic mice exhibited a reduced capacity to attack class-I-deficient bone marrow cells *in vivo* [49*]. Thus, exposure of the class-I⁺ NK cells to class-I-deficient cells throughout development prevented these NK cells from subsequently attacking the class-I-deficient cells.

Another mosaic mouse model employed B6 (H-2^b) mice harboring a transgene encoding the D^d class I molecule. Previous studies showed that NK cells from transgenic B6 mice that express D^d on all of their cells are self tolerant but can attack nontransgenic B6 cells [50], presumably because some of the D^d-transgenic B6 NK cells express D^d-specific but not H-2^b-specific inhibitory receptors. Of several D^d-transgenic lines tested, however, one exhibited variegated transgene expression. The NK cells and most other lineages in this transgenic line, called DL6, consist of a mixture of H-2^b cells that express D^d and H-2^b cells that do not express D^d. The NK cells that developed in these mosaic mice were mutually self tolerant [51**]. Thus, exposure of the D^d-expressing NK cells throughout development to non-H-2^b-expressing cells induced tolerance to the latter cells. Most interesting was the finding that the tolerant status of the D^d-expressing NK cells in the mosaic DL6 mice was rapidly reversible *in vitro*. When the D^d-expressing NK cells were separated from the mixture and cultured briefly *in vitro*, they recovered the capacity to attack H-2^b cells [51**]. These results suggest that some forms of NK cell self-tolerance, at least, are rapidly reversible *in vitro*. The mechanism of tolerance in these mosaic mouse models, however, has not been determined.

Conclusions

There has been significant recent progress in understanding both early events in NK cell development and the ontogeny of inhibitory-receptor expression. The successful development of *in vitro* culture systems for NK cell differentiation from progenitor cells is an encouraging milestone. These culture systems, and the parallel application of *in vivo* gene-knockout models, have already suggested a key role

for the cytokine IL-15 and should allow the rapid definition of additional requirements for differentiation of functional NK cells. A sophisticated understanding of the development of NK cell specificity, on the other hand, will require a more complete knowledge of the receptors involved and their ontogeny. While there is still relatively limited information as to how NK cells become self tolerant, evidence suggests a role for education processes that determine which receptors an NK cell expresses as well as other mechanisms that regulate the responsiveness of NK cells. It thus appears likely that several complementary mechanisms play a role in imposing tolerance, akin to the mechanisms that impose tolerance of T and B cells. Additional progress on these important issues should be rapid as new reagents and genetically manipulated animals become available.

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