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Natural killer cell education and the response to infection and cancer therapy: stay tuned

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Abstract

The functional capacities of natural killer (NK) cells differ within and between individuals reflecting considerable genetic variation. “Licensing/arming”, “disarming” and “tuning” are models that have been proposed to explain how interactions between MHC class I molecules and their cognate inhibitory receptors – Ly49 in mice and KIR in humans – “educate” NK cells for variable reactivity and sensitivity to inhibition. In this review, we discuss recent progress toward understanding the genetic, epigenetic and molecular features that titrate NK effector function and inhibition, and the impact of variable NK cell education in human health and disease.

NK education

Central to the role of the natural killer (NK) cell in tumor surveillance, pathogen control, and pregnancy is its ability to mount an immune response while maintaining tolerance to self. Regulating these functions is a diverse spectrum of NK cells, endowed with varied capacities for effector response through a process termed “education,” governed by receptor interactions with major histocompatibility complex (MHC) proteins (see glossary). Broadly, the education of an NK cell by a particular MHC molecule is defined by its capacity to sense downregulation of that same HLA molecule on an adjacent putative target cell to mount an effector response. As a result, the NK response to damaged or infected cells, whose appearance to the NK cell is termed as “altered self”, can vary based on each specific disease and patient.

The functional diversity of NK cells found within and between individuals is driven by germline-encoded ligands and their receptors (Tables 1 and 2). Notably, a large number of NK inhibitory receptors, Ly49 in mice and KIR in humans, recognize MHC proteins, and together play a major role in governing NK cell education. Interestingly, the Ly49 and KIR

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receptor families are independently evolved but functionally orthologous. Despite marked genetic and structural differences, inhibitory members of the Ly49 and KIR families perform a remarkably similar immunologic role: programming NK responsiveness via interaction with self-MHC[1,2].

In mice, chromosome 6 hosts the Ly49 locus, which encodes up to 16 inhibitory and activating lectin-like receptors[3]; in humans, up to 15 genes for killer immunoglobulin-like receptors (KIR) are encoded on chromosome 19[4,5]. The Ly49 and KIR genes are clustered into haplotypes, with particular genes in strong linkage disequilibrium[6,7]. Notably, the KIR haplotypes have been classified as A and B, characterized by enrichment for inhibitory and activating KIR, respectively[6]. The KIR and Ly49 receptors are expressed in a largely stochastic fashion by overlapping subsets of NK cells. Thus, within a given repertoire, an individual may harbour educated and uneducated NK populations.

(Educated) NK cells were initially characterized by their ability to recognize target cells that lack “self” MHC class I molecules (Figure 1)[8]. “Missing self” recognition is now known to require co-expression of activating ligands to stimulate NK cell reactivity[9]. While educated NK cells exhibit the highest responsiveness against MHC class I negative targets, they are also susceptible to inhibition by damaged cells that retain expression of “self” MHC class I. In mice, educated NK cells are considered to be those that exhibit inhibitory Ly49 molecules that bind co-inherited MHC ligands[10]; likewise, in humans, NK cells that express inhibitory KIR engaged by self MHC class I ligands (hereafter referred to as human leukocyte antigen, HLA) comprise the major educated population[1]. While exhibiting lower thresholds for reactivity against target cells with lowered or absent self-MHC expression, educated cells preserve tolerance to self and avoidance of auto-reactivity through inhibitory signalling upon recognition of self-MHC. Coexisting with educated NK cells are populations of “uneducated” NK cells, which lack receptors specific for self-MHC and are hyporesponsive to activation[1,2]. In reality, NK cell education exists on a continuum and individual NK cells exhibit graded levels of responsiveness that correspond with their quantitative sensitivity for inhibition by “self” class I molecules[11,12] (Figure 2).

Separate from the *Ly49* and *KIR* genes, other conserved MHC-binding receptors confer additional diversity in NK education and protection from autoreactivity. These include the C-type lectin inhibitory CD94/NKG2A heterodimer which binds to MHC class I sequences presented by the universally expressed Qa1 and HLA-E in mice and humans respectively; the paired immunoglobulin-like receptors (PIR), the leukocyte immunoglobulin-like receptors (LIR) and the signaling leukocyte activating molecule (SLAM) family receptors, which enable both activating and inhibitory reactivity of NK cells[13–16]. Variably expressed on the surface of individual NK cells, these additional receptors and their interaction with MHC and non-MHC ligands ensure a multi-layered and complementary system of immune tolerance and education of the NK population by providing additional signals for inhibition by healthy cells[17,18].

Models of NK education

Functionally, NK cell education is recognized in both mice and humans; however, its underlying mechanism has not been clearly determined. Unlike T and B cells, where

diversity is generated by recombination of DNA to establish unique receptors and tolerance to self is achieved by selective survival of developing lymphocytes, NK cells employ germline-encoded receptors to generate diversity and achieve self tolerance by counterbalancing reactive potential with sensitivity to inhibition by "self" MHC. The polymorphic, polygenic *KIR* and *Ly49* loci segregate independently from the genes encoding their MHC ligands[19]. The available evidence suggests that for the most part *KIR, Ly49* and *MHC* expression are fixed and independent features of their genotypes[12,20–22]. While any given inhibitory receptor is expressed on only a fraction of NK cells in the repertoire, it can be co-expressed with other inhibitory receptors on the same cell. Therefore, the education endowed through receptor-ligand interactions differs for each individual NK cell, establishing a relative setpoint for both reactivity and inhibition by self-MHC class I molecules[23,24]. Several models of NK education have been extensively reviewed elsewhere[25,26] and are summarized here (Figure 3).

Licensing and Arming

Both the licensing and arming models propose that the expression of inhibitory receptors complementary to self-MHC ligands endow NK cells with higher effector capabilities. In the licensing model, signalling through inhibitory receptors results in a "licensing program", whereby molecular machinery and activating receptors are mobilized to make the cell more receptive to activating input[2]. Supporting a role for inhibitory signaling in the education of NK cells, deletion of key pathway mediators, including SHP-1 (Src homology region 2(SH2)domain-containing phosphatase, encoded by *PTPN6*), SHIP-1 (SH2 domain-containing inositol 5′phosphatase, encoded by *INPP5D*) or SAP (signaling lymphocytic activation molecule (SLAM)-associated protein, encoded by *SH2D1A*), limits NK cell education, and leads to accumulation of immature NK cells[15,16,27,28]. Likewise, preventing inhibition through deletion of the cytoplasmic region in the Ly49A receptor or through point mutations in the cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM) critical for recruitment of SHP-1, precludes NK education[10,29]. Furthermore, licensing and inhibition utilize signaling through the same ITIM[29].

Similar to the licensing model, the arming model proposes the possibility that during education the inhibitory receptor stimulates a signalling program or acts as an adaptor to endow NK cells with an educated phenotype, however in this model the cascade is distinct from the inhibitory interactions triggered by healthy cells in the periphery[30]. For both licensing and arming, the events downstream of inhibitory signalling responsible for NK education remain unknown.

Disarming

The disarming model aims to resolve the seemingly paradoxical observation that inhibitory receptors are necessary for reactivity. It postulates that all NK cells are initially highly reactive, but lose function by activation-induced anergy unless they are rescued by inhibitory signalling triggered by engagement of self-MHC class I ligands[31]. Unlike the licensing/arming model that focuses exclusively on inhibitory receptors, the disarming model illustrates how the inhibitory signalling preserves the reactive potential of NK cells by dampening the effects of activating signals. In support of this model, persistent triggering of

the activating NKG2D receptor leads to hyporesponsiveness of NK cells to subsequent stimulation through all activating receptors[32]. Likewise, KIR2DS1^{+} NK cells in the presence of high levels of their HLA-C2 ligand, as occurs in HLA-C2 homozygous individuals, or Ly49H^{+} NK cells developing in the presence of transgenically expressed m157 ligand are hyporesponsive to their respective triggering ligands, supporting development of an anergic state due to chronic stimulation[33,34].

Rheostat

Allelic variation determines Ly49 or KIR expression independent of ligand availability[12,22], and co-expression of other MHC-specific receptors can further alter the extent of each cell's interaction with self-MHC. Incorporating this, the rheostat model postulates that the excitability of each NK cell is calibrated by its capacity for inhibition by self-MHC class I molecules[35]. Indeed, the "dose" of inhibition titrates NK effector potential: among NK cells that express a single receptor type, the density of inhibitory receptors correlates with the education and magnitude of responsiveness of each NK cell[21,23]; co-expression of two or more self-specific inhibitory receptors, or the availability of multiple cognate MHC ligands likewise increases NK education[11,36–38]. Applicable to both the licensing/arming or disarming models, the rheostat model proposes that an educated state is the result of quantitative interactions between receptor and ligand. As such, it introduces the concept that the education of the NK cell can be tuned upward or downward, depending on environmental MHC.

Tuning

The education of NK cells is neither static nor binary, but can be tuned to changes in environmental MHC occurring over short periods of time, as proposed by the discontinuity theory of immunity[37,39]. Supporting this are data from experiments employing antibodies to interfere with inhibitory signalling, or adoptive transfer in disparate MHC mouse models and mice transgenic for HLA, which reveal that NK reactivity can be calibrated upward in the presence of novel cognate ligand[40–42] and, in many cases, downward in the sudden loss of cognate ligand[41,43,44]. As a product of an ongoing process sensitive to changes in MHC, the ongoing tuning of NK cell responsiveness ensures the maintenance of tolerance, possibly to avoid autoimmunity and/or permit successful reproduction.

NK education was initially thought to represent a single developmental event, but experimental evidence now supports ongoing calibration of NK cell education to maintain tolerance to healthy "self" cells. The multiple models of NK education may not be mutually exclusive, and all accommodate the concept of tuning. While the mechanism(s) by which NK education is established (licensing vs disarming) and adjusted (rheostat/tuning) remain to be resolved, it is now understood that education is adaptable and reflects the ability of each NK cell to respond to "self" MHC. Important associations between education with NK development, protein transcription, and physical interaction in the cytoskeleton and cell membrane offer additional clues.

Molecular features of NK education

Phenotype and development

While education is a process separate from NK development, they are linked by virtue of acquisition of molecules fundamental to education. To study the stepwise acquisition of surface markers occurring during NK cell development, studies have relied on irradiation and reconstitution of syngeneic mice, allowing synchronous maturation[45]. NK cells first upregulate CD27 and NKG2D, followed by CD11b, NKG2A, SLAM family receptors and inhibitory Ly49[45,46]. Subsequent loss of CD27 leads to formation of a $CD11b^{+}CD27^{low}$ terminally-differentiated NK population[45,47]. Both the $CD11b^{+}CD27^{high}$ and $CD11b^{high}CD27^{low}$ populations are capable of missing-self reactivity[45]; therefore, education does not require terminal differentiation of NK cells.

In humans, hematopoietic cell transplantation provides an opportunity to study NK development and the acquisition of tolerance to self-MHC. The first NK cells developing after transplantation are developmentally immature, but responsive[48]. Analysis of telomere lengths revealed that human NK cells develop from a $CD56^{bright}NKG2A^{+}$ stage to a $CD56^{dim}KIR^{+}$ stage, which includes the educated population[49].

Recognizing the critical role of education for establishing NK responsiveness, identifying its characteristic phenotypic markers remains an important goal among NK biologists. Transcriptional profiling between educated and uneducated NK populations in mice[50] confirmed previously described skewing of the latter toward increased expression of unbound inhibitory receptors and demonstrated upregulation of killer cell lectin-like receptor G1 (KLRG1) molecules in the presence of MHC molecules. [23,51,52] More recently, microarray analysis identified upregulated clusters of co-expressed genes involved in cell death and cytolysis in educated NK cells, in contrast to genes associated with cytokine activity and binding in uneducated NK cells[53]. In mice, while the activating receptor DNAM-1 is not required for the acquisition or maintenance of MHC class I-mediated NK education, its expression is significantly correlated with the educated state and may be modified by an alternative maturation program [54,55]. Human educated NK cells may also be distinguished from their uneducated counterparts by expression of DNAM-1 and the open active conformation of LFA-1[56]. Despite their important observations, these studies failed to identify by phenotype or gene profiling unique markers identifying the mechanism underlying education. The lack of unambiguous transcriptional differences between responsive and hyporesponsive NK cells is nevertheless noteworthy and suggests a dynamic plasticity to NK responsiveness, consistent with the tuning model in the context of environmental MHC.

Education of NK cells by MHC class I molecules in *cis* and *trans*

In both mice and humans, the presence of MHC on the NK cell itself, in the hematopoietic compartment, or on stromal cells, contributes to NK education[40,42,57]. Ly49 and cognate MHC bind both in *cis* and *trans*, with both sources of MHC competing for the same binding site on the inhibitory receptor due to a flexible hinge region in the receptor stalk[58,59]. Constitutive binding of the inhibitory receptor to cognate MHC in *cis* restricts inhibitory

signalling by the same MHC ligand in *trans*, reducing the threshold for activation by co-expressed activating ligands on the target cell and providing a mechanism for tuning to environmental MHC. Experimental mutation of the stalk region permitting *trans* but not *cis* binding results in NK cells that can be inhibited, but lose their capacity for effector response[59]. Therefore, *trans* interactions may be involved in tolerance, but *cis* interactions are required for NK education[59].

A similar *cis* interaction between KIR and HLA has not been shown; however, we recently demonstrated that the acquisition of cognate HLA from *trans* sources is associated with increased responsiveness among human NK cells bearing cognate KIR receptors[42]. Supporting a potential role for *cis* interaction, educated human NK cells retain their functional potential despite transfer to an HLA-negative host, and knockdown of β2m leads to reduction in response capacity.

Stromal and hematopoietic sources of *trans* MHC ligands may differently contribute to the generation of NK cell function and tolerance. In bone marrow chimeric mice, expression of MHC exclusively by stromal cells leads to responsiveness of NK cells even when bone marrow cells lack MHC. In contrast, lack of MHC on bone marrow-derived or stromal cells is sufficient to engender tolerance to an MHC-negative allograft[60]. In “mosaic” DL6 mice, a novel MHC D^dL^d transgene introduced into B6 mice is expressed only on a subset of hematopoietic cells, leading to a repertoire of NK cells that exhibit different MHC molecules[61]. Fratricide between educated D^dL^{d+} NK cells and neighbouring cells bearing MHC H-2b but lacking the D^dL^d transgene was not observed, underscoring the concept that NK cells can be rendered tolerant to neighbouring cells with differing MHC; the NK cells from these mice remained capable of rejecting MHC-negative allografts and lymphoma cells. Moreover, separation of D^dL^d-positive from D^dL^d-negative cells for a period of 4 days was sufficient to restore reactivity against D^dL^d-negative cells by the educated D^dL^{d+} NK population. Hence, tolerance to neighbouring “non-self” MHC can be achieved without compromising NK cell effector function, but is reversible upon removal from the tolerizing environment.

Availability of activating and inhibitory receptors

To trigger degranulation, NK and target cells form an “immunological synapse” (IS) stabilized by adhesion molecules, which facilitates simultaneous activating and inhibitory interactions. Functional competence can be altered by the availability of adhesion molecules and activating receptors. In contrast to uneducated NK cells, where LFA-1 is maintained in a closed conformation, the open conformation of LFA-1 on educated NK cells fosters target:effector binding and development of the IS[62]. In mice, inhibitory Ly49 receptors and their confinement to the actin meshwork appears similar between responsive and hyporesponsive cells. In contrast, activating receptors are partitioned in signalling-favorable nanodomains or membrane rafts in responsive cells, whereas in hyporesponsive cells, they are confined to the actin meshwork[50]. Providing further illumination, MHC-engaged inhibitory KIR promote accumulation and co-clustering of the activating receptors 2B4 and CD2 at the IS, but block their recruitment into lipid rafts and therefore their ability to signal[63,64], instead triggering dissociation of the NK cell from its target[65]. Therefore,

by altering the availability of activating receptors, education enables IS formation and NK reactivity.

Beyond receptor-ligand pairs: additive education by multiple sources of inhibition

Although the majority of NK cells exhibit a single self-specific inhibitory receptor, many co-express NKG2A or multiple other receptors that can bind to class I molecules. In keeping with a “rheostat” model, co-expression of multiple receptors is associated with increased education[18,38]. Similarly, mouse NK cells bearing multiple self-specific Ly49 receptors or co-expressing NKG2A exhibit higher response capacity[37,66]. Receptor co-expression is mainly predicted by the product rule: the product of the expression frequencies of each receptor predicts the likelihood of co-expression, suggesting that the expression of each receptor is independent from that of other inhibitory receptors[18,66]. There is little evidence to support a purely ligand-driven expression model for inhibitory receptors; instead, deviations from the product rule likely reflect allele subtype variation and imprints from previous immunologic challenges[12,67–69].

A role for balancing NK effector potentials at the repertoire level has recently been described. KIR2DL1, KIR2DL2 and KIR2DL3 exhibit complementary features to enable recognition of both HLA-C1 and HLA-C2 subtypes[70,71]. KIR2DL1 and KIR2DL3 are in positive linkage disequilibrium on KIR-A haplotypes, and faithfully engage HLA-C2 and -C1, respectively[71]. KIR2DL2, characteristic of many KIR-B haplotypes, however, is not in positive linkage disequilibrium with KIR2DL1 or KIR2DL3, and demonstrates binding to both HLA-C subtypes[22]. Hence, both KIR-A and KIR-B haplotypes have evolved to enable recognition, education and inhibition by both HLA-C1 and –C2 epitopes[72].

NK cell repertoires have been classified based on their reliance on KIR or NKG2A for recognition of self HLA[18,73]. Dictating the relative contributions of KIR and NKG2A to education are the HLA molecules themselves; polymorphism in the HLA leader sequence controls the availability of peptides presented by HLA-E to NKG2A[74]. Maintaining a balance between activating and inhibitory signals, individuals with KIR-B haplotypes enriched for multiple activating receptors notably exhibit greater expression of the inhibitory receptor, LIR-1[75]. Thus, human NK education is tuned at the repertoire level, striking a balance of activating and inhibitory effector functions. Mouse NK cells can express NKG2A and PIR; whether these invariant molecules analogously buffer mouse NK cell repertoires has not been described.

Allele subtype variation: impacts on expression, affinity and binding

Allelic variation for both KIR/Ly49 and MHC diversifies surface protein expression, representation in the repertoire and receptor-ligand binding affinity, all of which can impact NK education. Although it is challenging to study the impact of Ly49 allelic differences in inbred mouse models, the available evidence supports the conclusion that the strength of binding between Ly49 molecules and different cognate MHC allotypes determines the magnitude of NK cell education[37].

Representing the most polymorphic receptor-ligand partnership in humans, *KIR3DL1* receptor and *HLA-B* ligand alleles exhibit significant phenotypic variation [76,77].

KIR3DL1 subtypes are expressed with no, low or high cell surface densities, and at predictable repertoire frequencies[12,78–81]. Likewise, HLA-B can be categorized into non-binding (Bw6) and binding (Bw4) subtypes, and further based on the amino acid at position 80 (Bw4-80I or -80T, respectively) [12,77]. With few exceptions, Bw4-80I alleles are expressed at a higher cell surface density than - 80T[12], and this dichotomy broadly predicts interactions with KIR3DL1 subtypes[77] (Figure 4).

Initial studies using a limited number of recombinant HLA-B and KIR3DL1 alleles in transfected cell lines revealed that low-expressed KIR3DL1 subtypes bind with high affinity to both Bw4-80I and Bw4-80T subtypes, while high-expressed KIR3DL1 subtypes bind preferentially to Bw4-80I[82,83]. However, these studies did not consider the important role of physiologic levels of expression of KIR3DL1 and HLA-B play in determining response. When primary NK cells are used instead of transfected cells, the density of both receptor and ligand, as well as the affinity with which they bind, contribute to the overall magnitude of NK reactivity against HLA-negative target cells[12]. Further studies revealed that the affinity of receptor-ligand binding can be influenced by the peptide presented by HLA, but whether these peptides adjust NK education is unclear[82,84,85]. Surprisingly, persistent surface expression of KIR3DL1 is not required to achieve or maintain education, as the “null” group of KIR3DL1 alleles which are retained intracellularly and expressed in only small amounts on the cell surface can, in combination with cognate HLA-Bw4, still educate the NK cell[86,87].

Among the inhibitory KIR2DL1/2/3 family members, a diversity is observed in binding affinity for HLA-C molecules and in surface expression, although the distinctions are less dramatic than those observed for KIR3DL1[71,75]. The KIR2DL1 subtype harbouring 245C is expressed at lower surface densities and is less sensitive to inhibition by HLA-C2 than the KIR2DL1 subtype with 245R[75,88]. KIR2DL2/L3 subtypes with 35E educated by HLA-C1 demonstrate greater missing self recognition, surface density and binding affinity than those with 35Q[22,75,89]. Whether KIR2DL1/2/3 diversity and NK education vary based on co-inherited HLA-C alleles has not been described, but may explain the additional functional variation observed among NK cells exhibiting these KIR.

Adaptive NK cells

Once established, the resting repertoire of NK cells is stable at steady state[90], and human cells expanded *ex vivo* maintain the education programmed *in vivo*[91]. Following infection with cytomegalovirus, however, specific subpopulations of NK cells expand and ultimately create a pool of “memory-like” or “adaptive” cells, capable of more rapid response to subsequent exposure to the virus[92,93].

The first evidence for adaptive NK cells was observed in mouse studies. Mice lacking the recombinase activating gene (RAG) and therefore lacking T and B lymphocytes, could be protected against a lethal dose of murine cytomegalovirus (MCMV) if previously exposed to the virus or given adoptive transfer of NK cells from an MCMV-experienced host[94]. Subsequent studies demonstrated that priming of mice with haptens enabled a faster, stronger hypersensitivity response to secondary homologous hapten challenge and other viruses, especially among educated populations of NK cells[95,96].

Human cytomegalovirus (HCMV) infections in humans are associated with expansion of a population of educated “adaptive NK cells” expressing the activating receptor NKG2C as well as self-specific KIRs[93,94,97,98]. This NKG2C+ population further displays downregulation of the transcription factor PLZF and variable silencing of the transmembrane adaptor protein FcεRγ, the tyrosine kinase SYK, and the intracellular adaptor EAT-2. Together, they represent a profound change in the NK repertoire, occurring as a result of epigenetic modification. Whether these epigenetic modifications broadly mark “adaptive” or memory NK cells in humans is unknown; however, many are shared with activated T and B lymphocytes after interaction with antigen-specific stimuli, suggesting that conserved evolutionary patterns enable adaptive NK functions. These include a reliance on RAG, a more rapid response to subsequent infection, and improved target specificity[94,99]. Whether the expanded and adaptive NK populations are specific to a particular antigen, as occurs for memory response in T and B lymphocytes and occurs in mice, has not been determined in humans.

NK education in human disease and therapy

Both educated and uneducated NK cells are retained and are functional in healthy immune repertoires, performing complementary functions to sense targets exhibiting a spectrum of class I expression (Figure 1). HLA-positive and HLA-negative phenotypes have been described in viral infections and cancer[12,100,101], and the diversity of NK cell function driven by independent assortment of *HLA* and *KIR* establish resistance against an array of disease phenotypes at the population level.

NK education in infection

A common viral strategy is to eliminate or diminish expression of HLA on the surface of the infected cell[102,103]. HIV notably induces downregulation of HLA-B, but not HLA-C molecules, making infected cells appear as “missing self” targets for educated $KIR3DL1^{+}$ NK cells[12,102]. Herpes simplex virus and HCMV, in contrast, induce downregulation of HLA-C molecules, creating a putative target for KIR2DL-expressing NK cells[103,104]. While HCMV clearly imprints the NK repertoire[93,105], giving rise in some individuals to educated “adaptive NK” populations that are highly efficient at ADCC[98], it is unclear whether these populations clear HCMV infection or prevent secondary CMV reactivation. Interestingly, the same terminally differentiated $NKG2C^{+}KIR^{+}CD57^{+}$ population expands in CMV-seropositive individuals upon exposure to hantavirus, HIV, or chikungunya virus[106,107], indicating a common mechanism underlying “adaptive NK” expansion between unrelated viruses. It remains to be seen if uneducated NK cells play the dominant role in controlling HCMV as they do in mice against mCMV[100].

For some viruses, the magnitude of NK education is directly proportional to the extent of viral control. Epidemiologic studies and subsequent functional investigations have revealed that allele subtype combinations of KIR3DL1 and HLA-B that convey the strongest education, such as the highly expressed high-affinity KIR3DL1-h and Bw4-80I combinations, are associated with the best control of HIV in patients not treated with antiretroviral agents[12,108]. In contrast, Bw4-negative individuals, in whom $KIR3DL1^{+}$

NK cells are uneducated, exhibit the most rapid progression to AIDS[12,108]. Thus, in downregulating HLA-B expression to avoid T cell recognition, the HIV virus makes itself vulnerable to NK recognition and clearance in a manner commensurate with hierarchies of KIR3DL1-dictated NK education. That NK education can be predicted by allele-encoded receptor-ligand interactions has important prognostic and management implications for HIV infected populations, potentially identifying super-controllers from progressors by genotype alone.

NK education and cancer

In cancer control, we and others have demonstrated a benefit of uneducated NK cells, whose activity is unaffected by the expression of HLA on the tumor cell surface. While the threshold for reactivity may be higher for the uneducated NK cell, the inflammatory milieu created by radiation, chemotherapy and infection in the setting of leukopenia augments their reactivity[109]. In HLA-matched hematopoietic cell transplantation (HCT), absence of HLA ligands for the donor's KIR ("missing ligand") is associated with lower relapse and improved survival in patients with acute myelogenous leukemia (AML)[110,111]. Early post-HCT, uneducated NK cells expressing KIR for non-self HLA are hyper-responsive, possibly due to their inflammatory *in vivo* environment. In this context, lack of inhibitory signals from cognate HLA leads to enhanced anti-leukemia surveillance and improved clinical outcomes. The benefit of uneducated NK cells in cancer is not restricted to HCT, however. Targeted antibody therapies, including anti-GD2 antibody for treatment of neuroblastoma[109] and anti-CD20 antibody for treatment of lymphoma[112] exhibit a greater benefit among patients whose NK cells lack at least one KIR ligand.

For educated NK cells in cancer patients, the presence of self-HLA ligand on the cancer cell may not necessarily mean a complete loss of NK effector function due to inhibitory KIR signalling. Hierarchies in NK education and inhibition, as programmed by allelic diversity in KIR and HLA have shown that some receptor-ligand combinations can approximate a missing-ligand benefit in HCT patients with AML[87]. For example, KIR3DL1 and HLA-B allotypes that engage with weak or no avidity are also poorly inhibited, leading to superior anti-leukemia activity and lower risk for disease relapse. These same weak/poor inhibitory KIR/HLA pairs are associated with a similar progression-free survival benefit is seen in patients with neuroblastoma receiving anti-GD2 monoclonal antibody therapy[113]. Taken together, these studies suggest that gradations of education also impact NK inhibition *in vivo* leading to clinically relevant outcomes for hematologic and solid tumors targeted both by NK cytotoxicity and ADCC. The implications of these findings extend beyond prognostic: 1) tumor targets, despite being classically labeled as low or non-expressing for HLA, express adequate HLA to inhibit NK cells in a hierarchical manner corresponding to educational status; 2) despite the powerful stimulus of CD16 engagement, educated NK cells can still be inhibited *in vivo*; 3) variable inhibition of KIR allotypes by HLA allotypes affords the opportunity to select HCT donors based on KIR to minimize inhibition and decrease risk for disease relapse.

Given their higher effector potential and lower threshold for reactivity, the educated NK cell population would be expected to be beneficial in patients with cancer if inhibition by "self"

HLA could be avoided. Indeed, a strong missing self benefit is observed in patients undergoing HCT from HLA-mismatched or haploidentical donors, when donors are educated by an HLA molecule absent in the recipient[114,115]. The notion that educated, uninhibited NK cells are superior effectors is further supported by our own study in patients with AML where combinations involving the “null” KIR3DL1 receptor, which educate NK cells but do not signal inhibition, were associated with the lowest relapse after HCT[116]. For the majority of educated NK cells, including “adaptive NK” cells, however, self-HLA expression on the tumor surface will limit cytotoxicity through KIR-mediated inhibition, unless blocked by an anti-KIR antibody. Transcriptional profiling indicates that certain tumors, such as head and neck tumors, may be particularly amenable to such a therapeutic approach[117].

More recently, clinical trials have focused on adoptive transfer of allogeneic NK cells from healthy donors, with superior outcomes in patients receiving highly alloreactive donor cells [118], or induced adaptive NK cells[119].

Concluding remarks and future directions

The ability to recognize and eliminate cells lacking self-MHC class I molecules is the defining hallmark of NK cells. Education establishes a spectrum of functional capacities that enable varying recognition and response to abnormal cells with diverse MHC expression, but further research is required to understand this intriguing process (see Outstanding Questions). Recognition that inherited gene- and allele-level variation leads to varied capacity for NK response suggests that *KIR* and *HLA* allele typing will play an increasingly important role in the prognosis and treatment of disease. Building on the success of a missing ligand benefit in transplantation for leukemia, we are undertaking a prospective clinical trial (NCT02450708) to select donors to maximize NK reactive potential while minimizing NK inhibition.

Multiple groups have now demonstrated the safety of adoptively transferred NK cells from matched or unrelated donors[120,121]. Good manufacturing protocols for rapid expansion of long-lived NK cells exist, making it feasible to use NK cells derived from related and/or third-party donors[121]. Although initial trials have yielded mixed results, the possibility exists to deliver NK cells whose function is tailored to particular disease phenotypes, to maximize the benefit of NK therapy[122]. Recalling that human NK education is maintained and even potentiated after adoptive transfer to a novel host HLA environment[42], consideration of NK education and the HLA of the patient may help to increase the precision of adoptive transfer strategies. Delivery of adaptive NK cells and/or antibodies to interfere with NK inhibition may further enhance the efficacy of adoptive cell therapies[123,124].

That NK cells are not aggressive to healthy cells represents another important advantage in their clinical application. Specifically, it may be possible to transfer “armed” and highly reactive NK cells, resulting in activity against diseased cells without off-target effects. Refining these protocols to instead induce “memory-like”[119] or “adaptive”[125,126] NK cells may circumvent this problem by allowing NK cells to remain reactive *in vivo* for longer. Notably, reduction in HLA expression has recently been observed in response to

checkpoint inhibition (anti-PD1) in patients with non-small cell lung carcinoma[127]. Whether this results in an improved target for educated NK cells and/or indicates adoptive transfer of educated NK cells with checkpoint inhibition remains to be investigated.

NK cell functional diversity is established by variations in education and likely reflects a long co-evolution between *HLA* and *KIR* genes in humans and *Ly49* and *MHC* in mice. Diversity in NK cell reactive capacities driven by NK education protect some individuals against a variety of infections and diseases, while leading to increased risk for others. Understanding the molecular drivers of NK cell education, function and inhibition, together with the MHC phenotypes of diseases will present opportunities for precision therapy of infectious disease and cancer.

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Glossary

Adaptive NK cells
Memory-like, self-renewing NK cells established after exposure to an antigen or pathogen that exhibit inhibitory receptors for self MHC ligands and respond to rechallenge with homologous antigen stimulation

Disarming
A model for NK cell education proposing that all cells are initially responsive, but lose responsiveness due to chronic activation unless rescued through inhibition by binding self MHC

Education
the process through which an NK cell is programmed for reactivity, calibrated by its ability to be inhibited by self proteins, principally HLA or MHC class I molecules

Human Leukocyte Antigen (HLA)
The set of MHC molecules expressed in humans

Licensing
A model for NK cell education proposing that NK cell sensitive to inhibition by self molecules will activate a program through which they are potentiated for reactivity

Major Histocompatibility Complex (MHC)
A set of highly-polymorphic, polygenic cell surface proteins exhibited in all vertebrates. Class I MHC proteins engage NK cells for education and define "self". In mice, MHC class I are also referred to as H-2 molecules

Tuning/Rheostat

A model for NK cell education proposing that the magnitude of NK cell reactive potential is quantitatively counterbalanced by inhibitory interactions between NK and healthy cells

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Trends Box

1. NK cell function is calibrated, through a process called “education”
2. The reactive potential of each NK cell is counterbalanced by its sensitivity to inhibition by self MHC class I molecules
3. Immunogenetic variability confers different education programs between individual subjects, leading to variable sensitivity to activating and inhibitory input
4. Differences in NK cell education, which can be predicted by NK immunogenetics, impact susceptibility and resistance to specific disease phenotypes

Outstanding questions

- What is/are the molecular mechanisms underlying NK cell education and the adjustment of NK cell function upon exposure to a novel MHC class I ligand environment?
- How are the programs and molecular features of human and mouse NK cell education different?
- How can NK cell immunogenetic diversity, including allele subtype diversity, be used a tool for precision medicine?

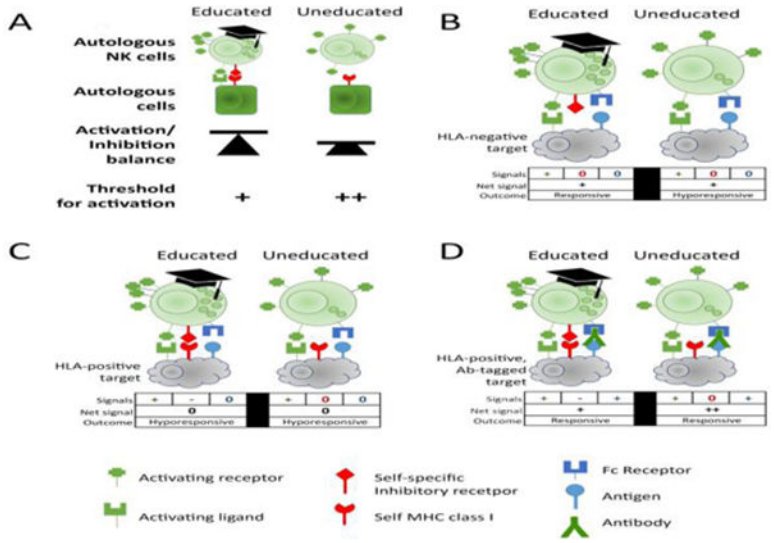

Figure 1. NK cell education and functional outcomes of NK:target interactions

(A) NK cells express variable combinations of inhibitory receptors. Only some NK cells will display inhibitory receptors capable of binding co-inherited MHC class I molecules. Cells bearing inhibitory *Ly49* or *KIR* molecules for self MHC class I molecules are educated and exhibit the lowest threshold for activation. NK cells that do not express inhibitory receptors for self-MHC class I molecules are “uneducated” and require higher activation signals (+) to become reactive, but are insensitive to inhibition (−) by self-MHC class I. Hence, education programs a different reactive threshold to each NK cell; shown are relative requirements for activating signals in representative educated and uneducated NK cells. (B) against an HLA-negative target cell expressing an activating ligand, educated NK cells are activated but as uneducated NK cells exhibit a higher threshold for reactivity, they are hyporesponsive against the same target. (C) Against an HLA-positive target cell, activation is nullified by inhibitory signaling through KIR and the educated NK cell is hyporesponsive. Uneducated NK cells are refractory to inhibition because they lack cognate inhibitory receptors for “self” class I molecules, but also require a high signal for activation. Without strong stimulation, uneducated NK cells are hyporesponsive to target cells. (D) Accessory activating factors, including pro-inflammatory cytokines (not shown) or antibodies, which trigger NK cells for ADCC, support activation of both educated and uneducated NK cells.

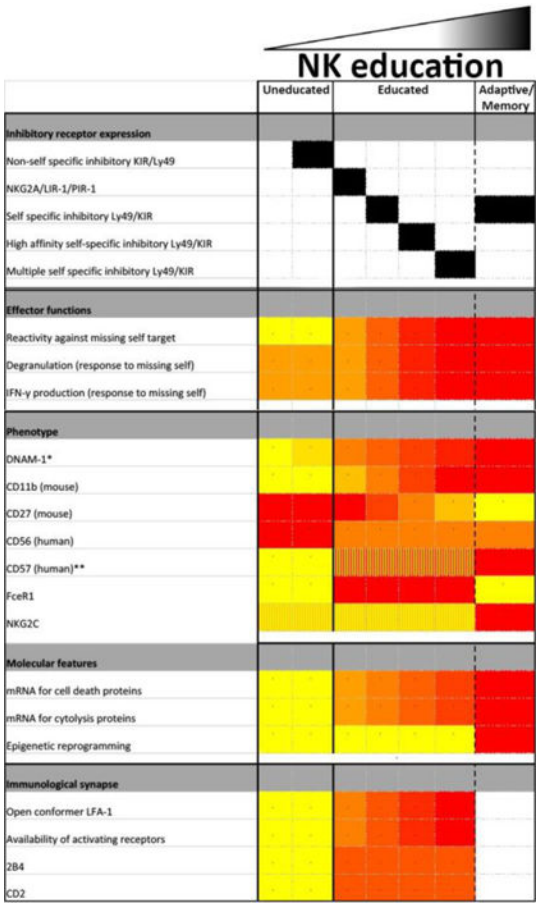

Figure 2. Molecular features of NK cell education

NK cell education increases with a cell's sensitivity to inhibition by "self" class I molecules and can be additive based on the co-expression of multiple receptor types. Shown is a schematic heatmap describing the relative expression and function of NK cells with increasing education from left to right. Potential receptor expression profiles associated with increasing education are presented at the top of the table (black boxes indicates expression). Factors are shown on a yellow (low) to red (high) heatmap and striped cells indicate factors that are not uniformly expressed on a population of cells or consistently changed with increasing activation. Where cells are white, data are not available. *DNAM-1 is required for the expansion of adaptive NK cells, but downregulated after their differentiation. **CD57 is expressed on a fraction of cells; this proportion increases with education.

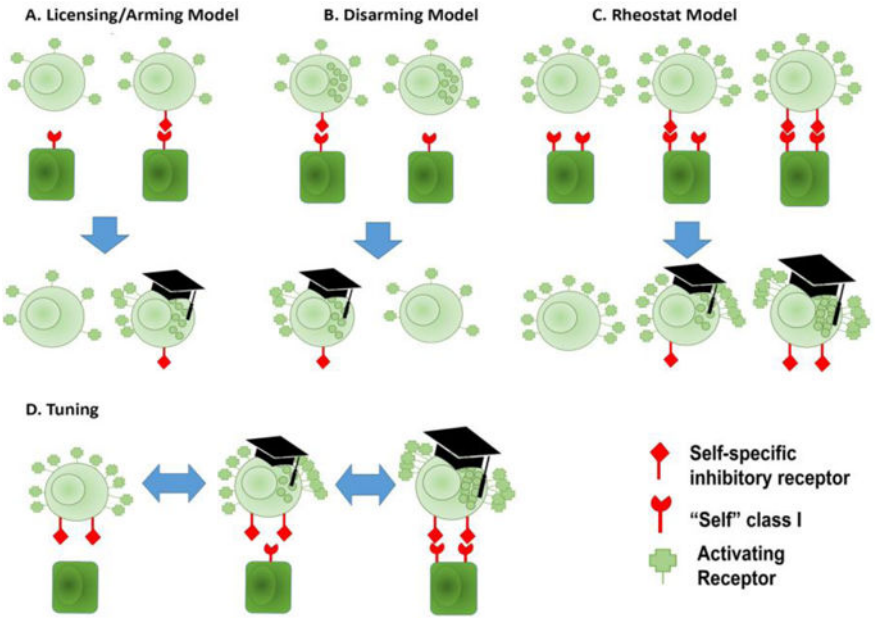

Figure 3. Models of NK education

Natural killer cells stochastically express activating and inhibitory receptors. The quantity of inhibitory input triggered by "self" MHC in each NK cell programs its education. Three models exist to describe the process of education. (A) The licensing/arming model postulates that developing NK cells capable of binding self-MHC class I molecules are endowed with higher effector potential, while those that cannot bind self-MHC class I molecules will be programmed for lower reactivity. (B) The disarming model postulates that all developing NK cells are initially capable of high effector responses, but only those that are capable of inhibition by self-MHC binding can rescue themselves from activation-induced anergy. As a result, those NK cells which are sensitive to "self" class I remain highly reactive, while those that cannot bind "self" class I lose effector potential, becoming comparatively hyporesponsive. (C) The rheostat model postulates that the avidity of the total interactions between inhibitory receptors and class I molecules tunes the reactivity of each NK cell. Those with fewer interactions with self-MHC class I binding exhibit the lowest effector potential, while those with intermediate and high numbers of interactions with self-MHC molecules exhibit intermediate and strong reactivity, respectively. (D) All three models are compatible with "tuning", wherein the reactivity of an NK cell can be adjusted up or down in response to changes in the local environment.

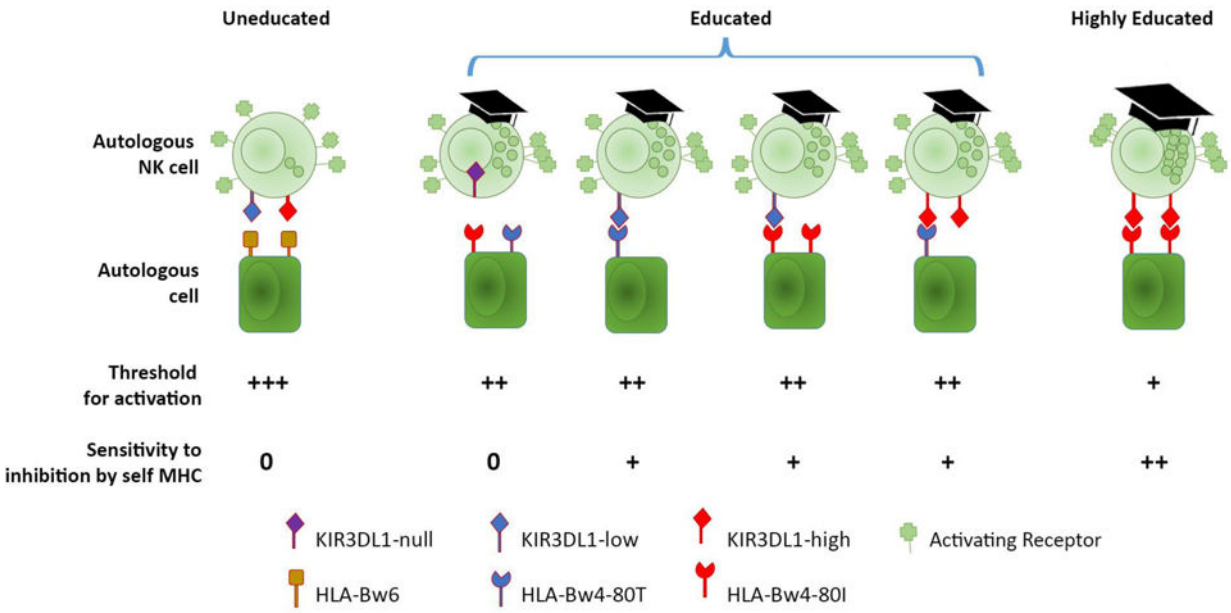

Figure 4. Graded education by allelic variation: the example of KIR3DL1 and HLA-B

KIR3DL1 is expressed as null (n), low (l) and high (h) -density alleles. Subsets of HLA-B exhibiting the Bw4 epitope act as ligands for KIR3DL1, with expression density and binding affinity correlating to the amino acid present at position 80: threonine (80T) or isoleucine (80I). Subtype combinations of KIR3DL1 and HLA-B generate a spectrum of reactive and inhibitory potentials among NK cells. Shown are the educating outcomes of various combinations of KIR3DL1 and HLA-B alleles. HLA-B molecules lacking the Bw4 epitope (Bw6) do not educate NK cells through any KIR3DL1 receptor. Both the Bw4-80I and -80T subtypes educate NK cells, with the highest education observed among subtype combinations of KIR3DL1-h and HLA-Bw4-80I. Intermediate education is conveyed by all other combinations of Bw4 and KIR3DL1, including the KIR3DL1-n subtype which is retained intracellularly but nevertheless conveys education to the NK cell. The intracellular retention of the KIR3DL1-n alleles enables education but protects NK cells from inhibition by cognate HLA-Bw4 presented in trans.

Table 1

The major NK receptor-ligand pairs in humans.

Human inhibitory receptor-ligand pairs		Human activating receptor-ligand pairs	
Inhibitory receptor	**Ligand**	**Activating receptor**	**Ligand**
KIR2DL1	HLA-C2 group (Lys^{80})	KIR2DS1	HLA-C2 group (Lys^{80})
KIR2DL2/3	HLA-C1 group (Asn^{80})	KIR2DS2	unknown
	HLA-C2 group (Lys^{80})		
KIR2DL5A/B	Unknown	KIR2DS3	unknown
KIR3DL3	Unknown	KIR2DS5	HLA-C2 (variable)
KIR3DL1	HLA-A and B alleles encoding the Bw4 epitope	KIR3DS1	HLA-F
KIR3DL2	HLA-A*03 and HLA-A*11	KIR2DS4	Subsets of HLA-C and HLA-A11
CD94/NKG2A	HLA class I signal peptides presented by HLA-E	KIR2DL4	HLA-G
LILRB1	HLA class I	CD16	Antibody Fc
KLRG1	Cadherins	CD94/NKG2C	HLA-E
SIGLECS	Sialic acid	CD94/NKG2D	MIC-A, MIC-B, ULBP1-6
NKRP1A	Lectin-like transcript-1 (LLT1)	CD94/NKG2E	HLA-E
		DNAM-1	CD155, CD112
		CD58	CD2 (LFA-2)
		NKp30	B7-H6, HCMV-pp65, heparin sulfate
		NKp44	Mixed-lineage leukemia-5 (MLL5), viral hemagglutinin, proliferating cell nuclear antigen (PCNA)
		NKp46	Complement factor P, viral hemagglutinin, heparin sulfate
		NKp65	Keratinocyte-associated C-type lectin (KACL)
		NKp80	Activation-induced C-type lectin (AICL)

Table 2

The major NK receptor-ligand pairs in mice.

Mouse inhibitory receptor-ligand pairs		**Mouse activating receptor-ligand pairs**	
Inhibitory receptor	**Ligand**	**Activating receptor**	**Ligand**
Ly49A	H-2D^{b}, D^{d}, D^{k}, D^{p} and H2-M3	Ly49D	MHC H-2D^{d}
Ly49C	H-2K^{b}, -K^{d}, D^{k}, D^{d}, D^{b}, D^{k}, m157	Ly49H	MCMV m157
Ly49F	H2-D^{d}	Ly49L	H-2K^{k}
Ly49G	H-2D^{d}, L^{d}	Ly49P	H-2D^{d}
Ly49J	H2-K^{b}	Ly49W	H-2D^{d}, D^{k}
Ly49I	H-2D^{b}, D^{d}, D^{s}, D^{q}, D^{v}, K^{b}, K^{d}, K^{s}, K^{q}, K^{v}, and m157	Ly49R	H-2D^{d},D^{k},L^{d}
Ly49J	H-2K^{b}	CD94/NKG2D	Rae-1, H-60a-c, Mult-1
Ly49O	H-2D^{b},D^{d},D^{k},L^{d}	CD94/NKG2E	HLA-E
Ly49V	H-2D^{b},D^{d}, K^{k}	NK1.1	MCMV m12
LILRB1	Overall MHC class I expression	NKp46	Complement factor P, viral hemagglutinin, heparin sulfate
PIR-B	MHC class I	NKR-P1	Clr-b
CD94/NKG2A	Qa1	PIR-A	MHC class I
CD94/NKG2E		DNAM-1	CD155, CD112