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Newtonian cell interactions shape natural killer cell education

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Summary: Newton's third law of motion states that for every action on a physical object there is an equal and opposite reaction. The dynamic change in functional potential of natural killer (NK) cells during education bears many features of such classical mechanics. Cumulative physical interactions between cells, under a constant influence of homeostatic drivers of differentiation, lead to a reactive spectrum that ultimately shapes the functionality of each NK cell. Inhibitory signaling from an array of self-specific receptors appear not only to suppress self-reactivity but also aid in the persistence of effector functions over time, thereby allowing the cell to gradually build up a functional potential. Conversely, the frequent non-cytolytic interactions between normal cells in the absence of such inhibitory signaling result in continuous stimulation of the cells and attenuation of effector function. Although an innate cell, the degree to which the fate of the NK cell is predetermined versus its ability to adapt to its own environment can be revealed through a Newtonian view of NK cell education, one which is both chronological and dynamic. As such, the development of NK cell functional diversity is the product of qualitatively different physical interactions with host cells, rather than simply the sum of their signals or an imprint based on intrinsically different transcriptional programs.

Keywords: *natural killer cells, major histocompatibility complex, repertoire development, cytotoxicity, differentiation, cell surface molecules*

Natural killer cell repertoire diversity: tolerance and functional flexibility combined

Natural killer (NK) cells express an array of germline-encoded receptors that determine their functional response against virus-infected and transformed cells (1, 2). Key receptors regulating NK cell function are the major histocompatibility complex (MHC) class I binding receptors: killer cell immunoglobulin-like receptors (KIRs) in humans and Ly49 receptors in mice. The KIR and Ly49 receptor families are both polygenic and highly polymorphic and each includes activating and inhibitory variants (3, 4). Whereas the latter have well-defined ligands in the polymorphic binding motifs on MHC class I molecules, the natural ligands for the former remains largely undefined, with only a few exceptions (5).

A unique feature of KIR and Ly49 receptors is their stochastic distribution of expression across the NK cell

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compartment (6, 7). Combined with genetic variability of the KIR/Ly49 locus at the levels of gene content, copy number variation, and allelic polymorphism, stochastic expression results in highly diverse NK cell repertoires in different populations. In human, the expression of KIR in combination with presence of one or more of four major MHC class I ligands (HLA-C1, HLA-C2, HLA-Bw4, and HLA-A3/A11), endow the majority of NK cells with at least one inhibitory KIR specific for a self-MHC molecule (8, 9). Despite the fact that the random distribution of KIR/Ly49 also leads to significant proportions of NK cells that altogether lack self-specific inhibitory receptors in both mice and humans (10–12), self-receptor expression still represents the major mechanism in the maintenance of self-tolerance. Partly compensating for the gaps in the repertoire left through stochastic expression, most KIR/Ly49-negative NK cells express CD94/NKG2A, an inhibitory receptor that binds to the ubiquitously expressed HLA-E (in humans) and Qa1 (in mice) (13–16). In addition to preserving tolerance, the random distribution of KIRs also tends to restrict the number of receptors expressed by each NK cell (13, 16). This is of particular importance as it provides the individual with a variegated repertoire that is capable of responding to pathogens that selectively downregulate specific class I MHC alleles.

It has been debated whether there are additional, cellular mechanisms involved in shaping the NK cell repertoire to further promote self-tolerance. Several models of positive and negative selection have been proposed including the sequential selection model and the two-step selection model (17, 18). Both models suggest that interactions with MHC during NK cell development results in selection of cells that express self-specific inhibitory receptors. These models have been substantiated by repertoire studies, both in mice and humans that showed a bias for expression of self-specific receptors, extending beyond those resulting from the expected combinatorial effects of a limited number of receptors and ligands (19, 20). However, an important distinction is whether this is the result of an intrinsic selection program that occurs during NK cell development or whether it is the product of repertoire skewing consequent to encounters with pathogen. Whereas Ly49 repertoires in mice seem to be intrinsically shaped under germ-free conditions (19, 21), evidence in the human suggest that repertoires are profoundly influenced by exposure to pathogens throughout life, particularly in the case of cytomegalovirus (CMV) infection. Thus, NK cell repertoires in individuals that are CMV negative are entirely stochastic and reflective of those found in cord blood (22, 23), which would preclude an instructive selection program during NK cell development or early differentiation. Expression of self-specific inhibitory receptors may instead protect against apoptosis, leading to skewing of the repertoire as an effect of cumulative survival under stress (24, 25). In the absence of a formal negative mode of selection, both mouse and human NK cell repertoires contain a significant fraction of cells that completely lack inhibitory self-specific receptors. The size of this population is around 10% in both human and mice (10, 11). To varying degrees this population is a mixture of cells that are immature and therefore less functional, and cells that are fully mature and contain effector molecules including perforin and granzymes (12, 26). To accommodate such cells and especially to avoid autoaggression, additional mechanisms are implicated. A major advance in the study of NK cells was the discovery that NK cells are functionally calibrated to the host MHC environment, so that cells lacking self-specific receptors are rendered hyporesponsive (10, 27).

Functional calibration of NK cell responses against self-MHC

The functional tuning of NK cells to self-MHC class I is termed NK cell education (28, 29). NK cells expressing inhibitory receptors for self-MHC class I display a strong missing-self response to targets that lack the very same MHC class I molecules (10, 12). Conversely, NK cells that lack self-specific receptors are hyporesponsive to stimulation with MHC class I-deficient target cells. Although we currently lack insights into the cellular and molecular mechanisms that regulate NK cell education, several models using different terminology (licensing, arming, disarming, rheostat) have been put forward to conceptually define the processes that determine the functional calibration against self-MHC (*Table 1*). The theoretical framework for each of these models and experimental data supporting them has been reviewed extensively elsewhere (30–32). Here, the intention is to elaborate on the context in which all of these models can possibly operate. The higher functional activity of educated cells suggests that NK cells must first achieve a state of activation conducive to a functional effector phenotype that either persists through inhibitory signaling (disarming/

Table 1. **Nomenclature**

Term(s)	Description
NK cell education	In this review, the term *education* refers to the functional calibration of NK cells against self-MHC class I, involving both uptuning through inhibitory input and downtuning through activating input. The dynamic functional tuning of the cell during education acts independently of the epigenetic and transcriptional changes occurring during NK cell differentiation. Furthermore, in contrast with previously used definitions (28, 29), changes to the repertoire, in terms of increasing/decreasing frequencies of specific cell populations, are not considered as elements of the NK cell education process. Although interactions with self-MHC class I may influence the NK cell repertoire, the mechanisms behind such effects, including imprints of viral infection, are probably distinct from those that set the functional potential of the cell
Licensing, arming and disarming	Licensing is the term used to describe the process starting with inhibitory signaling through ITIMs to the enhanced functional responses to stimulation through activating receptors (32). It makes no assumptions concerning the mechanisms involved other than the need for inhibitory signaling through ITIMs to generate the functional phenotype. Two distinct models describing the downstream effects have been proposed. The stimulatory licensing model infers a conversion of the inhibitory signal into an instructive positive regulator of effector function. The latter is largely synonymous with the *arming* model (17). The inhibitory licensing model suggests that the inhibitory input through MHC binding receptors interfere with positive signals from activation receptors. In the absence of such inhibitory input, cells get anergized by too much activation signals. This latter variant is developed further in the *disarming* model (17). Thus, the disarming model shifts focus to address the processes leading to hyporesponsiveness of NK cells lacking self-specific receptors. Unopposed continued activation of NK cells that fail to express inhibitory receptors leads to exhaustion and inability to respond to subsequent challenges
Theory of discontinuity	This theory explains how the immune system calibrates to its environment and is tuned to sense changes occurring at relatively short time scales (48). It does not attempt to explain the mechanism behind NK cell education, rather the context in which it operates. Therefore, it is compatible with all models of NK cell education

NK, natural killer; MHC, major histocompatibility complex.

inhibitory licensing model) or where the inhibitory signal itself changes the nature of the cell expressing it (arming/stimulatory licensing model). To date, a multitude of studies have explored how different receptor configurations and the strength of their interactions with cognate MHC ligands can influence downstream functional responses, as determined both *in vitro* and *in vivo* (13, 33–37). A growing body of evidence is emerging that demonstrates how these functional states are dynamically tuned in different environments (38–40). However, we currently have limited insights into the nature of the cellular interactions and the time scales that occur upstream of the signaling events connected to education in such studies and the mechanisms that regulate the functional potential of the cell over time. Moreover, although models such as the rheostat (30) includes a contextual aspect of NK cell functionality and describe dynamic features of NK cell education, the current models have yet to fully integrate the parallel functional maturation of NK cells occurring as a result of cellular differentiation, exposure to homeostatic cytokines, and pathogenic drive.

Need for prior sensitization after all?

NK cells are commonly referred to as innate lymphocytes that display strong cytolytic potential in the absence of prior sensitization. However, recent developments in NK cell biology have challenged this view (41). The functional potential of the cell is the product of multiple interactions with other cells under exposure to homeostatic cytokines and is exemplified in the need for priming of naive NK cells through trans-presentation of interleukin-15 (IL-15) by dendritic cells (42, 43). These interactions are likely to occur continuously over long time-scales, probably months or even years, and shape the functional potential of the cell in parallel with the intrinsic epigenetic remodeling of the cell that occurs during differentiation (44–46). However, this integrated and time-dependent functional model raises many unknowns. In particular, we lack information about spatiotemporal aspects of the cellular interactions. Where do they take place? Which cell types are involved and what is the frequency and duration of the contacts? Here, we attempt to delineate how physical cellular interactions shape the functional potential of the cell in a manner that provides a basis for NK cell education, superimposed on the transcriptionally regulated cellular differentiation program. The imprint of these cellular interactions, in terms of functional potential, bears resemblance with Newtonian classical mechanics and the reaction of an object to an external perturbation (47). The continuous calibration of the cytotoxic potential through Newtonian interactions serve to maintain tolerance at steady state and determine the response of the

individual cell to sudden changes in the environment (discontinuity) (48), for example the loss of an MHC allele. Before dissecting the continuous cellular interactions that ultimately shape the functional potential of the cell, we review the currently available data that defines the educated state.

Functional tuning of NK cells in mice and humans

Although the description of hyporesponsive NK cells lacking self-specific receptors and the concept of NK cell education are relatively recent, the influence of MHC class I on NK cell functionality had already been well-established in earlier studies. Mice and humans with substantially lowered MHC expression due to a deficiency in the transporter associated with antigen presentation (TAP) had normal numbers of phenotypically mature yet hyporesponsive NK cells (49–52). Using mouse strains with disparate MHC settings as well as using single chain MHC transgenic mice, Kim *et al.* (27) were the first to show a direct positive role for inhibitory Ly49 receptor interaction on the capacity of NK cells to respond to ligation of activating receptors. Reconstitution of NK cells from bone marrow cells engineered to express a self-reactive Ly49 receptor led to increased functional responses, provided that the cognate ligand was present in the mouse strain. Deletion of the cytoplasmic tail or mutation of the immunoreceptor tyrosine-based inhibition motif (ITIM) abrogated the increase in functional potential, suggesting that education is dependent on continuous input through the inhibitory receptor itself (27).

Fernandez *et al.* (10) characterized Ly49 repertoires in B6 mice and noted that approximately 10–13% lacked self-MHC specific receptors. This subset of potentially autoreactive NK cells was hyporesponsive to cross-linking of activating receptors or stimulation with MHC-deficient tumor cells. On the other hand, they responded normally to infection with *Listeria monocytogenes in vivo*, suggesting that the poor response to receptor-ligation was not due to a general inability of the cells to become activated and secrete IFN-γ. Shortly after these initial studies, Anfossi *et al.* (12) reported the existence of similarly hyporesponsive subsets in the human.

Using refined multi-parameter flow cytometry panels the function of NK cell subsets expressing various KIR/Ly49 receptors and combinations thereof have been examined (13, 33–37). These studies revealed that the functional potential of the cell is shaped by the strength of the inhibitory input, including variation in binding strength caused by allelic polymorphism of the receptor and the ligand. For example, the missing self response of KIR2DL3 single positive NK cells was relatively stronger in donors carrying the HLA-Cw*07 (C1) compared to those educated by the weaker interaction with HLA-Cw*1402 (C1) (13). Conversely, KIR3DL2 single positive NK cells were hyporesponsive even in individuals harboring the proposed cognate ligands HLA-A3/A11 (11, 13). KIR3DL2 binding to HLA-A3 was shown to be dependent on presentation of EBV-derived peptides (53). Thus, in non-infected individuals or during latency this interaction is either non-existing or of weak affinity and therefore incapable of educating of KIR3DL2-positive NK cells. Likewise, KIR3DL2 binding to HLA-F during conditions of cellular stress does not seem to impose functionality in this subset at steady state (54).

Co-expression of multiple inhibitory receptors to self-MHC class I boost the response in a near additive manner (13, 33, 34). Ultimately, the effect of expressing multiple inhibitory receptors may reach a plateau, at least in some contexts where expression of an additional receptor had no influence on the magnitude of the response (33, 55, 56). Notably, cells expressing two allelic variants of KIR2DL3 had higher total densities of KIR2DL3 but were not more functional than the subset expressing the strongest allele (55). Similarly, variation in ligand densities does not seem to influence education (35, 57, 58). Whereas strong inhibitory interactions potentiate the missing self response, much like a Newtonian reaction to a potent action on the cell, NK cells expressing the activating KIR2DS1 were found to be hyporesponsive in donors homozygous for the cognate C2 ligand (59, 60). Thus, in terms of signal strength in the ability to interact with class I MHC, both in mice and humans, NK cell education appears to occur along a continuum rather than digitally as an all or nothing response (30).

A particularly interesting aspect of this model is the dynamic change in functionality that occurs in response to change in the host MHC environment. Thus, mature educated NK cells transferred from MHC-sufficient to MHC-deficient mice lose their functional responsiveness within 4 days (39, 40). Conversely, transfer of hyporesponsive NK cells expressing a non-self inhibitory receptor to an educating environment led to uptuning (resetting) of their functionality, independently of *in vivo* proliferation. Information concerning which cell type(s) provide the educating ligand in the new host remains sparse but studies in chimeric mice as well as in humans undergoing stem cell transplantation have

provided some insight and suggest that both hematopoietic cells and stromal cells may contribute to NK cell education. Supporting a role for donor-derived hematopoietic cells, donor MHC determines the education status of the NK cells following transplants where the whole hematopoietic environment is engrafted (61–63). In contrast, NK cells transferred in isolation rapidly adapt to the new host under the influence of recipient MHC (39, 40). Using mice with inducible expression of MHC, Ebihara *et al.* (38) elegantly showed that the strongest educating impact was mediated by hematopoietic cells rather than stromal cells and that neither homotypic interactions between NK cells nor *cis* interactions were capable of providing educating signals. Notably, this does not exclude that NK cells are capable of providing some degree of educating signals to themselves in *cis* or to neighboring NK cells in trans (64, 65), since it is entirely possible that the cells were not present in sufficient numbers to interact and so dominantly influenced by cellular interactions with host cells in this model. Early observations in mice where MHC was expressed in a mosaic fashion demonstrated that tolerance of the whole NK cell compartment could actually be maintained by as few as 20% MHC-negative host cells (66). These data suggest that the thresholds for uptuning are higher than those required for downtuning NK cell functionality, or that the kinetics of the two events differ in such a way that uptuning is unable to progress past an effective threshold before cells are downtuned by interactions with MHC class I deficient host cells. Overall, tolerance seems to be favored over maintenance of high functionality.

Receptor-binding to non-classical MHC class I molecules is likely important for balancing the overall functionality of the NK cell repertoire (13, 16). Interaction of Ly49A with the non-classical MHC molecule H2-M3 was shown to promote missing self-recognition acting both in isolation and in synergy with Ly49A-H-2D^{d}-mediated education (67). Stratified subset analysis also revealed a role for NKG2A in NK cell education (11, 13, 26, 37, 68). Thus, NKG2A^{+} NK cells are functional even in the absence of KIR/Ly49 and act additively to the education mediated by KIR/ly49-MHC interactions. Since HLA-E/Qa-1 are ubiquitously expressed, NKG2A^{+} NK cells are typically educated in all individuals. This may be particularly relevant in the context of stem cell transplantation where NKG2A^{+} NK cells have been shown to dominate the functional NK cell repertoire during the first 3 months (61, 69). Recent evidence suggests that dimorphism at position 2 (P2) (methionine versus threonine) significantly influenced the strength of the NKG2A-HLA-E interactions and the functional response of NKG2A^{+} NK cells to target cells lacking HLA-E (70, 71). Notably, NK cells expressing NKG2A but not those expressing self KIRs are functional in the fetus (72). This remarkable finding opens up for the existence of multiple mechanisms to endow NK cell with functional potential. A remaining outstanding challenge is to decipher the cellular mechanisms for KIR-mediated education that are lacking in the fetus yet emerge shortly before or during birth to mediate education of NK cells in cord blood (23).

In search of a cellular and molecular marker of education

Attempts to define a molecular signature for NK cell education by transcriptional mapping have been inconclusive (73). Similarly, attempts to define differences in upstream activating signaling pathways have failed (29). Thus, selective deficiencies in signaling molecules may cause alterations in NK cell repertoires and specific loss of function variants but cannot account for the broad hyporesponsive phenotype of NK cells that lack self-specific inhibitory receptors. However, using spot variable fluorescence correlation spectroscopy, Guia *et al.* (73) suggested that the educated phenotype correlates with a unique organization of receptors in the cell membrane. Activating receptors, including the natural cytotoxicity receptor NKp46, were suggested to rearrange from an actin cytoskeleton meshwork into nanodomains upon education (73). Such membrane organization of activating receptors may facilitate signaling and, thus, influence conjugate formation as well as the ability to respond to receptor-ligation during the effector phase (74).

Although morphological correlates with NK cell education may hold clues to the underlying biology, it remains technically challenging to use these characteristics to identify the educated cell in downstream assays. In the absence of available phenotypic markers, studies into NK cell education have largely been dependent on probing the functionality of different subsets *in vitro* or *in vivo* to resolve the educational status of the NK cell subset (29, 75). The requirement for functional read-outs as a means to resolve NK cell education has led to a bias toward examining differences in the integration of proximal signaling during the effector phase of NK cell responses, and away from attention to spatiotemporal aspects of NK cell regulation. Furthermore, the majority of such assays are quantitative in the sense that they measure the frequency of responding cells rather than qualitative aspects of the response of individual cells. Clearly, the lack of a marker together with the lack of a transcriptional signature has been a major hurdle for deciphering the

mechanisms behind NK cell education. Much like Heisenberg's uncertainty principle, stimulation of the NK cell during monitoring may abrogate important features that hold clues as to the pre-existing heightened functional potential of the cell.

DNAM-1 as an intrinsic marker of the functional potential of educated NK cells?

Phenotypically, hyporesponsive NK cells appear to be mature, with similar levels as educated NK cells for every species of activating receptor tested (10, 12, 76). The only exceptions are the higher relative expression of KLRG-1 in educated mouse NK cells and the correlation with DNAM-1 expression in humans (10, 77). Careful assessment of these molecules may provide insights into the upstream cellular interactions that shape the education status of the cell. Adoptive transfer of NK cells into a new ligand environment lead to dynamic changes in KLRG-1 expression (40). Although KLRG-1 is linked to T and NK cell differentiation (78), no other phenotypic markers defining the differentiation states in NK cells appear to change specifically as the NK cell rheostat is adjusted, indicating that the expression of KLRG-1 somehow marks the educated NK cells. A potential limitation of KLRG-1 is its differential regulation on human NK cells where expression is inversely correlated with the number of expressed inhibitory KIRs and with differentiation (76). In contrast with KLRG-1, DNAM-1 is tightly linked to the expression of self-specific receptors in both human (12, 77) and in mice (Benedict Chambers, personal communication). A reductionist model system, based on expression of single and multiple ligands in *Drosophila* cells (79), was used to establish DNAM-1 as a cell-intrinsic marker for NK cell education, independent on interactions with its ligands CD155 and CD112 (77, 80).

DNAM-1 is a co-activation receptor and adhesion molecule that is involved in LFA-1-mediated synapse formation in NK and T cells and critical for the recognition of many tumor types (81–84). Intriguingly, recent studies indicate that DNAM-1 is also involved in differentiation of adaptive NK cells (85) and defines two functionally distinct subsets of mouse NK cells. Notably, in mice DNAM-1^+ NK cells were relatively immature and could differentiate into more mature DNAM-1 negative NK cells (86). This appears to differ from the human, where the most differentiated NK cells, defined by expression of self-specific KIRs and CD57, expressed the highest levels of DNAM-1 (77). A poorly defined subset of DNAM-1^- NK cells has been observed in patients with cancer, and although this subset was induced through receptor-downregulation by stimulation of NK cells with CD155 expressing targets (87–90), it remains a possibility that this population contain cells with the unique maturation profile of DNAM-1^- NK cells described in mice (86).

DNAM-1 is physically associated with the open conformation of LFA-1 in mouse T cells at the immune synapse, thereby facilitating the early signaling events during effector-target cell interactions (91). LFA-1 is an essential component of immune synapse formation (92–94) and polarization of cytotoxic granules (79). In the absence of LFA-1/ICAM-1 interactions, NK cell activation leads to non-polarized degranulation and limited killing. Educated NK cells were recently shown to form more frequent and stable conjugates, which were dependent on the conformational change in LFA-1 (74). Therefore, we examined the spatio-temporal dynamics of DNAM-1 and LFA-1 in the cell membrane during the effector phase. Strikingly, DNAM-1 and LFA-1 expression were tightly coordinated in educated NK cells, potentially providing a mechanism to regulate cytotoxic responses (77). Thus, DNAM-1 expression defines functional states and links NK cell activation to the downstream engagement in functional immune synapses with target cells (95).

Is there a qualitative definition of the educated state?

Although we commonly think of education as a process that changes the qualitative state of the individual cell, there is very limited evidence supporting this notion. The functional tuning of a given subset is primarily reflected in the size of the responding fraction within that subset (IFN-γ, CD107a TNF) or the *in vivo* rejection efficiency of the population as a whole. Although there is some evidence for qualitative changes occurring at the single cell level, i.e. increased mean fluorescence intensity of IFN-γ (33), further studies using imaging platforms that allow visualization of single NK-target interactions are required to address differences in the kinetics and efficiency of the lytic hit at the single cell level (96). While this issue may seem trivial, it is indeed very important to resolve. As pointed out above, educated NK cells form more conjugates with targets compared to hyporesponsive NK cells. This is likely mediated through increased inside-out signaling to LFA-1 following receptor ligation resulting in an open conformation state of LFA-1 (74). Intriguingly, polarization of cytotoxic granules was not qualitatively different in hyporesponsive NK cells in the few conjugates that did form, suggesting that the effector

program itself is unaffected. Given that the ability to form conjugates *per se* influences the result of all target cell-dependent *in vitro* read-outs, as well as *in vivo* rejection models, quantitative differences can partly be accounted for in the capacity of cells to form cell-to-cell interactions. In this respect, the coordinated expression of DNAM-1 and LFA-1 in educated NK cells provide one important clue, since the differential expression of DNAM-1 could influence target cell conjugation and thus many of the down-stream functional outcomes. However, we still lack a precise determination of exactly how inhibitory input translates into modulation of the level of DNAM-1. Furthermore, educated NK cells are more functional also in DNAM-1/LFA-1 independent assays that bypass the need for conjugation, such as those based on antibody-coated plates (75). Thus, it is likely that education encompasses qualitative changes at the single cell level that define cell intrinsic differences in functional potential beyond the increased capacity to form conjugates with targets. Indeed, using single cell imaging in nanowells (97), we recently noted qualitative differences associated with education, manifested as increased fractions of lytic events following target cell conjugation (E. Forslund and B. Önfelt, unpublished data).

NK cell education through series of well-defined cellular events?

In the absence of a transcriptional program that regulates protein expression, there lies the possibility of an as yet undiscovered mechanism that acts on the cell to instruct effector potential. Regardless of the preferred model, arming or disarming, it is believed that the hyper- or hypo-responsive states, respectively, must be linked to different thresholds for stimulation through activation receptors. Although it is natural to think that the mechanism behind NK cell education must be novel, discrete, and complex, it is also possible that the solution is in fact very simple and based entirely on multiple and already well-defined cellular interactions. In this case, it may be possible to define the simplest solution that fit with all currently available data. Given there is no unknown mechanism behind NK cell education, which key steps are likely to be involved in determining the functional state of the cell? Which fragments of known facts need to be fitted together to define a process that increase (or decrease) effector function of cytotoxic lymphocytes in an MHC-dependent manner?

There is robust experimental evidence for (i) cytokine-driven acquisition and maintenance of effector function during normal homeostatic cellular differentiation (42, 98, 99), (ii) the need for signaling (or lack of signaling) through inhibitory Ly49/KIR (27, 100), and (iii) a role for deterministic cellular interactions with host educator cells (38–40). To decipher how these fundamental qualities define the functional phenotype, we need to consider the influence of inhibitory Ly49/KIR on cellular interactions in the context of self during homeostasis. The known outcomes of this process are (i) increased (or decreased) expression of DNAM-1 (12, 77), (ii) decreased levels of self-specific Ly49/KIR (56, 101–103), and (iii) dynamically tuned responsiveness to subsequent challenges (12, 13, 33, 39, 40). In the next section, we consider NK cell homeostasis and differentiation, which forms the basis for dynamic functional tuning by self-specific receptors.

NK cells reach out for self during differentiation

NK cell differentiation, a template for functional diversification

The homeostasis of NK cells is largely unexplored. Early work using *in vivo* deuterium labeling suggested that NK cells have a very quick turn over with a median turn over time of 14 days (104). We now know that this notion may be revisited based on stratified analysis of the NK cell compartment using markers that define transitioning phenotypes from naive $CD56^{bright}$ NK cells to terminally differentiated NK cells (76, 105, 106), the resolution of which will only increase with emerging technologies (107). The development of the mature NK cell repertoire is a cumulative process revealed by the pooling of cells at discrete stages based on receptor expression (76). Since for the majority of receptors the expression is digital, discrete stages of differentiation can be ascribed to expression of functional receptors at different stages of effector cell progression. The naive ($CD56^{bright}$ $CD16^{-}$) and immature NK cells tend to express NKG2A, whereas the most mature cells tend to lack NKG2A and natural cytotoxicity receptors (NCR), acquire CD57, and express KIR in combination with other inhibitory receptors (such as ILT2 and siglec7). Mature $CD57^{+}$ NK cell subsets accumulate over time in an age-dependent manner (108). This gradual NK cell differentiation is a striking feature during immune reconstitution following stem cell transplantation and manifested in a shift from predominance of $NKG2A^{+}KIR^{-}$ cells to mature cells with KIR repertoires resembling those of the donor (76, 109).

NK cells expressing self-specific inhibitory receptors have been shown to resist apoptosis (24, 25), suggesting that

there is a range in the relative age of cells that is connected to their state of differentiation and education status, leading to the accumulation of developed cells over time in response to the tides of infection (22). As with memory T cells, it is likely that functionally mature NK cells are retained through either homeostatic cell division in response to cytokine such as IL-15, the effect of which attenuates through differentiation (76), or low level activating signals mediated by activating receptors that recognize ligands expressed at rest or intermittently at regular intervals (48).

The writing on the (cell) wall: receptor expression occurs randomly under epigenetic drive

In the same sense, functional development within the cell is also a cumulative process that develops under the shifts in positive and negative signaling, both epigenetically and in the normal cell-to-cell contacts that occur under homeostasis. The accumulation of multiple KIRs on differentiated cells is indicative of progressive epigenetic remodeling through sustained or repeated stimulation (110, 111). In both T and NK cells, the sequential upregulation of KIR appears to occur independently of self-MHC recognition (16, 112). Instead, the KIR expression on NK cells is genetically hard-wired, stochastically regulated, and highly stable over time (22, 113, 114). Yet, the functional NK cell repertoire is highly adapted to the MHC environment. Therefore, the acquisition of KIR during NK cell differentiation takes the cell on different trajectories based on whether the KIR interacts with its cognate ligand or not (*Fig. 1*).

Cytokine stimulation rapidly induces a robust NK cell effector phenotype even in naïve and receptor negative cells (76, 115, 116), suggesting that all NK cells have the intrinsic ability to reach a cytolytic phenotype given sufficient stimulation, even from early stages of differentiation and in the complete absence of educating input. This argues against inhibitory receptor mediated 'arming' as an absolute driver of NK cell function. However, it is the educated cells that retain the capacity to deliver a cytotoxic response in the absence of additional stimuli under homeostasis (i.e. it is the educated cells that have the capacity for 'spontaneous' reactivity).

Primary drivers of NK cell differentiation, most likely homeostatic cytokines such as IL-15, provide the basis for NK cell functional development, which is then diversified by inhibitory interactions affecting the functional potential and maturation rate of the cells. This is reflected most profoundly in the functional heterogeneity associated with expression of KIR. Differentiation occurs in a manner that is influenced by education but not absolutely dependent upon it (76). It is possible to detect receptor negative and hyporesponsive cells even at the most terminal stages of differentiation ruling out an absolute effect of education on NK cell differentiation. However, the frequencies of educated NK cells gradually increase with differentiation, suggesting an increasing effect

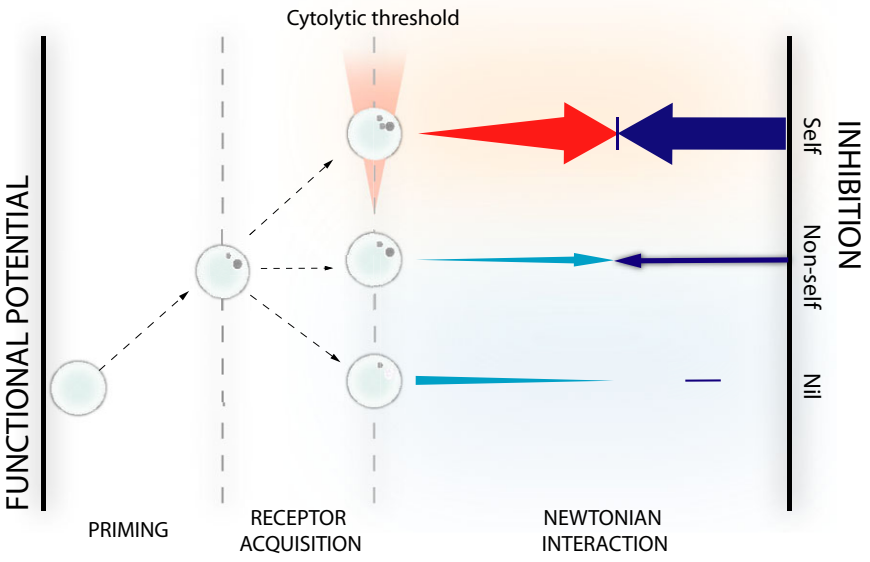

***Fig. 1.* Functional impedance builds killer potential.** The immune trajectory taken by differentiating natural killer (NK) cells develops as a function of effector priming, such as interleukin-15 (IL-15) stimulation, and concomitant expression of inhibitory receptors, which determine the persistence of effector priming through variable interaction with self-ligands. In this manner, NK cells develop a functional effector potential in proportion to the strength of inhibitory signaling mediated through receptors that recognize their ligand.

of both specific (educating) and general inhibitory signaling on persistence of differentiated cells (25). This is particularly apparent in mice and in healthy donors with past CMV infection (22, 24). Adaptive NK cells, expanding in response to acute CMV infection display a strong bias for expression of self-specific receptors (22, 117, 118). Notably, however, the near perfect random distribution of KIR in differentiated subsets from CMV-negative individuals suggests that the link between education and differentiation occurs in response to external influence (22), possibly related to cellular stress/starvation during enhanced proliferative cues (25). Indeed, adaptive NK cells in patients with deficiency in the TAP show polyclonal, random KIR repertoires (V. Beziat and K. J. Malmberg, unpublished data). Thus, NK cells differentiate normally and are fully capable of undergoing virus-driven proliferation in the context of adaptive NK cell responses independently of education (119).

Time for self-reflection

Education occurs within the context of differentiation and provides functional flexibility by acting on a shorter timescale. The interconnection between the two also suggests that there is a point in NK cell development at which the role of education is most prominent. The degree to which education influences the functional capacity diminishes as the cells become more differentiated (J. P. Goodridge and K. -J. Malmberg, unpublished data), possibly due to the incorporation of accumulating inhibitory interactions. Notably, this flexibility in function is tightly connected to the read-out used to monitor the educating impact (*Fig.* 2). The capacity to degranulate is less influenced by NK cell differentiation than TNF-α and IFN-γ production, the latter being imprinted epigenetically in terminally differentiated NK cells (44, 46). Thus, for CD107a responses, inhibitory input through KIR remains important throughout NK cell differentiation, whereas the difference in cytokine responsiveness is most prominent at more naïve stages. Furthermore, education through NKG2A is best monitored by measuring CD107a whereas KIR-mediated education induces robust IFN-γ responses (69).

The uncoupling of education from differentiation is also reflected in the manner these processes contribute to NK cell immunity. Using the conceptual framework of the discontinuity theory of immunity, which operates under the assumption that the immune system is geared to detect sudden changes in the host (48), education can be viewed as a process that provides the cell with a capability of detecting negative discontinuity (such as loss of ligand)

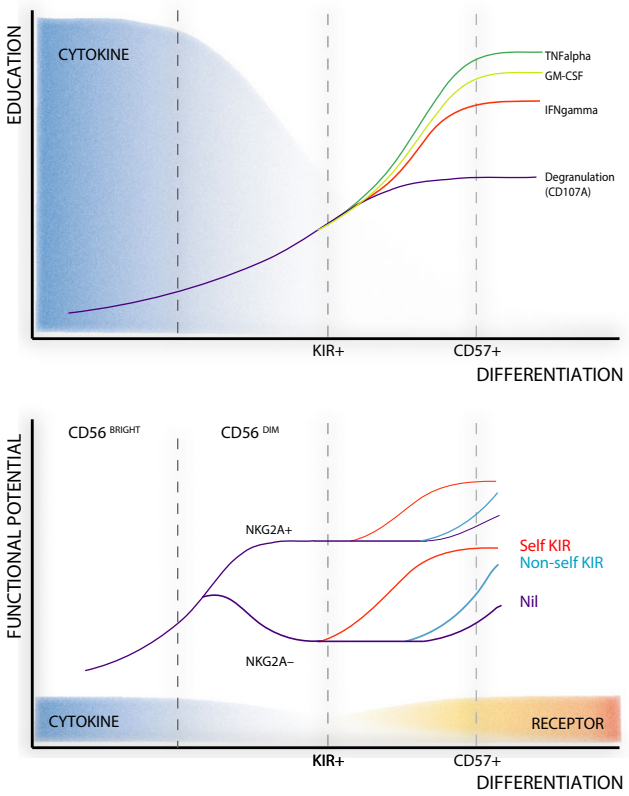

***Fig.* 2. Natural killer (NK) cell education is embedded within the broader context of NK cell differentiation.** Differentiation from the naive CD56^{bright} subset begins with broadly stimulating input, reflected in the higher responsiveness of naive NK cells to cytokine (blue field). As the cells mature responses become increasingly specific and receptor driven. The functional profiles of maturing NK cells, linked to increasingly specific activating signals, develop over time. While degranulation is apparent from very early stages, receptor-driven TNF-α production is epigenetically tied to later stages of differentiation (upper). In parallel, differentiating NK cells also accumulate increasingly specific inhibitory signaling, the effect of which can be stratified by recognition of self (lower).

whereas differentiation occurs as a spectrum of responses to positive discontinuity (such as infection, cytokine expression). Thus, education primarily influences the responsiveness of NK cells to missing-self, with a minor influence on cytokine- or antibody-dependent cellular cytotoxicity (ADCC)-induced responses, which dominate the responses of naive and terminally differentiated NK cells, respectively (*Fig.* 2). With mounting inhibitory signals that accumulate throughout differentiation, effector function becomes subject to progressively greater and increasingly specific stimulation, such as ADCC or synergistic stimulation through combination of receptors (120), to release stored functional potential. In the next section, we discuss the cellular interactions and the inhibitory input that tune the functionality of the cell at any given state of differentiation.

A Newtonian view of NK cell education

NK cells circulate in the continuous presence of their natural ligands, as opposed to adaptive cells that respond to very specific stimulus and require time to develop their functions. In addition to functional priming by cytokines, the development of NK cell education also requires physical cell-to-cell interactions with host cells (38–40). NK cells must retain the capacity to form regular cell-to-cell contacts, whilst simultaneously preserving their heightened functional potential. It is therefore very likely that the ability of NK cells to form contacts, and the bearing that inhibitory signaling has on either the rate at which this happens, or the outcome of signaling that results from each cell-to-cell interaction are connected to NK cell education. Although we have limited insights into the nature of the cellular interactions that determine NK cell education, class I MHC ligands expressed by hematopoietic cells appear to make a dominant contribution, while stromal cells appear to make a minor contribution (38). Normal homeostatic cell-to-cell interactions may therefore represent the principle determinant for the maintenance of NK cell reactive potential. These interactions operate within the context of the cumulative effects of differentiation and are reflected in the phenotype of the NK cells observed under homeostasis.

DNAM-1 and KIR expression intensities: two pieces of the puzzle

Cytokine receptors and NCR are progressively downregulated throughout NK cell differentiation, but the same downregulation is not reflected as a difference between educated and hyporesponsive NK cells. Thus far, only two unique phenotypic features of educated NK cell have been defined: increased levels of DNAM-1 (12, 77) and decreased expression of self-specific Ly49/KIR (56, 101–103). It has still to be determined whether the differential expression of DNAM-1 is due to increased expression on educated NK cells or downregulation on hyporesponsive NK cells. However, the distribution pattern in the context of differentiation, where it is expressed at high levels in the $CD56^{bright}$ cells, and at its lowest in receptor negative ($NKG2A^{-}KIR^{-}$) $CD56^{dim}$ cells argues in favor of downregulation, given its positive correlation with inhibitory receptors in mature cells (77). Using a reductionist approach with *Drosophilia* insect cells transfected with PVR, we showed that the expression of DNAM-1 is rapidly downregulated upon interaction with its ligand (88). These data support the notion that DNAM-1 downregulation on NK cells reflects the frequent and cumulative 'Newtonian' interactions between NK cells and host cells. DNAM-1 may therefore act as a gauge for the net interaction between NK cells and their environment.

In considering quantitative and/or qualitative differences during physical cell-to-cell interactions, it may be possible to account for downregulation of DNAM-1 and KIR/Ly49 in hyporesponsive and educated NK cells, respectively, through the kinetics and accumulating downstream effects of such interactions (*Fig.* 3). Therefore, determination of the manner in which KIR/Ly49 inhibitory signaling may influence transient, non-cytotoxic interaction is a key to understanding the development and persistence of NK cell functional potential. A central question is whether a difference in kinetics due to the upstream termination of synapse formation by self-KIR blocking progression of the synapse and conjugate formation (effectively limiting cell-to-cell contact), or that the rate of contact is equivalent and inhibitory signals abrogate downstream signaling mediated by activating receptors.

Synapse progression and its possible influence on education

Most experimental studies into the immune synapse compare the cytolytic synapse (educated NK cells versus MHC class I negative allogeneic targets) and the inhibitory synapse (educated NK cells versus allogeneic targets expressing self-MHC ligands). In the context of the upstream phase of NK cell education, it is important to distinguish classical NK cell interactions with target cells from those with normal healthy cells. Although educated NK cells appear to form more conjugates with MHC-deficient targets (74), it is less clear whether the same cells are affected in their capacity to form conjugates with normal healthy cells *in vivo* and whether these are qualitatively different (conjugation time, progression of the synapse, triggering of exocytosis).

Synapse formation between an NK cell with any other cell progresses as a function of the sum of receptor signaling (121). However, surprisingly little definitive information relates to the initiation of contact between an NK cell and a cell of interest, particularly for NK cells at rest, and the molecules that mediate this process. One reason may be the difficulty in tracing intermittent or short-term interactions that occur between normal healthy cells in an *in vitro* setting. Initiation of the T-cell synapse is driven predominantly through TCR recognition of pMHC, which in association with adhesion receptors such as LFA-1, form a tight junction between the cells (122). Likewise, the initial

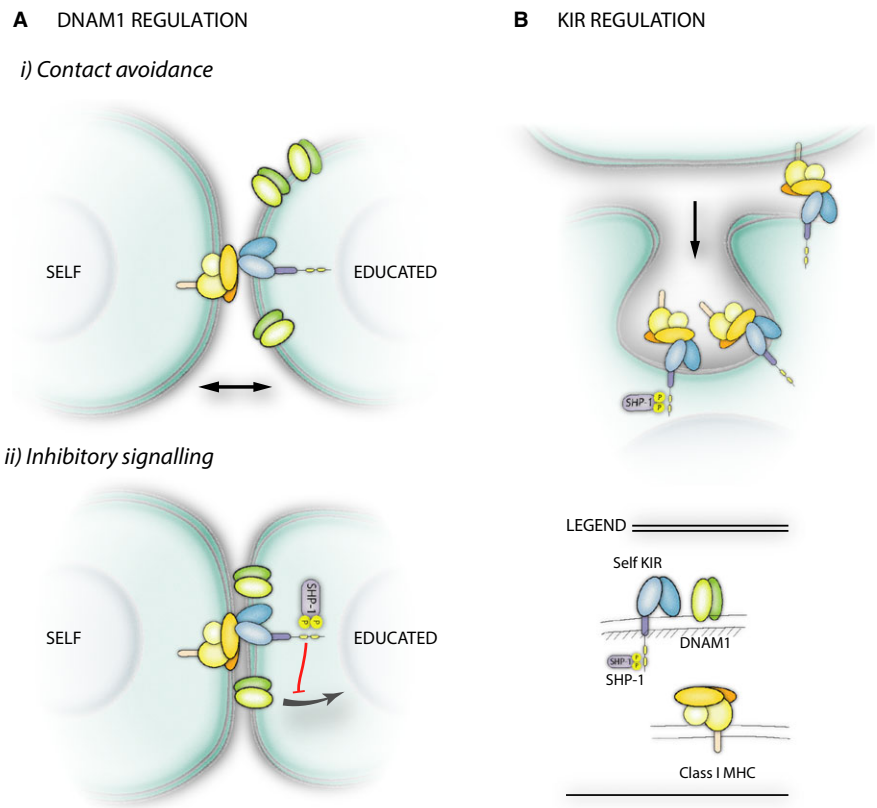

***Fig. 3.* Phenotypic hallmarks of Newtonian natural killer (NK) cell interactions.** Phenotypic differences between hyporesponsive and educated NK cells are reflected in the surface expression DNAM-1 and KIR/Ly49. The basis for these differences may hold important mechanistic clues into NK cell education. (A) The downregulation of DNAM 1 is dependent on whether it occurs upstream or downstream of NK cell synapsing. (i) Inhibitory termination of early events leading to synapse formation and progression would effectively prevent recruitment, internalization, and degradation of DNAM-1. (ii) Assuming that cell-to-cell contacts are qualitatively similar in educated and uneducated subsets, inhibitory signals may instead directly prevent ligand-mediated DNAM-1 internalization that occurs in activating synapses that lack inhibitory signals. (B) Downregulation of KIR in the presence of self-ligands may be the result of cell-to-cell transfer or internalization following receptor-ligation in *trans*. Selective downregulation of self-specific KIR indicates qualitatively different interactions with host cells. It is still open whether inhibitory signaling persists from within the cells after internalization.

recognition events that occur when an NK cell first contacts another cell, given sufficient stimulation, are focused on stabilizing the contact between the two cells (123).

Inhibitory signaling is best characterized for the effect it has on preventing the structural changes that occur in the reorganization of actin and the cytoskeleton that coordinates a stable interface between the cells. In doing so polarization of the lytic machinery to the synapse is disengaged, although granule convergence still occurs (124). The role of inhibitory signaling in disengaging immune synapse progression is well-established, and centralizes around dephosphorylation of Vav-1 (125), which prevents (WASP-mediated) actin polymerization and stabilization of the cell-to-cell contact (126). The effect that inhibitory signaling has on cell-to-cell interaction is such that NK cells expressing self-specific NK cell receptors may be able to retain their functional potential through contact avoidance, whereas those cells without no such inhibition to prevent adherence progress further into synapse formation. The net effect of multiple such transient disarming conjugates (TDC) would be the progressive downtuning of effector potential (disarming). It is tempting to speculate that positive signaling through DNAM-1 during the interaction with host cells triggers the unpotentiated release of effector molecules, and the downregulation of DNAM-1, thereby gradually inducing the hyporesponsive state of the cell.

These continuous Newtonian cell interactions could also account for the well-established downregulation of self-specific Ly49 and KIR (56, 101–103). The level of downregulation partly reflects the strength of the receptor-ligand binding and was used to define the educating impact as determined by *in vivo* rejection of MHC class I negative cells (56). The basis for receptor downregulation is currently unknown, however, given that NK cells bearing self-receptors circulate

in the continuous presence of their ligand, it is tempting to speculate that the downregulation is the product of increased rate of internalization and/or transfer between cells (trogocytosis). Exchange of receptor in the presence of its ligand has been observed in both directions (127). Whether the inhibitory receptor downregulation contributes to intracellular persistence of signaling remains an open question. In T cells, persistence of TCR signaling was found to occur within the cell in a dynamin-dependent manner, influencing the metabolic program (128).

Persistence of signaling within the cell may be highly relevant when taking into account the broader kinetics of immune progression and maintenance of potentiated cells. Notably, it was found that β-arrestin-2 is also a requirement for inhibitory signaling through KIR (129). While the authors explored the influence of β-arrestin-2 on KIR internalization in the short-term, it was seen through the perspective of constitutive internalization, and not in response to ligation. KIR internalization in response to ligand binding demonstrates that natural constitutive receptor recycling is impeded in the presence of the ligand. KIRs require phosphorylation of the ITIMs to transmit their inhibitory signals, in the same way as activating receptors require phosphorylation (130). Receptor phosphorylation is associated with their internalization and recycling or degradation (131, 132).

Regardless of whether it is an increase in internalization and retention of signaling within the cell or transfer of receptors and ligands between cells, these possibilities suggest that the influence of inhibitory interaction extends beyond simple cell-to-cell contact in a manner that may inherently alter the cell. The net cumulative effect of multiple iterations can therefore form the basis for the discrete modes of functionality observed for educated NK cells. In this context, receptor internalization and integration of signaling inside the cells could account for the threshold effects on education in conditions with varying receptor-ligand densities (35, 55, 57, 58). Inside the early endosomes, the quality rather than the quantity of Ly49/KIR-MHC complexes may determine the signaling strength.

The leaky cell hypothesis

The functional priming of cytotoxic lymphocytes clearly begins with cytokine stimulation (42, 43). The upregulation of inhibitory receptors that occurs in parallel with activation (133) suggests that functional priming of cells can be maintained through a balanced interaction between inhibition and homeostatic stimulation. This implies that certain receptors in combinations would produce ideal functional phenotypes. NK cells expressing self-specific inhibitory receptors are able to retain this functional potential during transient, repeated interactions with host cells. Similar homeostatic factors acting on NK cells lacking self-specific receptors would produce no accumulation of effector potential (*Fig.* 4). Importantly, these interactions should occur at a sufficiently high frequency to ensure that self-receptor negative NK cells do not cross the effective functional threshold at which effector potential renders the killer cell capable of eliminating host educator cells during subsequent cell-to-cell contacts. Repeated cycles of priming and Newtonian interaction would ultimately result in both accumulation of effector function in the short-term and gradual movement of the cells through the greater cycle of differentiation in the long-term. In essence, such a model would suggest that multiple transient interactions with host cells continuously trigger a weak sub-cytolytic release of effector molecules (the leaky cell hypothesis). Accordingly, tonic homeostatic cytokines fail to restore functionality due to the loss of potential during continuous Newtonian interactions with host cells.

Notably, this scenario harmonizes with the disarming or inhibitory licensing models, as well as the rheostat model. Upon transplantation of cells to a new MHC environment bearing new cognate ligands, the same homeostatic forces would allow the hyporesponsive cell to develop and retain functional potential by the gradual accumulation of function through repeated cycles of priming and Newtonian interactions (*Fig.* 4). Conversely, transfer of fully potent cells to an MHC-deficient or mismatched environment, is expected to trigger a relatively immediate release of effector potential, possibly associated with limited tissue damage, followed by a contractive phase characterized by the inability to restore function due to continuous disarming cell-to-cell contacts. It is important to note that in this sense hyporesponsiveness may not be a molecular imprint of anergic state by an as yet unknown mechanism. The phenotype, in terms of expression of DNAM-1, KIR/Ly49, and possibly also the dynamic organization of receptors at the nanoscale level, could simply be a natural consequence of cellular interactions with a predictable behavior of all involved receptors and signaling pathways, that over time result in tangible differences in functional state at rest.

Education in space and time

A crucial role for cell contact dynamics in instructing the downstream functionality of the cells was elegantly shown in

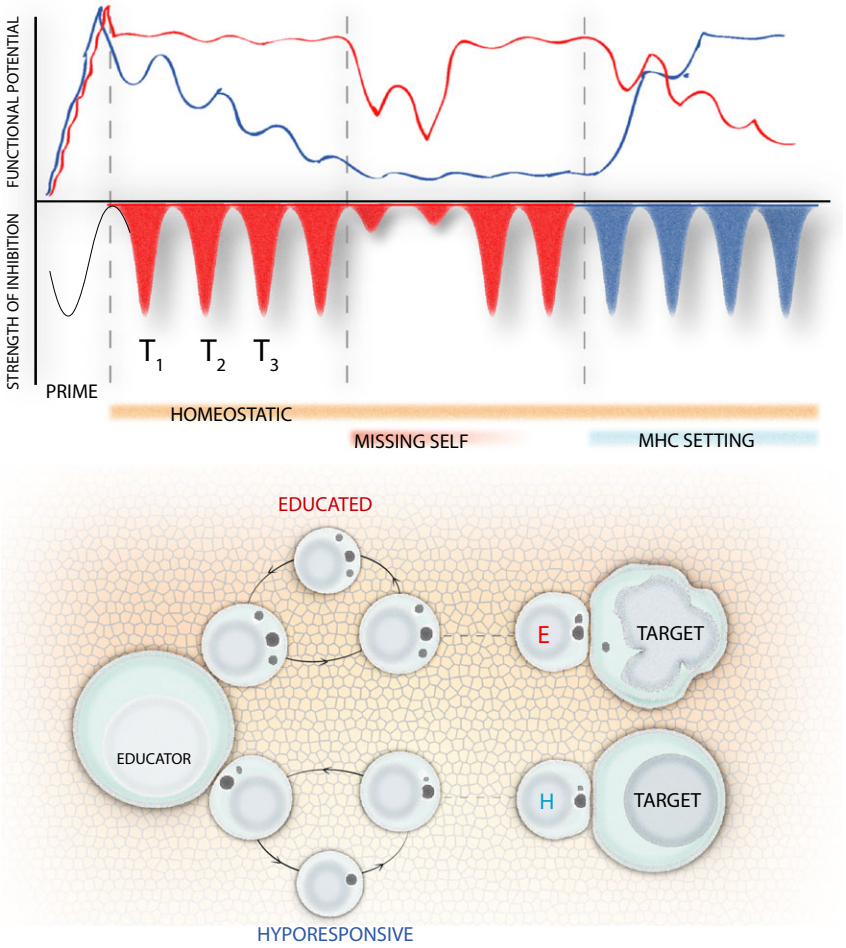

***Fig. 4.* Conservation of functional momentum.** A stepwise and cyclic sequence for natural killer (NK) cell effector potential begins with the functional priming of NK cells predominantly by cytokines, perhaps through trans-presentation by host cells expressing self-ligands. Upon priming, transient homeostatic interactions (T_1, $T_{2...}$) between NK cells lacking self-specific receptors and host cells results in gradual attenuation of effector potential. Conversely, the functional potential may be sustained during similar transient homeostatic interactions with host cells under the influence of self-specific inhibitory receptors (upper). Through cumulative cycles of priming and inhibition, educated (E) NK cells develop sufficient functional potential to mediate effective target cell cytolysis, such as the detection of missing self. Hyporesponsive (H) cells does not retain cytolytic potential above a given threshold and therefore do not mediate effective cytolysis. Transition of NK cells to a new major histocompatibility complex (MHC) environment allows the hyporesponsive cell to retain its potential upon each interaction and gradually become potentiated.

patients with deficiencies in perforin (Prf) and granzyme (Gzm) B (134). The quanta of IFN-γ secretion were dependent on the length of the contact between cytotoxic lymphocytes and their targets. Although the effects noted in Prf-null and Gzm-null effector cells relied on differential kinetics in target cell killing, it is possible that more subtle differences in cell-to-cell conjugates may have similar effects, provided that the number of iterations is sufficient. The downstream phenotypes are likely generated as the result of multiple, perhaps thousands of interactions, taking place over time-scales ranging from days to months. As such, several aspects of the Newtonian cell interactions need to be addressed, including the nature of the cells that provide different educating cues, their anatomical location (bone marrow, lymph nodes, or circulation), ligand-specificities [DNAM-1, signaling lymphocyte activation molecule (SLAM)-family receptors (135)], and the minimal time between cell-to-cell interactions required to continuously disarm NK cells lacking self-specific receptors between cycles of priming. The degree to which NK cells interact with their environment, especially within the periphery, likely has tremendous impact on stability and functional state of the NK compartment over time. Single-cell platforms hold promise to provide critical information about the degree to which non-lytic cell-to-cell interactions can influence the functionality of effector cells. However, a major technical limitation in the ability to study transient cell-to-cell interactions is that resting cells generally do not form long lasting or functional conjugates in the absence of prior stimulation, making

quantification of the net effects of cell-to-cell interactions difficult.

The vast majority of historical studies in the human into both phenotype and function have focused on the circulating NK cells derived from the blood. The cell-to-cell interactions that define the NK cell repertoire are likely very different when considering the blood versus complex tissue. In blood, interactions would be predicted to be faster and more frequent while in the tissue cell-to-cell interactions can persist over very long periods, and are influenced much more by the dynamic nature of infiltrating cells and the ever-changing contexture of a tissue's microenvironment. Another important aspect is to what extent the different niches provide signals (e.g. IL-15) for the priming of NK cell effector function and differentiation (136). Notably, T cell differentiation does not occur in the periphery, and is shaped through secondary ICAM-1 mediated interactions with neighboring lymphocytes (137). Deciphering these cellular events in different tissues may provide new insights into the contribution of NK cells and their various effector mechanisms in clinical contexts. In this respect, decidual NK cells show accumulation of self-specific inhibitory receptors (138), express high levels of effector molecules but do not typically degranulate in response to missing-self (139). Instead, decidual NK cells seemed to be geared toward releasing cytokines and chemokines, possibly as a result of divergent priming programs in this environment. The transient interactions that occur between circulating cells and the epithelium are likely very different in nature from the interactions that occur between cells in the tissues. Blood cells are more attuned to immune surveillance than tissue bound or infiltrating cells, as indicated by their reduced expression of chemokine receptors and accumulated phosphatase signals.

Another important future area of research concerns the homeostatic drivers of the functional state, i.e. the relative contribution of cytokines, activating receptors and perhaps weak agonistic antibodies to NK cell priming. It will be essential to decipher the location where priming, differentiation and education take place. Given the downregulation of effector function in NK cells under the continuous presence of stimulatory ligands (140, 141), it is unlikely that the same triggers of NK cell cytotoxicity also drive the initial development of effector function. There is an abundance of evidence that the development of an effector (and particularly an effective lytic) phenotype occurs under cytokine stimulation (142). That is not to say that function does not develop further under additional (receptor) stimulus, but on-site stimulation activating receptors are unlikely to account for either the stability or breadth of functional phenotype in the entire NK cell compartment.

Concluding remarks

Spatiotemporal aspects of Newtonian cell-to-cell interactions in the presence and absence of self-specific receptors are critical and unexplored elements in shaping the steady state functionality of NK cells. Although much remains to be discovered across all facets of such interactions, our prediction is that no single key mechanism or driver can explain the near digital functional phenotype associated with the expression of self-specific inhibitory receptors. In general terms most, if not all, mechanisms involved in the steps leading to NK cell education are known. Through careful integration of the various elements that promote NK cell development and education, a holistic approach to the timing and precise role that education plays in broader immunity can be developed. This begins with priming and development of effector function by cytokines, abrogation of target cell conjugation or intrinsic changes to the cell transmitted by inhibitory receptors, initiation of the effector response through activating receptors, including but most likely not restricted to DNAM-1, and exocytosis of cytolytic granules. All of which is superimposed on the transcriptionally and epigenetically regulated functional changes associated with NK cell differentiation.

A key challenge is to decipher the biology of the TDC between recently primed (naturally under homeostasis) self-receptor negative NK cells and host educator cells. The degree to which inhibitory signaling can limit contact, versus the degree to which it influences progression of the synapse toward cytolysis and how either accounts for the functional phenotype of the cell is a critical unmet element in NK cell biology. Inhibitory signaling clearly affects cells in the short-term by controlling their state of activation, particularly in the formation of immune synapses, but may also affect cells in the long-term by controlling their rate of proliferation, the kinetics of effector stimulation and return to rest. It is possible that effects of KIR inhibition and SHP-signaling involve metabolic fine-tuning of lysosomal biogenesis or exocytosis and as such represent novel mechanisms.

The Newtonian view of NK cell education represents a striking example of the theory of discontinuity, whereby immune cells iteratively calibrate their functional potential against their environment. Through well-established cellular and molecular programs, these interactions shape the long lasting function of the NK cell, resulting in a repertoire that is both self-tolerant and has a refined ability to respond to both positive and negative discontinuity.

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