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Review

The dynamics of natural killer cell tolerance

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Abstract

Natural killer (NK) cells are important mediators of resistance against tumor growth and metastasis. NK cell reactivity is regulated by a balance of signals from activating and inhibitory receptors. While reactivity against tumor cells is beneficial, it is essential that NK cells do not attack normal tissue. The distinction between tumor cells and normal cells is partly made at the level of activating receptors: transformation often results in induction of ligands for such receptors. In addition, NK cells discriminate self from non-self using MHC class I-binding inhibitory receptors. Host MHC class I molecules regulate development of NK cell reactivity and tolerance, a process that is not well understood. Recent data suggest that functional maturation may not be a binary phenomenon: quantitative aspects, with regards to avidity and frequency in interactions between developing NK cells and normal cells, may be important for the generation of NK cells that are 'tuned' to optimally sensing the absence of self-MHC class I. In this article, we discuss models for development of NK cell reactivity and tolerance. Our understanding of this process may have significant implications for the use of NK cells in cancer therapy.

Keywords: Natural killer cells; MHC; Tolerance

Contents

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1. Basics of NK cell recognition

The missing-self-hypothesis states that NK cells are able to sense, and become triggered by, the absence of self-MHC class I molecules [1,2]. The discovery and characterization of the MHC-specific inhibitory receptors Ly49, killer immunoglobulin-like receptors (KIR) and CD94/NKG2A provided the molecular explanation for how this may happen. The KIR and Ly49 families of receptors bind to MHC class Ia (classical) molecules in humans and mice respectively and the CD94/NKG2A heterodimer binds to the class Ib (non-classical) molecules HLA-E in humans and Qa-1^b in mice [3–10]. These class Ib molecules associate with, and their cell surface expression depend on, leader peptides from class Ia molecules. They thus serve as a measure of the expression of class Ia molecules in the cell [11–13]. Hence, NK cells can sense MHC class I expression in two different ways, directly with class Ia molecules via KIR or Ly49 receptors and indirectly with class Ib molecules via CD94/NKG2A. Engagement of inhibitory receptors leads to recruitment of tyrosine phosphatases that dephosphorylate intracellular signal transduction molecules and thereby inhibit signaling downstream of activating receptors.

Characterization of activating receptors has further added information to the picture of how NK cells can recognize different types of target cells. A well studied activating receptor is NKG2D, ligands for which are major histocompatibility complex class I-related chain (MIC)-A and -B and UL16-binding proteins (ULBPs) in humans [14–16]. In mouse, members of the retinoic acid early induced protein-1 (RAE-1), H60 and murine UL16-binding protein like transcript (MULT)-1 bind NKG2D [17–21]. These ligands are not expressed to any great extent on normal cells, but are upregulated in virus-infected and transformed cells. The DNA damage pathway has been demonstrated to mediate induction of NKG2D ligands in response to genotoxic stress [22]. Ly49H, and potentially NKp46, recognize pathogen encoded ligands, m157 from mouse cytomegalovirus and viral haemagglutinin respectively [23–26]. In addition, NKp46, as well as other activating receptors such as NKRP1-C, have as yet unidentified ligands [27–29]. The activating receptors associate with adaptor molecules such as KARAP/DAP12, DAP10, FcεRIγ or CD3ζ, which mediate signals into the cell [29–31]. There are also examples of target cells that are susceptible to NK cell lysis, but where neither the activating receptor, nor its ligand is known. One such example is NK cell-mediated rejection of beta$_2$-microglobulin $(\beta_2 m)^{-/-}$ bone marrow or spleen cells by normal mice *in vivo* [32–35]. This rejection highlights the fact that normal hematopoietic cells express activating ligands for NK cells, and that mechanisms are needed to prevent killing of endogenous cells *in vivo*.

NK cells thus use an array of activating and inhibitory receptors in interactions with endogenous cells and potential target cells. Signals from these receptors are integrated by the NK cell and the balance between activating and inhibitory signals decides if the NK cell will react or not. It is thus important that the NK cell is appropriately 'tuned' and able to become activated by cells altered by for example transformation and still not react against normal endogenous cells.

2. Models for how NK cell tolerance could be achieved

In the adaptive immune system, much is known about how self-tolerance is achieved. T and B cells express a single randomly rearranged antigen receptor of any possible specificity and in a clonal fashion. An efficient way to ensure self-tolerance in those systems is clonal deletion of cells with a self-reactive antigen receptor. There are also peripheral mechanisms to take care of autoreactive cells that have escaped clonal deletion. For example B cells with a self-reactive antigen receptor can get anergic by downmodulation of this receptor, modulation of activating signaling pathways, induction of inhibitory receptors, or recruitment of phosphatases [36,37]. Similarly, T cells encountering antigen in absence of co-stimulation, can become anergic via downregulation of the T cell receptor and/or T cell receptor signaling pathways as well as via upregulation of inhibitory receptors [36,38]. However, the circumstances are different for NK cells. Instead of one dominant activating receptor, NK cells express a whole range of both activating and inhibitory receptors that are all germline encoded. The activating receptors are in many cases expressed on all NK cells, while the MHC-specific inhibitory receptors are expressed on overlapping subsets of NK cells. Thus, in contrast to the situation for T and B cells, discrimination between self and non-self in NK cells is done at the level of inhibitory receptors rather than by activating receptors.

Ligands for the inhibitory receptors are the highly polymorphic and rapidly evolving MHC class I proteins. The inhibitory receptors themselves display limited polymorphism and are specific for only one or a couple of MHC alleles each. In addition, the inhibitory NK cell receptors are not genetically linked to their ligands. It is thus clear that there is a need for somatic processes ensuring that NK cells do not attack normal endogenous tissue. In support of such a role for somatic processes are the findings showing that MHC class I expression of the host regulates the specificity and function of NK cells. Mice lacking MHC class I expression have NK cells, but they are unable to kill MHC class I deficient target cells [33,34,39]. Furthermore, introduction of an MHC class I transgene expressed on all cells of the mouse resulted in induction of NK cell reactivity against transgene-negative target cells [40–44]. There are several mechanisms by which the interaction between inhibitory receptors and host MHC class I alleles could result in a reactive, yet self-tolerant, NK cell population (Fig. 1).

2.1. Negative selection of NK cells

One way to avoid autoreactivity caused by NK cells would be to delete all cells lacking self-specific inhibitory receptors. If such negative selection was used, one would predict that mice or humans lacking expression of MHC class I molecules, i.e. inhibitory ligands, due to genetic mutations in the genes for class I heavy chains, β_2m or the transporter associated with antigen processing (TAP), would be devoid of NK cells or have dramatic changes in their NK cell repertoires. The fact that individuals lacking MHC class I expression carry normal numbers of NK cells and display surprisingly "normal" repertoires of activating and inhibitory receptors, argue against this possibil-

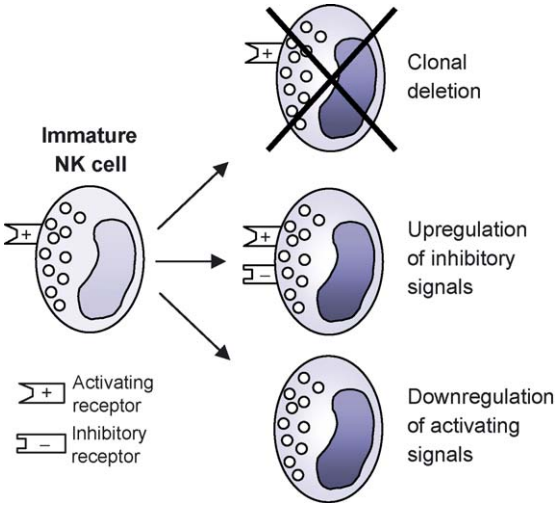

Fig. 1. Possible mechanisms whereby the NK cell population may be rendered tolerant to endogenous cells. An immature NK cell (left) with a potential for autoreactivity: it may express activating receptors but lack inhibitory ones or it may express inhibitory receptors for which there are no ligands in the host. Such cells may be clonally deleted and thereby removed from the NK cell repertoire (top). Alternatively the NK cell may be kept but become tolerant by adaptation. Upregulation by inhibitory signals (middle) may be achieved by new expression of inhibitory receptors specific for MHC class I or for other types of ligands. Downregulation of activating signals (bottom) could involve downregulation of activating receptors from the cell surface or downregulation of intracellular signaling molecules. This is collectively illustrated in the figure as loss of the activating receptor.

ity [33,39,45–50]. Thus, tolerance is more likely to be achieved by adaptation of the NK cells.

2.2. Adaptation of NK cells through upregulation of inhibitory receptors

Adaptation could be attained by different mechanisms. One possibility would be that an NK cell lacking self-specific inhibitory receptors would switch on new inhibitory MHC-specific receptors continually until a match with a ligand was found. This is one variant of the "at least one" hypothesis, postulating that each NK cells would have to express at least one self-MHC-specific inhibitory receptor to become functionally mature [51,52]. This would lead to the prediction that mice with different MHC haplotypes would have NK cells with very different repertoires of inhibitory receptors expressed. Furthermore, in the absence of MHC class I expression, all possible inhibitory receptors would be expressed on all NK cells. Inhibitory Ly49 receptors are expressed in a stochastic and cumulative fashion [53] and while some differences in the Ly49-repertoires between hosts expressing different alleles of MHC class I molecules have been observed [46,47], these repertoire differences are not large enough to fit with the notion that this mechanism is the major one to accomplish NK cell tolerance.

It is also possible that NK cells may switch on inhibitory receptors with other specificities than MHC class I to become self-tolerant. CD48/2B4 interactions have been suggested to provide a tolerance mechanism additional and non-redundant to MHC dependent NK cell tolerance [54,55]. In addition, NK cells can be inhibited via the receptor NKR-P1D by cells expressing ocil/Clr *in vitro* [56,57]. The importance of these types of inhibition for achieving self-tolerance in the NK cell population will require further studies.

2.3. Adaptation of NK cells through downregulation of activating receptors or pathways

Another possible adaptive change to secure self-tolerance in NK cells without self-specific inhibitory receptors would be to downregulate activating receptors or signaling pathways and by this maintain hyporesponsiveness. NK cells in $\beta_2m^{-/-}$ mice are completely unable to kill MHC class I-negative cells *in vitro* and *in vivo*. They do, however, have the capacity to kill tumor target cells through activation via defined activating receptors such as the NKG2D and Ly49D receptors *in vitro*. Killing of those target cells occur at significantly reduced levels [33,39,58], which may be explained by a functional impairment due to downregulation of activating signaling pathways. Neither the NKG2D receptors, nor Ly49D, are downregulated at the surface of $\beta_2m^{-/-}$ NK cells, which suggest modulations of signaling pathways rather than downregulation of the receptors themselves. However, it should be kept in mind that the activating receptors regulating recognition of normal cells are still unknown, leaving the possibility that such receptors would be regulated by downmodulation. Taken together, most experimental data are consistent with an education mechanism based on modulation of activating receptors or signaling pathways.

3. NK cells developing in MHC mosaic mice become tolerant to all MHC phenotypes present

An experimental mouse model that has provided some insight into the regulation of NK cell self-tolerance is a mouse strain expressing an H-2D^d (called D^d hereafter) transgene in a mosaic fashion [59]. The D^d transgene was maintained on a C57BL/6 (B6; H-2K^b, D^b) background and was expressed on only a fraction of the cells in peripheral blood. This fraction varied between 10 and 80% in different individuals. The fraction of D^d-positive cells was similar in T cells, B cells and NK cells derived from peripheral blood, spleen, lymph nodes and thymus. A similar pattern was observed when analyzing cultured fibroblasts derived from these mice, only a fraction of the cells expressed the transgene.

We had previously shown that transgenic mice expressing the same D^d transgene on all cells developed missing-self-reactivity towards cells lacking the D^d molecule [40–42]. In contrast, in the mosaic mice, around half of the cells lacked expression of the transgene and no missing-self-reactivity against D^d-negative cells was observed *in vivo* or *in vitro*. This indicated that the D^d-negative cells dominantly induced NK cell tolerance to this phenotype [59]. Similar conclusions were also reached in a study by Wu and Raulet, in which fetal liver chimeras between $\beta_2m^{-/-}$ and β_2m^{+} mice in different combinations were analyzed for rejection of $\beta_2m^{-/-}$ bone marrow grafts. The presence of $\beta_2m^{-/-}$ cells in the hematopoietic and/or non-hematopoietic compartment resulted in tolerance to such grafts, suggesting a

dominant effect by the presence of $\beta_2m^{-/-}$cells [60]. In addition, in a study of mice transgenic for a D^d gene modified to be excised by Cre recombinase, two expression phenotypes were investigated: mosaic D^d expression in hematopoietic and non-hematopoietic tissue, resembling the previously described mosaic mouse strain, and expression on all hematopoietic but not on all non-hematopoietic cells. NK cells in both strains of mice were unable to reject D^d-negative bone marrow grafts *in vivo*, demonstrating that hematopoietic and non-hematopoietic D^d-negative cells could dominantly induce tolerance to this phenotype [61].

The results in the three studies suggested that there were mechanisms to ensure tolerance to all MHC class I phenotypes present in the animal. This tolerance could be induced by hematopoietic as well as non-hematopoietic cells. It was further possible to rule out simple tolerance mechanisms, such as the possibility that MHC class I molecules expressed by the NK cell itself was the only determinant for the specificity of that NK cell. If this were the case, NK cells expressing D^d would always kill cells lacking D^d, which was not observed in the mosaic mice. Likewise, one could rule out the possibility that NK cell specificity would be decided on the basis of the first cell-cell interaction the NK cell experienced. In that case, all NK cells that first met a D^d-positive cell would not develop tolerance to negative cells and would be autoreactive. It therefore became clear from these results that the tolerance mechanisms operating in the mosaic and chimeric mice must be based on interactions with multiple surrounding cells. Data from the mosaic mice also corroborated data arguing against educating mechanisms based on major changes in the inhibitory receptor repertoire, since no changes that could be coupled to self-tolerance were observed in mosaic mice [59,62,61]. These phenotypic data were, however, consistent with the notion that potentially autoreactive NK cells could be kept alive, but in a hyporesponsive state.

4. Tolerance may be reversible and dependent on the continuous presence of tolerizing cells

When D^d-positive and D^d-negative splenocytes from mosaic DL6 mice were separated and then IL-2-activated, tolerance to D^d-negative cells was broken in the D^d-positive NK cell population [59]. This result has implications for the discussion of different models for tolerance. It strongly argues against clonal deletion, at least as the sole mechanism for tolerance. One may argue that during 4 days of culture in IL-2, new clones could be selected and expanded. However, tolerance could be broken also if D^d-negative and positive cells were cultured together for 4 days and separated only during the last 6 h (our unpublished data). Clonal expansion and selection during such a short time is unlikely. This result therefore supports models for reversible adaptation mechanisms where potentially autoreactive NK cells would be kept in the mouse as functionally impaired, or with a changed specificity. A similar conclusion was reached by Ioannidis in the study of cre-D^d transgenic mice [61] as well as by Kung and Miller in a study of H-$2^{b/d}F_1 \rightarrow$ H-2^b and H-$2^{b/d}F_1 \rightarrow$ H-2^d bone marrow (BM) chimeras, where specific tolerance to the host phenotype was detected *in vivo*. After culture in IL-2, NK cells from the chimeric mice gained ability to kill host type lymphoblasts [63]. It may be argued that these reactivities results from unphysiological culturing conditions. However, IL-2 culture alone was not enough to break tolerance in NK cells from our mosaic mice; removal of D^d-negative cells followed by isolated culture of D^d-positive cells for a couple of hours was essential [59] (and unpublished data). Furthermore, in co-culture experiments, where spleen cells from D^d-transgenic mice (with complete expression) were cultured together with D^d-negative B6 spleen cells in the presence of IL-2, there was a gradual decrease in the D^d-negative population, dependent on NK cells in the D^d-positive population. No such decrease was observed in cultures of spleen cells from mosaic mice (our unpublished observations). Taken together these results argue that in cultures from the mosaic mice tolerance was maintained also in the presence of IL-2, but only as long as the tolerizing cells were present.

5. NK cells that do not receive inhibitory signals via self-MHC are hyporesponsive

As already mentioned, NK cells in $\beta_2m^{-/-}$ mice, that fail to receive inhibitory signals during NK cell development, are unable to kill MHC-negative cells *in vitro* and *in vivo* but retain a reduced capacity to kill some tumor cell targets [33,39,58]. Recently, Fernandez et al. have studied an NK cell subset lacking self-specific inhibitory receptors in normal B6 mice (K^b, D^b) [64]. Specifically, these NK cells lacked expression of Ly49C/I specific for K^b and NKG2A specific for Qa-1^b in conjunction with the D^b leader peptide. This subset of NK cells showed a reduced reactivity, not only to syngeneic B6 lymphoblasts *in vitro*, but also to MHC class I negative lymphoblasts ($\beta_2m^{-/-}$ or $K^b/D^{b-/-}$). This reduced reactivity was evident in measurements of intracellular IFN-γ production as well as in cytotoxicity assays. Furthermore, in assays where NK cells were stimulated by antibody-mediated crosslinking of the activating receptors NKR-P1C, NKG2D or Ly49D, a reduced reactivity was observed in the NK cell population lacking self-specific inhibitory receptors compared to NK cells expressing such receptors [64]. Thus, also in mice expressing MHC class I, some NK cells may fail to receive inhibitory signals and develop a hyporesponsiveness similar to that in mice completely lacking MHC class I molecules.

In another study Kim et al. reached similar results [65]. NK cells expressing self-specific inhibitory Ly49 receptors were able to produce cytokines like IFN-γ and TNF-α in response to triggering of activating receptors by immobilized monoclonal antibody (mAb). In contrast, NK cells from MHC class I-negative mice, or NK cells lacking self-specific inhibitory receptors, produced less cytokines. In an experiment utilizing mice transgenic for a single chain MHC class I molecule, composed of K^b heavy chain, β_2m and peptide (covalently linked), they demonstrated reactivity specifically in the Ly49C$^+$ NK cell subset but not in Ly49C$^-$ NK cells. Furthermore, a functional immunoreceptor tyrosine-based inhibitory motif (ITIM) in the intracellular domain of the Ly49 molecule was required for NK cells to gain reactivity via interactions with MHC class I expressing cells [65]. Hence, NK cells that had interacted with MHC class I

via inhibitory receptors during development were reactive as mature, while NK cells failing such interactions became hyporesponsive.

Das and Saxena studied the dependence of the inhibitory interaction using a different approach. In a study of NK cells developing from bone marrow precursors *in vitro* in the presence of IL-2, they showed that blockade of the interaction between Ly49C and its H-2^b ligands using mAbs inhibited development of NK cells able to kill susceptible YAC-1 tumor cells [66]. In addition, we found that among NK cells from long term bone marrow cultures, only the subpopulation expressing Ly49 receptors (Ly49C/I, -A or -G2) were able to distinguish between β_2m^+ and $\beta_2m^{-/-}$ lymphoblast target cells [67]. These results highlight the importance of the inhibitory interactions for NK cell development and function, and suggest that NK cells that have not been engaged in such interactions during development become hyporesponsive.

6. How could the activation potential in NK cells be modulated?

The studies described above show that hyporesponsive NK cells are not observed only in mice that are manipulated in some way, such as those lacking expression of MHC molecules altogether or expressing MHC molecules in a mosaic fashion. Also in normal mice, there are hyporesponsive NK cells present. What molecular interactions could decide whether an NK cell becomes functionally active or hyporesponsive? There are at least two possibilities: first, engagement of inhibitory receptors could in itself stimulate the NK cell to become functionally active. Secondly, engagement of inhibitory receptors could balance signals from activating receptors and prevent hyperstimulation that would otherwise lead to induction of hyporesponsiveness (Fig. 2). An important distinction between the two models is that in the first model, the starting point is an immature NK cell with no, or low, activation potential. Furthermore, an interaction between this NK cell and a cell lacking cognate inhibitory ligand is a null event, and will not result in any signaling in the NK cell. Only interactions with educating cells expressing the proper MHC class I ligand will give rise to a signal that allows the NK cell to become fully functional. In the latter model, the starting point is instead an NK cell with high activation potential. Here, interactions with MHC class I ligand-positive cells will result in no net signaling, while interactions with MHC class I ligand-negative cells will result in induction of hyporesponsiveness due to insufficient balancing of the activating signals by inhibitory signals. Can available data distinguish between the two models?

6.1. Direct activation via inhibitory Ly49 receptors only

The first model states that the interaction between the inhibitory receptor and its ligand in itself results in a positive signal to the developing NK cell, leading to induction of full activation potential of the mature NK cell (Fig. 2A). In the absence of this signal (due to lack of the MHC class I ligand or its receptor) the NK cell would remain hyporesponsive. This model is similar to the 'licensing model' proposed by Kim et al. [65,68].

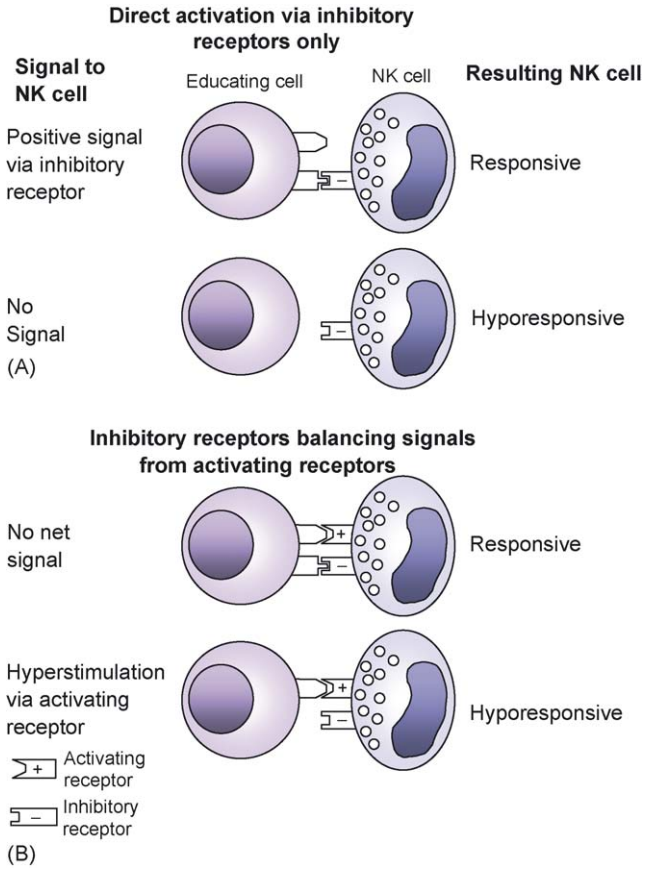

Fig. 2. Models for modulation of activation potential in NK cells. (A) Direct activation via inhibitory receptors only. This model assumes that immature NK cells have no or low activation potential. An interaction engaging the inhibitory receptor (top) will be translated into a positive signal that will allow the cell to mature into a functionally responsive NK cell. In contrast, in absence of inhibitory receptor engagement (bottom), the NK cell will receive no signal and remain in a stage of low activation potential (hyporesponsive). (B) Inhibitory receptors balancing signals from activating receptors. This model postulates that the starting point for an immature NK cell is full activation potential. NK cells are interacting using both inhibitory and activating receptors. In an interaction engaging both types of receptors (top) the signal from the inhibitory receptor will balance the signal from the activating one. This will result in no net signaling in the NK cell, the high activation potential will be kept and the cell will be functionally responsive. In an interaction that will only engage the activating receptor and not the inhibitory one (bottom) the activating signal will not be balanced resulting in hyperstimulation of the NK cell. This will induce a decrease in activation potential and hyporesponsiveness in the NK cell.

This model is somewhat counterintuitive since the engagement of inhibitory receptors in mature NK cells, and phosphorylation of ITIMs recruits SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1), and SHP-2 has been shown to turn the NK cell off rather than stimulate it [69]. There are, however, some experimental data suggesting that engagement of Ly49 receptors could have a stimulatory effect. It has been suggested that SHP-2 could contribute to activating rather than inhibitory signaling [70]. SH-2 containing inositol phosphatase (SHIP) has been shown to associate with Ly49 receptors [71], in addition, it has been demonstrated that inhibitory KIR intracellular domains could associate with the regulatory p85α subunit of the phosphatidylinositol 3-kinase and possibly contribute to stimu-

latory signals [72]. Studies by Kim et al. suggest however that at least SHP-1, p85α and SHIP are not involved in the functional maturation of NK cells while SHP-2 remains uninvestigated [65].

Tolerance to all MHC phenotypes was induced in mosaic and chimeric mice [59–61,63]. In such animals, NK cells are exposed to interactions with both inhibitory ligand-positive and ligand-negative cells, and the effects of the ligand-negative interactions seem to dominate. If only inhibitory ligand-positive cells were able to mediate developmentally relevant signals in the NK cells, one has to postulate that the stimulatory effect of such interaction is reversible and short lasting. Thus, the NK cell would require continuous interactions with ligand-positive cells to induce and maintain its activation potential. In an environment where ligand-positive and ligand-negative cells co-exist, for example in mosaic mice, the continuous signaling would be interrupted by interactions with ligand-negative cells and responsiveness lost. The results that 20% D^d-negative cells in mosaic mice was enough to achieve tolerance but that tolerance could be broken by complete removal of D^d-negative cells [59], would further suggest that the effect of interrupting inhibitory signaling is longer-lasting than the effect of the inhibitory interaction itself, but still reversible. Thus, several conditions have to be introduced to fully explain the data from mosaic mice within this model only. We do, however, not rule out the possibility that direct activation via inhibitory receptors could act in concert with other mechanisms.

6.2. *Inhibitory receptors balancing signals from activating receptors*

An alternative model states that NK cells may interact via inhibitory and activating receptors during development (Fig. 2B). Raulet and co-workers have termed this model the ‘tolerance induction’ or ‘disarming’ model [64,73]. It is similar to the model we initially put forward to explain our data from mosaic mice [59]. The model assumes that ligands for one or several activating receptors are expressed on educating cells. While the nature of such cells is not known, the NK cell susceptibility of several hematopoietic cells in the bone marrow argue that cells surrounding NK cells during development express activating ligands. This model postulates that the starting point for an immature NK cell is full activation potential. In interactions with cells expressing cognate inhibitory ligand, the signal from the activating receptor(s) would be balanced by an inhibitory signal in a similar way as for mature NK cells. This would have no impact on the NK cell, but maintain its activation potential. In contrast, cell interactions in the absence of inhibitory signals would lead to hyperstimulation of the NK cell, resulting in induction of hyporesponsiveness.

Interpretation of tolerance data from mosaic and chimeric mice within this model would suggest that interactions with inhibitory ligand-negative cells would induce hyporesponsiveness in NK cells independently of the presence of ligand-positive cells. Since hyporesponsiveness could be reversed, its maintenance would have to depend on recurring (but not necessarily continuous) interactions with ligand-negative cells. Thus, this model can directly explain the data from mosaic mice.

Induction of hyporesponsiveness as described above may be similar to what happens in mice transgenic for NKG2D ligands. In such mice, NK cells downmodulate NKG2D expression and become nonresponsive against cells expressing NKG2D ligand [74,75]. In one study this nonresponsiveness was extended also to other cell types [75]. An important feature of NKG2D is that signaling through this receptor is strong enough to override MHC dependent inhibition, thus, NK cells become hyporesponsive despite Ly49–MHC class I interactions in NKG2D ligand transgenic mice. The principle could however very well be true for other weaker activating receptors in the absence of inhibitory interactions via MHC specific receptors.

Data from other transgenic models also argue for the importance of maintaining a balance between activation and inhibition during NK cell development. Mice deficient in the src family kinase fyn demonstrated reduced reactivity against targets lacking self-MHC class I, and reduced usage of Ly49 receptors [76]. Furthermore, phospholipase C-γ2 (PLC-γ2)-deficient mice have a pronounced reduction of IFN-γ production and cytotoxicity in response to stimulation of a range of activating receptors. In addition, the proportions of NK cells expressing Ly49 receptors (inhibitory and activating) were reduced [77,78]. This may suggest effects of activating signals mediated via fyn and PLC-γ2 on NK cell development which also extend to changes in NK cell reactivity. Kim et al. has shown that NK cells go through a proliferative development stage after acquiring inhibitory NK cell receptors, and that NK cells expressing self-specific inhibitory receptors preferentially proliferate [65,79]. These results may suggest that signaling through inhibitory receptors during development lead to upregulation of activating pathways and proliferation of NK cells. The data are consistent with the model postulating a direct positive signal through the inhibitory receptor, as well as the model where balance between activating and inhibitory signals result in a positive outcome for the NK cells.

Taken together, available data favors a model where inhibitory receptors balance the signaling from activating receptors during NK cell education. It does not, however, formally exclude a model in which inhibitory receptors directly mediate positive signals to the developing NK cell.

7. Extending the complexity: quantitative aspects on NK cell tolerance and education

There is evidence suggesting that functional maturation of NK cells may not be a binary phenomenon, but instead quantitatively determined. Thus, education may not result in only functionally sufficient or hyporesponsive NK cells, but also a whole range of reactivities in between. This quantitative thinking could be applied both when it comes to the strength of each inhibitory interaction, determined by the avidity between receptors and ligands, and to the number or frequency of cell–cell interactions.

7.1. Quantitative differences in the avidity of the inhibitory interaction affect NK cell development

We recently evaluated the capacity of different MHC class I alleles to educate NK cells in missing-self-recognition [44]. Analysis of genetically engineered mouse strains, expressing single MHC alleles (K^b, D^b, L^d and D^d), demonstrated that all tested MHC alleles could educate a missing-self-response. However, the efficiency (termed educating impact) differed between the MHC alleles: while K^b and D^d educated strong responses, L^d and D^b educated weak ones. This could be due to avidity to inhibitory NK cell receptors and/or the percentage of NK cells expressing cognate receptors and thereby being able to respond. D^d is a ligand for the inhibitory receptors Ly49A and Ly49G2 [3,4]. K^b is a ligand for Ly49C and I [5,80,81]. Interactions between these receptor–ligand pairs have been studied in detail. For L^d and D^b, the situation is less clear. L^d is a suggested ligand for Ly49G2 and D^b is suggested to be a weak ligand for Ly49A and Ly49C [4,82]. This probably mirrors the educating impact of the different alleles. Importantly, in a phenotypic analysis of NK cells in the respective mouse strains, no differences in the levels or percentage of cells expressing a panel of activating receptors and maturation markers was observed [44]. Thus, there were no differences in number of mature NK cells that could explain the differences in educating impact. Instead, the explanation may lie in a difference in the reactivity within each individual NK cell.

The results could be interpreted in the following way (Fig. 3). Signals delivered by self-MHC alleles via inhibitory receptors may determine the NK cell's activation potential in a quantitative manner. Thus, there would be a direct correlation between the strength of the signal delivered by the inhibitory receptor during development and the activation potential, or responsiveness, of the mature NK cell. A high avidity inhibitory ligand would deliver a strong signal and thereby induce a stronger reactivity to MHC class I-negative cells. In contrast, a low avidity inhibitory interaction, or in an extreme case the absence of an interaction, would induce weaker reactivity to MHC-negative cells. The NK cell would thus be 'tuned' to optimally sensing the absence of self-MHC class I. This quantitative regulation of the NK cell's activation potential could occur by interactions using the inhibitory receptor only (Fig. 2A), or in balance with activating receptors (Fig. 2B). However, to optimally tune activation potential, an interaction involving relevant activating receptors seems the most likely alternative (Fig. 3).

Adding to the quantitative aspects of NK cell function, NK cells from mice expressing several MHC alleles were more efficient in killing MHC class I negative cells than NK cells from mice expressing few MHC alleles (our unpublished data). This result could be interpreted to mean that more NK cells are educated in such mice, but the data may also suggest that each NK cell may be educated by more than one MHC allele simultaneously and thereby be more efficient. Thus, inhibitory signals received from interactions with more than one MHC allele would result in a stronger reactivity against class I deficient target cells.

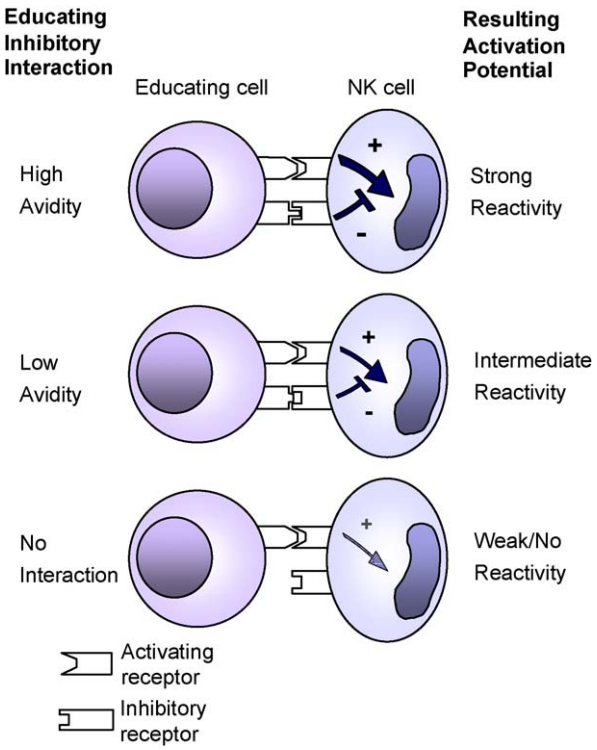

Fig. 3. Quantitative adaptation of activation potential in NK cells. The avidity of the educating inhibitory interaction regulates the activation potential of the NK cell. The figure shows three examples of what is likely to be a continuum. High avidity inhibitory interaction (top) will give a strong signal to the NK cell. This will lead to high activation potential and thereby strong reactivity in the mature NK cell. A low avidity interaction (middle) will lead to intermediate reactivity and no inhibitory interaction (bottom) will lead to low or no activation potential/reactivity and a hyporesponsive NK cell.

7.2. Frequency of interactions determining NK cell tolerance

Data from the MHC class I mosaic and chimeric mice suggest that several cell–cell interactions are required to functionally mature an NK cell. If the reactivity of the NK cell is determined in a quantitative manner rather than a binary one, the number or frequency of interactions with cells of a certain phenotype may also be important for the outcome of an individual developing NK cell. In our population of mosaic mice, the fraction of cells expressing D^d varied between individuals and all mice tested, with as little as 20% D^d-negative cells, were tolerant to D^d-negative lymphoma or bone marrow grafts [59]. These results favor a model where cells lacking an appropriate MHC ligand play a dominant role inducing tolerance.

Although not obvious initially [59], quantitative issues became apparent in this model when we studied mixed bone marrow chimeras generated by lethal irradiation of D^d-transgenic mice followed by reconstitution with D^d-negative and D^d-positive BM cells mixed in different proportions. These BM chimeras, carrying D^d-negative cells only in the hematopoietic compartment and in defined numbers, allowed us to analyze quantitative requirements for induction of tolerance to D^d-negative cells. Chimeras demonstrated a stable chimerism for (at least) 90 days after reconstitution, suggesting that NK cell

tolerance had been induced to D^d-negative cells. Furthermore, when challenging mice with subcutaneous inoculations of low numbers of D^d-negative lymphoma cells (RMA), 21 out of 22 BM-chimeras with more than 20% D^d-negative hematopoietic cells accepted the tumor cells. However, chimeras with lower percentages of D^d-negative cells rejected RMA grafts to a higher extent (rejection in 7/24 chimeras) [83]. Similarly, IL-2 activated spleen cell populations from chimeras with around 40–50% D^d-negative cells did not kill B6 lymphoblast target cells, while a higher level of lysis against D^d-negative lymphoblasts was observed with effector cells from chimeras carrying 0–20% D^d negative cells. These data suggest that tolerance was not complete in NK cells exposed to limited interactions with D^d-negative cells. Limiting numbers of tolerizing cells in mixed chimeras may induce a "weak tolerance" that was sufficient to prevent harm to normal cells (stable chimerism), but that still allowed reactivity against lymphoma cells *in vivo* and lymphoblasts *in vitro*. Lymphoma cells and lymphoblasts are likely to express higher numbers of activating ligands, which could explain why tolerance was more easily broken towards such cells.

One interpretation of these results is that NK cell tolerance induction may be a quantitative process where multiple interactions with tolerizing cells progressively reduce reactivity to such cells. Thus, in mixed BM chimeras with few tolerizing cells, the number or frequency of interactions may not be sufficient to achieve a complete tolerance, resulting in weak reactivity to syngeneic cells with elevated levels of activating receptors.

8. Can available data be reconciled?

There are at least two scenarios for NK cell development that best reconciles most of the available data. First, there may be two parallel processes, one that ensures NK cell reactivity and one that ensures self-tolerance. These processes would both involve activating as well as inhibitory receptor interactions similar to those depicted in Fig. 3. However, they may be active during different stages of NK cell maturation, at separate sites in the body and/or involve distinct cell types interacting with NK cells. The first process would be quantitatively regulated by the avidity of inhibitory interactions with surrounding cells and regulate the activation potential of the NK cell. It may be active preferentially during development of NK cells and allow selective proliferation of NK cells receiving signals through their inhibitory receptors. The second process, ensuring self-tolerance, would also be regulated by activating and inhibitory interactions such that absence of inhibitory signaling would induce hyporesponsiveness in the NK cell. Importantly, in case of conflicting signals (like in mosaic or chimeric mice) the mechanisms inducing hyporesponsiveness would be dominant. The tolerance process would be active during the entire life span of the NK cell and may be quantitative and reversible, such that hyporesponsiveness would be incomplete when inhibitory ligand-negative cells are sparse, and lost when such cells were completely removed. NK cells would then revert to the activation potential determined by the first process during development.

In a second scenario, a single process ensures both reactivity and self-tolerance. The interactions may again be similar to the model depicted in Fig. 3, but in this scenario we introduce the condition that the default status of an NK cell is full activation potential. This potential would be effectively downregulated by interactions lacking strong inhibitory signals, in a quantitative manner, such as in the absence of high avidity inhibitory ligand on some or all surrounding cells. Thus, few interactions with ligand-negative cells would be enough to make the NK cell fully hyporesponsive but only for a certain amount of time. Removal of inhibitory ligand-negative cells would lead to regained activation potential. The latter scenario would explain preferential proliferation of NK cells receiving inhibitory signals during development by stating that this proliferation is induced by default, but that it can be prevented by the absence of inhibitory signals.

9. Implications for reactivity against tumor cells

Collectively, the data suggest a fine-tuned regulation of NK cell reactivity and self-tolerance. This would be very important to maintain self-tolerance but still ensure reactivity against altered endogenous cells that have undergone transformation. Such cells likely upregulate activating ligands to different extents, rendering them more NK cell susceptible. Experiments comparing NK cell-dependent rejection *in vivo* of MHC-deficient spleen cells with that of the MHC-deficient tumor cell RMA-S, showed that the latter was more efficiently rejected [35]. This suggests a higher expression of ligands activating NK cells on tumor cells than on normal lymphocytes. The data described in Section 7.2 of this article also support this notion. In mixed bone marrow chimeras, generated by reconstitution of irradiated D^d-transgenic mice with a mix of D^d-negative and D^d-positive BM cells, there was reactivity against D^d-negative RMA tumor cells *in vivo* while the degree of chimerism was maintained [83]. This argues that tumor cells are more efficient in triggering NK cells and that accordingly, NK cell tolerance can be more easily broken towards tumor cells than towards normal cells. In another study, blocking of self-specific inhibitory NK receptors with F(ab)′2 fragments resulted in inhibited development of syngeneic leukemia *in vivo*. In addition, syngeneic leukemia cells could be selectively purged, by NK cells, from co-cultures with syngeneic BM cells. This supports the notion that breaking of tolerance towards tumor cells may be more easily achieved, presumably due to additional activating ligands on tumor cells [84,85].

In human bone marrow transplantation of acute myeloid leukemia patients, patients receiving haploidentical transplants, in a KIR–HLA ligand mismatch combination where NK cells of the graft had the potential to kill leukemia cells in the host, showed a markedly reduced risk for leukemia relapse compared to patients receiving a transplant without KIR–HLA mismatch. NK cells may thus have a beneficial antileukemic effect induced by missing-self-recognition [86,87]. Interestingly, donor-derived NK cells lost reactivity to the host phenotype within 3 months after transplantation suggesting development of tolerance [86].

As apparent from the examples in the previous sections, the benefits of utilizing NK cells in tumor-therapy is becoming clear. Knowledge regarding NK cell tolerance and its molecular regulation is of great importance for improving cancer therapies involving NK cells and will particularly serve to enhance efficiency of tumor elimination protocols that spare normal tissue.

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