Hanging in the balance: natural killer cell recognition of target cells

Natural killer (NK) cells kill certain tumor cells and virus-infected cells directly. Until recently, little was known about how they recognize their targets. Now, several candidate NK receptors have been identified, some of which may have carbohydrate ligands. Some of the receptors deliver positive signals, others negative signals. Thus NK cells seem to balance many different inputs to decide whether to kill a target.

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Natural killer (NK) cells are a population of lymphocytes that have the inherent capacity to kill certain tumor cells and virus-infected cells. For many years, little was known about how NK cells recognize and interact with their specific targets. Recently, there have been dramatic and fundamental changes in our understanding of how NK cells recognize target cells. Indeed, the problem is no longer that we lack candidate NK receptors, but that we seem to have too many of them.

In general, the newly described molecules that modulate NK function appear to segregate into two basic categories: those that activate lysis and those that inhibit it. This is an oversimplification, however, as some appear to do both in different circumstances. Some of the candidate receptors bind to class I molecules of the major histocompatibility complex (MHC), and generally deliver negative signals to the NK cell; others bind to poorly characterized target cell receptors and deliver positive signals. Several of these receptors, of both the negative and positive signaling types, are C-type lectins and would thus be expected to recognize carbohydrate ligands in a Ca^{2+}-dependent manner. Here we summarize the recent progress in defining NK interactions with target cells. For convenience, we separate the receptors that appear to deliver negative signals, in general, from those that seem generally to deliver positive signals. Both may be important in NK recognition; an NK cell may balance several positive signals against negative ones to determine the outcome of an interaction with a given target cell [1].

Turn-offs: NK cell receptors binding to MHC class I molecules

It has long been known that expression of class I molecules from the major histocompatibility complex (MHC) can protect target cells against NK-mediated lysis. The initial observation was that a lack of class I expression on target cells increased the susceptibility to NK killing (reviewed in [2] and [3]). Two possible mechanisms for this phenomenon have been put forward. First, recognition of class I may turn off the lytic capacity of the NK cell. Thus, normal cells, which should not be targets for NK lysis, would be protected from NK killing by the expression of appropriate (or 'self') class I MHC molecules. However, tumors or virus-infected cells may lose or reduce their expression of class I MHC to avoid lysis by CTLs. Such cells would be lysed by NK cells since they fail to deliver a negative signal [2]. Alternatively, class I MHC may not be recognized by NK cells directly, but may hide or block access to a ligand bound by NK cell receptors [2]. Loss of class I MHC would thus allow recognition of the putative ligand, causing the target to be lysed.

Recent developments strongly suggest that the first hypothesis is correct. There are now several well characterized NK cell receptors that bind to specific class I molecules and inactivate the NK cell (summarized in Table 1). These are discussed in more detail below.

Ly49s — lectins that recognize class I MHC

Ly49s are dimeric, Type II glycoproteins belonging to the C-type lectin superfamily [4,5]. Two other families of NK cell specific proteins, the NKR-P1s, which also appear to be NK receptors (see below) and the NKG2s, of unknown function, are also C-type lectins. These three protein families have been classed together as Group V of the C-type lectins [5].

To date, eight genes encoding Ly49s have been identified in the mouse (Ly49A–H) [4,6–8], and three members of this gene family have been cloned in the rat (J. Ryan and W.E. Seaman, personal communication). No human homologs of Ly49s have yet been reported. Expression of one member of the Ly49 family probably does not preclude the expression of others; it is known, for example, that Ly49A and Ly49C can be co-expressed [9]. These lectin molecules appear to recognize class I MHC. The importance of carbohydrate in recognition is discussed below.

Class I MHC molecules are polymorphic. There are three main loci encoding these molecules in both mouse and human, with up to 70 different alleles at each locus. Different members of the Ly49 family appear to bind different subsets of class I MHC molecules. For example, murine Ly49A binds to the murine class I MHC molecule H-$2D^d$, and NK cells that express Ly49A cannot lyse target cells that express H-$2D^d$ [10–13]. Indeed,

interactions between Ly49A and H-2D^d may inactivate NK cells completely. As well as recognizing their targets via specialized and poorly understood mechanisms, NK cells can also use a more general mechanism to destroy targets that are coated with antibody, in a reaction called antibody-dependent cellular cytotoxicity (ADCC). Ly49A$^+$ NK cells are also unable to mediate ADCC against target cells carrying H-2D^d, suggesting that the presence of H-2D^d does not simply prevent the recognition of the target [10,13]. Binding of a Ly49 molecule to a class I MHC molecule does not seem to be the only requirement for inactivation, however. Ly49A also binds to a second class I MHC molecule, H-2K^b, with an affinity comparable to that for H-2D^d [11], but H-2K^b does not protect a target cell from lysis [10,13]. It will be of considerable interest to determine which MHC class I molecules are recognized by which members of the Ly49 family, and which of these interactions inactivate NK cells.

Although, in general, most Ly49 molecules appear to inactivate NK cell lysis, some may deliver activating signals. For instance, there is some evidence that cross-linking of Ly49D or Ly49C triggers NK lysis [14,15].

p58s/NKATs — receptor candidates from the immunoglobulin superfamily

Human NK cells express at least two 58-kDa molecules (p58) that appear to interact with human class I MHC molecule of the HLA-C alleles. These appear to inhibit NK lysis [16,17], although, again, certain p58 molecules may activate NK function. Recently, several p58 molecules have been independently cloned by two groups [18,19]. These molecules are part of a new NK gene family provisionally termed NKAT (NK-associated transcripts) [18], which may have as many as six members. These putative NK receptors belong to the immunoglobulin superfamily, and are unrelated to the C-type lectins. Thus, disparate types of NK receptors may interact with class I MHC molecules, with similar functional consequences.

Using NK cell-specific monoclonal antibodies, a molecule known as NKB1 has recently been identified that recognizes the Bw4 epitope of several forms of the human class I MHC molecule HLA-B. This interaction results in a loss of lytic function [20,21]. Sequence data on NKB1 are not available, but as the NKAT3 and NKAT4 genes encode molecules with the same molecular mass as deglycosylated NKB1 protein, and also appear to recognize Bw4, it seems likely that NKB1 will prove to be encoded by NKAT3 and/or NKAT4.

CD94 (Kp43) — a window onto signaling

CD94 (Kp43) is a 70-kDa disulfide-linked dimer expressed on human NK cells and certain subsets of T lymphocytes [22]. CD94 has recently been cloned, and found to be a member of the C-type lectin superfamily although it is not a Ly49 homolog [23]. It recognizes the class I MHC molecules HLA-B7, -B8, -B14 and -B27 [24]. Crosslinking of CD94 transduces signals resulting in

Table 1. Class I MHC–NK cell receptor interactions that result in loss of NK cell lysis of target cells.[a]

NK cell receptor	Class I MHC reactivity						
Human	**HLA-A**		**HLA-B**			**HLA-C**	
NKB1	–		B*1513[b] B*2403 B*2501 B*2705 B*5101 B*5801			–	
p58	–		–			Cw*3	
NKAT-1	–		–			Cw*0401 Cw*1503	
CD94	–		B7 B8 B14 B27			–	
Mouse	**H-2^b**	**H-2^d**	**H-2^k**	**H-2^p**	**H-2^q**	**H-2^r**	**H-2^s**
Ly49A	+ (K)	+ (D)	± (D)				
Ly49C[c]	+	+	+				+

[a]The class I MHC alleles recognized by the different NK receptor candidates are listed. In the mouse, it is often not known which class I gene product is recognized; instead, what is known is that one of the three class I molecules of a particular strain can be recognized. For Ly49A the specific class I MHC molecules recognized are known, and are noted in parentheses.
[b]All HLA-B molecules recognized by NKB1 contain the Bw4 epitope.
[c]Not determined if binding results in protection from NK lysis.

phospholipase D activation and release of TNF-α, but does not induce Ca^{2+} mobilization or phosphatidylinositol hydrolysis, two events commonly associated with receptor-mediated cell signaling [25]. There are some NK clones, however, in which lysis is activated, not inhibited, by CD94 crosslinking; in these cells, intracellular Ca^{2+} levels do rise. Thus, it may be possible to identify the point at which the positive and negative signaling pathways diverge by comparing NK clones that have different responses to CD94 crosslinking.

Structural requirements for NK recognition of MHC class I

The class I MHC molecule is composed of a polymorphic heavy chain complexed noncovalently with an invariant β2-microglobulin (β2m) light chain. The heavy chain contains sites for N-linked glycosylation (one site in humans, two in mice) and a 'groove', defined by two α-helices (α1 and α2), in which peptides that are processed from proteins made within the cell are presented [26,27]. Since some of the class-I-specific receptors on NK cells belong to the C-type lectin family, it is of obvious interest to determine whether recognition of class I involves oligosaccharide binding, and if so, by which receptors. Further, it is of interest to determine whether the specific peptide bound to class I affects NK recognition. It is known that T-cell receptor/class I interactions are exquisitely specific for peptides presented by class I, but much less is known about whether NK receptors are similarly affected.

A limited amount of work has been done towards determining whether specific carbohydrate expression is required for NK recognition of class I MHC. Early work done with bulk populations of fresh NK cells demonstrated that carbohydrate expression was not required for class I to inhibit NK lysis [28]. However, now that it has become clear that multiple receptors with different targets exist, it is no longer enough to study the behavior of a bulk NK population; we must ask, instead, whether the function of individual NK clones or NK receptors is oligosaccharide-dependent. Recently, Gumperz *et al.* [21] determined that oligosaccharides were not of significance for inactivation of NK clones carrying the NKB1 receptor by HLA-Bw4$^+$ target cells. Thus, the NKB1 receptor does not appear to recognize carbohydrate moieties expressed on class I MHC. However, as discussed above, NKB1 is suspected to be a member of the immunoglobulin superfamily and therefore would not be expected to recognize carbohydrate. In contrast, the Ly49 C-type lectins examined to date do appear to recognize carbohydrates, although it is not yet known if this is important in the physiological response of NK cells to class I MHC-bearing target cells. Ly49A has a functional lectin domain and binds fucoidan [29]. Some complex N-linked glycans expressed on class I contain fucose [27] and these might serve as ligands for Ly49A, but it has not been directly determined whether the binding of Ly49A to class I is dependent upon an associated oligosaccharide. Interestingly, Ly49C also appears to bind fucose, since binding of Ly49C to class I MHC has been found to be inhibited by fucoidan and by fucosidase treatment [9].

Since the carbohydrate recognition domains of Ly49A and Ly49C have only ~50% homology, it is somewhat surprising that they have such similar specificities. To accomodate these data, Brennan *et al.* [9] have postulated that Ly49s may recognize class I MHC as a combination of a carbohydrate moiety and associated protein. It is similarly speculated that the L- and P-selectins bind to a combination of carbohydrate and protein [30]. It has been shown that there is heterogeneity in the oligosaccharide moieties expressed on different class I molecules [27]. This leaves open the possibility that other members of the Ly49 family (or perhaps other NK-expressed C-type lectins) may bind class I in the context of different carbohydrates.

Cell-surface class I MHC molecules generally carry peptides in their peptide-binding groove [26]. The peptide forms an essential part of the complex recognized by the T-cell receptor. It is, therefore, of significant interest to determine if the peptide is also involved in NK receptor interactions with class I MHC. Several observations suggest that peptides may be important. Early studies determined that expression of both the α1 and α2 domains were required for protection against NK mediated killing, suggesting that an intact peptide-binding cleft is necessary [28]. By extrapolation, it could be inferred that peptide itself might affect NK recognition. This could occur in two ways: first, peptide may confer a required conformation to the class I molecule; or, second, the identity of the individual peptide itself may be important. The situation is complicated, however, by the fact that peptides are required to stabilize the class I MHC molecule, so that class I molecules that lack peptide are poorly transported to the cell surface and have a short half-life once there.

Malnati *et al.* [31] reported data supporting the possibility that specific peptides might be recognized by NK cells. They observed that human NK clones inhibited by the class I MHC molecule HLA-B27 were differentially sensitive to single amino acid substitutions in the peptide-binding site of HLA-B27. Such substitutions may alter the repertoire of peptides that can bind to the peptide-binding site. More importantly, these clones were also differentially inhibited depending on which specific peptide was presented. In this system, however, the receptor used by the NK cell was not defined. In contrast, Correa and Raulet [32] demonstrated, in the murine system, that virtually any peptide presented by H-2D^d was sufficient to inactivate Ly49A$^+$ NK cells, as long as the peptide allowed high levels of class I expression.

The reasons for the differences in the results regarding peptide specificity in these two models are not yet clear. Many factors may affect these results, including species differences, the use of heterogeneous populations of cells,

differences in affinity of the various peptides for class I, or differences in the receptor that is used for recognition. Perhaps the specificity of Ly-49 binding is dependent only on carbohydrate, not peptide, and peptide is only important to ensure high levels of class I MHC expression on the surface of the cell. It is also plausible that the human NK receptor recognizing HLA-B27 is NKB1. We have already argued that NKB1 may be a member of the immunoglobulin superfamily and not a C-type lectin [20,21]. It is not unreasonable to think that NK receptors belonging to the immunoglobulin superfamily (to which the T-cell receptor belongs) might be somewhat similar to the T-cell receptor in their specificity, binding to a molecular complex that is partially composed of peptide. Clearly, resolving the role of peptide in NK recognition will be of fundamental importance for understanding how class I MHC modulates target cell susceptibility.

Turn-ons: NK cell receptors for target cell ligands

There is a growing number of candidate receptors that may activate NK cells in response to target cells. In defining such receptors, several aspects of NK cell biology must be kept in mind. There is no evidence for DNA recombination in NK cells, unlike the situation in T-cells or antibody-producing cells. This statement is based largely on the observation that NK cells develop and function normally in SCID and RAG-deficient mice [33,34], which lack the mechanisms required for recombination of T-cell receptor and antibody genes. Furthermore, clones of NK cells do not have fine specificity, as different clones recognize multiple types of targets [35]. Although the number of NK receptors listed in this article may seem large, the number pales in comparison with the vast repertoire of specificities that are generated by combinatorial rearrangement in T-cells and antibody-producing cells. How, then, is differential recognition of target cells by NK clones acheived?

It has been suggested that NK recognition involves the coordinated interaction of a number of receptors providing activation signals. It is important to distinguish between receptor–ligand interactions that increase lysis, and those that contribute to specificity; for example, it is clear that certain adhesion molecules such as LFA-1 and CD2 contribute to the conjugation of NK cells to target cells, and may promote NK cell activation [36]. But it is apparent that molecules of this type do not contribute to the selectivity of NK recognition, and instead enhance lysis triggered by other receptors. The selectivity is likely to reflect how many of the receptors on a particular NK cell match the ligands available on a target cell. As described above, some NK selectivity may be due to 'turning off' the NK cell. But it is obvious that NK cells must also have receptors that activate lysis of appropriate targets as there is some selectivity in their target cell preference. A number of candidate receptors of this type have recently been defined.

NKR-P1s — lectin 'turn-ons'

Among the first activating NK receptors to be identified were the NKR-P1s; these are dimeric, 60–80-kDa signal transduction molecules that are members of the C-type lectin superfamily [37,38]. There are three mouse NKR-P1 genes [39,40], and probably five in the rat (J. Ryan and W.E. Seaman, personal communication; P.M. Appasamy and W.H.C., unpublished data). Only one human NKR-P1 homolog has been reported so far [41]. Although there are some species differences, in general NKR-P1s are expressed on essentially all NK cells and some T-cells [41–43]. Most of the available data indicate that NKR-P1s are involved in activation of NK cells [37,38,44]. However, as with the class I binding molecules, there is evidence to suggest that they can also deliver inactivating signals [41,45].

Rat NKR-P1A (rNKR-P1A) has been reported to bind neoglycoproteins expressing amino sugars (e.g., N-acetylgalactosamine), in a Ca^{2+}-dependent manner, with very high affinity [46,47]. Soluble rNKR-P1A also binds to multiple target cell associated oligosaccharides, particularly chondroitin sulfate glycosaminoglycans [47]. The NK cell line RNK-16 responds to YAC-1 target cells with increases in intracellular Ca^{2+} and the production of inositol phosphates. These responses were inhibited by soluble rNKR-P1 [47], suggesting that the interaction of NKR-P1 with oligosaccharides on target cells may be involved in activating YAC-1 lysis. The interesting question of whether different members of the NKR-P1 gene family have different oligosaccharide-binding characteristics remains to be addressed.

Once again, the situation is far from clear. In one study, mutant clones of the rat RNK-16 cell line which lack expression of all NKR-P1s could still efficiently lyse YAC-1 targets, but were deficient in lysing a different target cell line, IC-21 [48]. Re-expression of NKR-P1A in the NKR-P1 deficient cells partially restored their ability to lyse IC-21, suggesting that NKR-P1 was involved in killing of IC-21. In another study, some rat NK clones spontaneously lost expression of all NKR-P1s, but maintained the ability to lyse YAC-1 and IC-21 target cells [49]. The discrepancies between these studies indicate that much additional work needs to be done before the physiological functions and ligands of NKR-P1 are understood.

NK-TR1 — a non-lectin receptor with a carbohydrate ligand

The identification of NK-TR1 was unusual. The classic human NK cell target, K562, carries a carbohydrate epitope, which is thought to be involved in NK recognition of this cell line. To identify the receptors that bind to carbohydrates, an anti-idiotype (anti-id) antiserum was raised against a monoclonal antibody that recognizes this epitope [50]. Since the monoclonal antibody and the putative NK receptor are similar in that they bind the carbohydrate epitope, it was hoped that the anti-id would recognize the NK cell receptor as well as the monoclonal antibody. The anti-id indeed recognized NK cell associated proteins of 150 and 80 kD [50]. Subsequently, an NK-TR1-specific cDNA was isolated and found to encode a unique 150 kD protein containing a

cyclophilin-like domain [51]. Although NK-TR1 is believed to recognize an oligosaccharide, there is no apparent C- or S-type lectin domain in NK-TR1.

The first evidence that NK-TR1 was a 'turn-on' receptor came from the observation that blocking NK-TR1 inhibited NK cell lysis of K562, while cross-linking of NK-TR1 activated NK lytic activity [50]. More recently, it was reported that transfecting the RKN16 cell line with an antisense copy of the RNA for NK-TR1 dramatically reduced the levels of lysis of NK-sensitive targets and *Vaccinia* virus infected cells. However, there was no effect on antibody-dependent lysis [52]. These data all strongly suggest a role for NK-TR1 in NK cell interactions with tumor cells such as YAC-1 cells, and, interestingly, with virus-infected cells.

2B4s — turn-ons from the immunoglobulin superfamily

2B4, a 66 kD monomer expressed on all NK cells and on T-cells that show NK-like cytotoxicity in mice, appears to be important in activating the lytic machinery of these cells [53,54]. The expression of 2B4 in mouse strains corresponds to the expression of NKR-P1s [53,54], but it is not known if 2B4 and NKR-P1s may somehow act in concert. 2B4 is a novel member of the Ig supergene family, with some homology to human LFA-3 and rat CD48; there are several closely-related 2B4 genes [55]. Although no ligand for 2B4s has yet been reported, the data available strongly suggest that these molecules are an important element of the NK cell recognition repertoire.

p38 — a new entry in the lists

The latest candidate receptor for activation of NK cells, p38, has recently been defined using biochemical and antibody data [56], but has not yet been cloned. Antibody to p38 activates a variety of NK cell functions including cytotoxicity, Ca^{2+} mobilization, and production of interferons, but, curiously, does not induce granule exocytosis [56]. Blocking p38 inhibits the lysis of tumor target cells by freshly isolated NK cells; p38 may also be important in the activity of T-cells that mediate non-MHC restricted cytotoxicity [56].

Summary

Significant advances have been made in defining NK cell receptors involved in the recognition of tumors or virus-infected target cells (Fig. 1). It is now clear that NK cells have many ways to recognize class I MHC molecules, and that these receptors directly deliver signals that inhibit lytic function. The redundancy in this protective effect, and the fact that similar negative signals are mediated by highly disparate molecules, is striking.

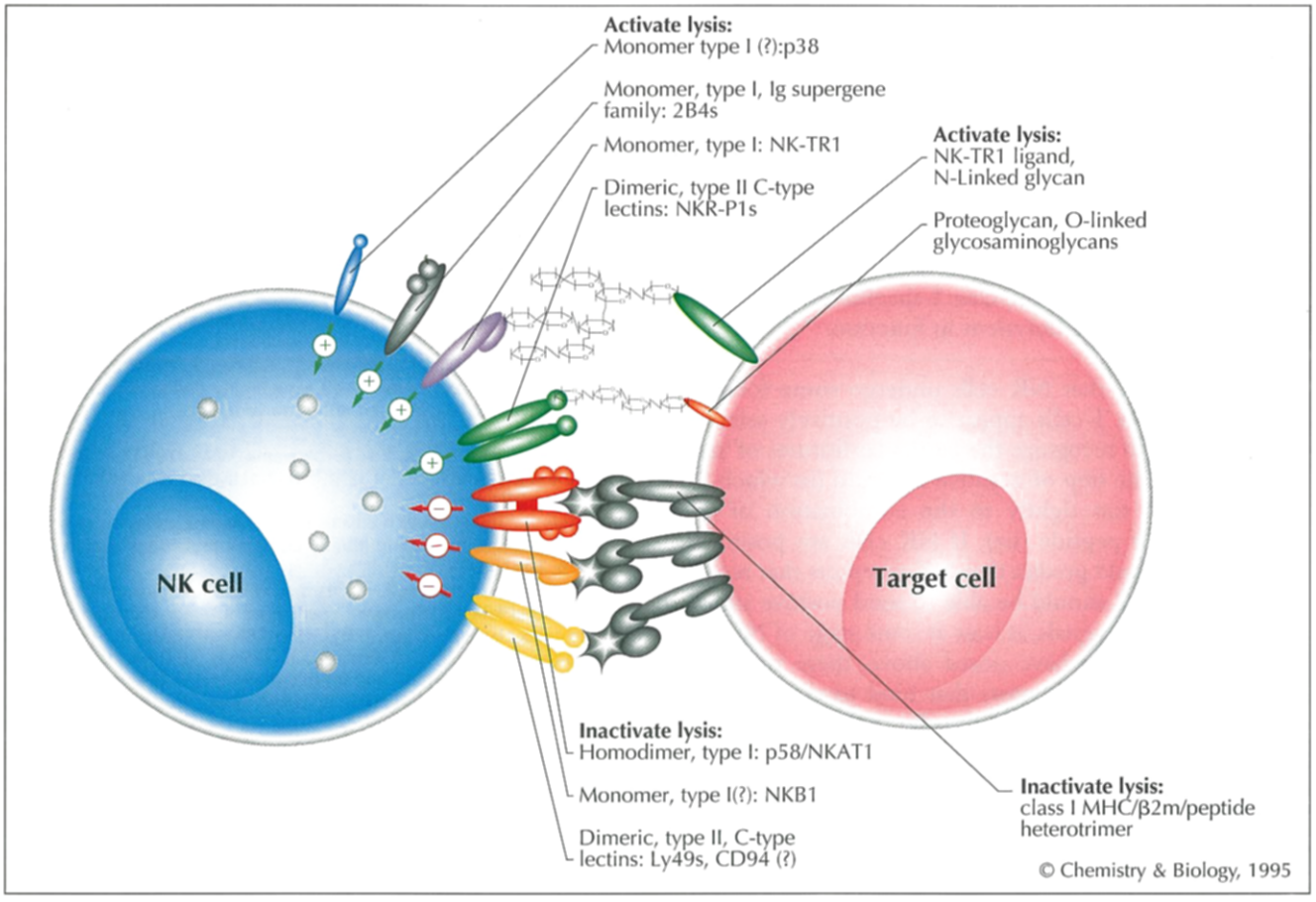

Fig. 1. NK activation is a product of many recognition steps, some of which send positive signals, others of which send negative signals. The NK receptors and target cell ligands discovered to date are summarized here. Many receptors can deliver both positive and negative signals (see text); the signal shown here is the signal that appears to be dominant for each receptor.

These results underscore the importance of preventing NK cells from attacking normal cells. Since NK cells are actively lytic without apparent prior sensitization, they would seem to be in a continual state of readiness to destroy target cells. Thus, it is crucial to provide redundant mechanisms for controlling reactivity against normal self.

The ability of NK cells to react against 'missing self' (i.e. lack of class I) in general, rather than against specific foreign antigen, fits teleologically with what is known of NK biology. Cytotoxic T-cells primarily recognize viral peptides presented in the binding groove of class I MHC molecules; to recognize all the possible varieties of invading viruses, T-cells require a vast repertoire of receptors, too large to be encoded in the genome, which is generated by DNA rearrangement. But T-cells, despite their power, have an Achilles' heel — if a virus should manage to prevent class I MHC expression on the surface of its host cell, the T-cell would have nothing to recognize. NK cells provide a crucial fail-safe mechanism for the immune system by recognizing cells that have lost class I MHC expression.

It is not yet clear whether the NK cells present in a given animal are selected to be unable to recognize the cells in that animal. T-cells that react with the combination of class I MHC with self peptide are eliminated early in their development; it is unclear whether NK cells follow a similar pattern. There is some evidence, however, that the expression of a specific class I MHC allele in a strain of mice affects either the number of Ly49-expressing cells, or the level of Ly49 expressed [57,58]. Additional experimentation will be necessary before it is clear whether this is correct. Any notion of NK cell selection must also be reconciled with the data suggesting that some of the class I-specific NK receptors (e.g. CD94) may be ambivalent in function.

It is also possible that NK cells undergo some form of positive selection. T-cells appear to be positively selected (for the ability to recognize the particular features of 'self' MHC) before the step of negative selection (in which T-cells that bind too tightly to the combination of self MHC with self peptide die). If NK cells are positively selected, one would predict that NK receptors that recognize class I MHC should be able to send positive signals as well as negative signals, at least at some stage of NK cell development. As we have noted above, there is indeed evidence that some receptors that bind to class I MHC can send positive signals, despite the general perception that NK cells recognize targets that lack class I MHC. In this context, it is interesting that NK cells from class-I-deficient mice seem to be less lytically efficient than NK cells from normal mice [59]. Furthermore, NK cells that reject B6 marrow develop in B6 animals transgenic for foreign class I [60]. This suggests that the foreign class I molecule induced the development of NK cells which recognize (and are turned off by) this molecule, and therefore perceive the normal B6 cells as lacking self.

In addition to the negative regulatory receptors that influence NK cell recognition, there are now clearly defined receptors,and multigene familes of receptors, that seem to be able to activate NK cells. Some of these receptors, for example, NKR-P1 and NK-TR1, may recognize oligosaccharide determinants on target cells. In general, however, very little is known about the nature of the ligands that interact with NK-activating molecules. It could be speculated that some will be conserved molecules, since NK cells of different species are similar in function and can recognize targets across species barriers. Many additional experiments will be necessary to clarify what the true ligands for NK cells are and how these affect NK activity.

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