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Activation and self-tolerance of natural killer cells

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Summary: Natural killer (NK) cells are regulated by numerous stimulatory and inhibitory receptors that recognize various classes of cell surface ligands, some of which are expressed by normal healthy cells. We review two key issues in NK cell biology. How do NK cells achieve tolerance to healthy self-cells, despite great potential variability in inhibitory and stimulatory receptor engagement? How is the disease status of unhealthy cells translated into changes in ligand expression and consequent sensitivity to NK cell lysis? Concerning the second question, we review evidence that ligands for one key NK receptor, NKG2D, are induced by the DNA damage response, which is activated in cells exposed to genotoxic stress. Because cancer cells and some infected cells are subject to genotoxic stress, these findings suggest a new concept for how diseased cells are discriminated by the immune system. Second, we review studies that have overturned the prevalent notion that NK cells achieve self-tolerance by expressing inhibitory receptors specific for self-major histocompatibility complex class I molecules. A subset of NK cells lacks such receptors. These NK cells are hyporesponsive when stimulatory receptors are engaged, suggesting that alterations in signaling pathways that dampen stimulatory receptor signals contribute to self-tolerance of NK cells.

Keywords: *DNA damage response, hyporesponsiveness, innate immunity, natural killer cells, tolerance, tumor immunology*

Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system that have an important role in the elimination of pathogens and tumors (1). NK cells contribute to the defense against parasites and intracellular bacteria, and they are critical for controlling several types of viral infection (2–6). The NK cell response is immediate, in contrast to responses of the adaptive immune system, and is believed to play its most important role during the first few days of infection with pathogens. The anti-tumor cell activity of NK cells, which was the basis for the original discovery of the cells, is well established *in vitro* and in several *in vivo* models (7–10). As discussed herein, it has been proposed that they play a role in the surveillance and elimination of spontaneous tumors.

NK cells do not express the specialized genes required for rearrangement of the T- and B-cell antigen receptor genes (11).

Nonetheless, NK cells exhibit a clear capacity to discriminate normal and diseased cells as well as self- and foreign cells. Our current understanding of the nature of NK cell receptors, the mechanisms that govern their expression, and the polymorphism and expression of the corresponding ligands provides much insight into the discriminatory power of NK cells, though much remains to be learned. It has become clear that NK cell recognition is governed by numerous receptors, some stimulatory and others inhibitory, and that the outcome of an NK cell–target cell interaction is governed by a process that integrates all these disparate signals and determines whether the NK cell is ultimately activated, cytokines are produced, and/or the target cell is killed (12–16). Despite the complexity in the recognition process, NK cells maintain self-tolerance, that is, they do not attack healthy self-cells, and are able to distinguish infected cells or tumor cells from normal self-cells (14). While we are far from a complete understanding of these processes, recent findings have added much to the picture. This review describes research, performed by our group and others, aimed at understanding how NK cells acquire self-tolerance, yet are able to discriminate normal cells from diseased cells.

Before addressing this newer research in detail, background information on NK cell recognition is discussed. Many of the inhibitory receptors expressed by NK cells are specific for major histocompatibility complex (MHC) class I molecules. Inhibitory receptors play a central role in 'missing self-recognition' by NK cells, that is, the capacity of NK cells to attack cells that lose or downregulate expression of MHC class I molecules, which are expressed by nearly all normal cells (17, 18). Lowered or absent MHC class I expression often occurs in tumor cells and infected cells, presumably as a means for these cells to evade an adaptive immune response. This absence can lead to or contribute to the susceptibility of the diseased cells to lysis by NK cells. NK cells attack even normal cell types, however, such as bone marrow cells and lymphoid cells, when these cells lack MHC class I molecules as a result of targeted mutations (19–22). *In vivo*, even resting lymphocytes are attacked if they lack self-MHC class I molecules (22).

Missing self-recognition occurs when inhibitory MHC-specific receptors on NK cells fail to be engaged. Inhibitory MHC class I-specific receptors include members of at least three families of proteins: the lectin-like Ly49 family present in mice, rats, and other species, but not in humans (23); the killer cell immunoglobulin-like receptor (KIR) found in humans, other primates, and other species, but not mice (12, 24); and the CD94/NKG2A receptor shared by all species so far examined (25, 26). Individual receptors bind with varying affinities to distinct MHC class I molecules as a result of allelic polymorphism of MHC class I molecules (24–30). Furthermore, a largely random process confers each NK cell with expression of only a subset of the 10–20 inhibitory receptors encoded in the germ line of each animal (14, 31, 32). Hence, individual NK cells typically express only three to five such receptors, and any combination seems to be possible (31–34). In addition, several inhibitory receptors exist that are specific for non-MHC class I ligands. One example is the 2B4 receptor, which is specific for CD48, and another example is the NKR-P1B and NKR-P1D receptors, which bind to Clr-b/Ocil, a member of a distinct family of lectin-like cell surface glycoproteins (35–37).

Whereas inhibitory receptors modulate NK cell activation, stimulatory receptors must be engaged in order for NK cell activation to occur. Thus, certain normal (non-diseased) cell types, such as bone marrow cells and lymphocytes, express stimulatory ligands for NK cells. For example, rejection of BALB/c bone marrow cells in F1 hybrid BALB/c × C57BL/6 mice depends on the expression of NKG2D ligands on these bone marrow cells (38). In other cases, MHC class I molecules (39) and other unidentified ligands (40) serve as stimulatory ligands on normal healthy cells. However, the fact that the normal bone marrow cells also express MHC class I molecules prevents them from being lysed by syngeneic or autologous NK cells (19). Hence, downregulation of MHC class I molecules alone, without alterations in stimulatory ligands, may render a cell susceptible to lysis by NK cells. In infected or transformed cells, however, the expression of stimulatory ligands is often induced or upregulated (41, 42). Strong stimulatory signaling resulting from increased levels of stimulatory ligands can often overcome inhibitory signals provided by recognition of normally expressed MHC class I molecules (43–48). Hence, susceptibility of diseased cells to lysis by NK cells may arise as a result of elevated levels of stimulatory ligands, even if MHC class I molecules are not downregulated. The greatest susceptibility arises when diseased cells undergo both changes, i.e. when the cells downregulate MHC class I and upregulate stimulatory ligands.

Some stimulatory receptors have been implicated in the recognition of tumor cells and virus-infected cells. One such receptor is the high-affinity Fc receptor, which enables NK cells to kill antibody-coated target cells (49). Several other receptors that enhance NK cell activation bind to constitutively expressed self-ligands and may serve as coreceptors in NK cell recognition (12). In addition to these receptors, other stimulatory receptors play a more direct role in discriminating diseased cells from normal cells. One class of stimulatory receptors appears to recognize ligands encoded directly by pathogens. For example, mouse cytomegalovirus encodes an MHC-like protein that

binds to the stimulatory receptor Ly49H in certain mouse strains, and the stimulatory receptor NKp46 reportedly recognizes molecules expressed at the surface of cells infected with influenza virus or vaccinia virus (50–52). MHC class I molecules are recognized by another class of stimulatory receptors, including a subset of KIRs in humans and Ly49 receptors in mice (53, 54). The function of these stimulatory MHC-specific receptors in the context of disease is uncertain, but it is possible that they help NK cells respond to diseased cells that lose expression of an inhibitory ligand for NK cells. A third category of stimulatory receptors, including NKG2D and probably NKp46, NKp44, and NKp30, recognize self-ligands that are expressed poorly by normal cells but are upregulated on the surface of diseased cells (13, 42). The term 'induced self-recognition' was coined to refer to the latter recognition strategy (55).

The purpose of this review is to describe the status of ongoing studies that address two key issues that arise in considering NK cell recognition. One issue concerns induced self-recognition. If some of the stimulatory ligands that NK cells recognize are encoded by the host genome and upregulated specifically in diseased cells, what information does the cell use to perceive its diseased status, and how is this information used to induce ligand upregulation? We discuss recent efforts to define the pathways that upregulate expression of ligands for the NKG2D receptor, currently the best characterized example of induced self-recognition. The second question concerns missing self-recognition. As described above, the inhibitory receptors that recognize MHC class I molecules are inherited independently of the MHC ligands and are expressed in a largely random fashion by NK cells. In addition, the MHC class I ligands they recognize are highly polymorphic. Given this randomness, do NK cells arise that fail to express inhibitory receptors specific for self-MHC class I molecules? If so, how are they prevented from attacking normal self-tissues? Or put another way, how is the potential autoreactivity of NK cells normally prevented?

Induced self-recognition and the NKG2D immunoreceptor

NKG2D is expressed on virtually all NK cells and is usually called an NK receptor (42, 44, 46, 56). However, it is also expressed by various T-cell subsets. NKG2D is expressed by all $CD8^+$ T cells in humans (46) but only by activated and memory $CD8^+$ T cells in mice (56). $CD4^+$ T cells in the mouse do not express NKG2D, even after short-term activation *in vitro* (56), but it may be upregulated on these cells under other conditions. In humans, NKG2D is rapidly upregulated after activation of $CD4^+$ T cells *in vitro* (57), and it can be detected on activated $CD4^+$ T cells present in the synovial fluid of arthritis patients (58). NKG2D is also expressed by subsets of natural killer T cells and γ/δ T cells (46, 56).

Target cell recognition by NK cells often requires NKG2D engagement *in vitro*, although other stimulatory receptors are usually also involved. In the case of interleukin-2 (IL-2)-cultured NK cells or NK cells from animals recently exposed to substances that activate NK cells, such as double-stranded RNA (poly I:C), cross-linking the NKG2D receptor with antibody or with NKG2D ligands presented on transfected cells is sufficient to induce production of inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (56, 59, 60). In conventional $CD8^+$ T cells, NKG2D is believed to provide a costimulatory signal, which enhances the responses of T cells activated through the T-cell receptor (56, 61, 62). In cases of chronic T-cell activation *in vivo*, as may occur in chronic inflammation such as in the intestinal mucosa of patients with celiac disease, $CD8^+$ T cells are believed to become independent of a requirement for T-cell receptor-mediated activation, such that NKG2D-mediated activation is a sufficient trigger to activate the cells (63).

In NK cells and T cells, NKG2D associates with DNAX-activating protein of 10 kDa (DAP10), a signaling adapter molecule that recruits and activates phosphatidylinositol 3-kinase (PI3K) after receptor ligation (64). Ligation of the DAP10-associated form of NKG2D induces cytotoxicity of NK cells and of cytokine-activated $CD8^+$ T cells but is insufficient to trigger cytotoxicity in many other $CD8^+$ T cells, where it instead enhances T-cell activation (56, 61, 62, 65–67). In mouse but not in human NK cells, NKG2D also associates with DNAX-activating protein of 12 kDa (DAP12), a distinct signaling adapter molecule that induces both cytotoxicity and cytokine production (65, 68, 69).

NKG2D expression on the surface of NK cells is upregulated by cytokines such as IL-15 (59). In some instances, IL-15 also alters signaling by NKG2D in T cells, such that NKG2D engagement becomes sufficient to activate $CD8^+$ T cell-mediated cytotoxicity (70). Hence, the local cytokine environment can regulate NKG2D functionality. Another level of NKG2D regulation, however, is at the level of expression of cell surface ligands for the receptor, and this process has been the main focus of our efforts to understand the principles of induced self-recognition.

NKG2D ligands

The ligands for NKG2D are the MHC class I chain related (MIC) and retinoic acid early transcript 1 (RAET1) protein families

(reviewed in 42). These proteins are distant relatives of MHC class I proteins, but unlike class I proteins, they do not associate with β2-microglobulin (β2m) or bind antigenic peptides or other cargo. The *MIC* gene family members, *MICA* and *MICB*, are localized within the human leukocyte antigen (*HLA*) gene complex and are expressed in most mammalian species except mice. In contrast, both mice and humans express the *Raet1* gene family. The *Raet1* genes are not part of the MHC but rather map to mouse chromosome 10 or the syntenic segments located on human chromosome 6. Human RAET1 proteins, also called UL-16 binding proteins (ULBPs), share only 25% or less of their amino acids with MIC proteins. There is also considerable sequence variation within the RAET1 family, with some pairs exhibiting as little as 35% amino acid sequence identity. Mouse Raet1 proteins include three subfamilies of proteins called Rae1, H60, and Mult-1, which have a similar structure but are only approximately 30% identical in their amino acid sequences in pairwise comparisons.

Expression of NKG2D ligands

In healthy humans, MICA and MICB are detected only on gastrointestinal epithelial cells, possibly due to the close contact of these cells with intestinal microbes (71). However, expression of MICA and MICB is upregulated on many tumor cell lines and primary tumors, particularly those of epithelial origin (72). Similar to MIC proteins, mouse and human Raet1 molecules are poorly expressed on the surface of normal cells but are commonly upregulated on transformed and infected cells (44, 45, 73–76). The upregulation of NKG2D ligands on transformed or infected cells suggests that cells have sensing mechanisms that recognize changes associated with infection and transformation and activate pathways that upregulate cell surface expression of NKG2D ligands. Expression of MICA on various intestinal epithelial cell lines is substantially upregulated under conditions of 'stress' such as heat shock and viral infection (71, 77, 78). Heat shock transcription elements have been identified in the promoters of the MIC genes and may account for stress-induced MIC upregulation under some circumstances (71).

Our efforts were initially directed toward understanding the signals that upregulate mouse Raet1 proteins in tumor cells. Given that nearly all established tumor cell lines express Raet1 proteins, we were surprised to find that certain cell lines that were transformed in cell culture did not express Raet1 proteins (79). These findings suggested that transformation *per se* is not sufficient to induce ligand expression. Interestingly, we found that treating these transformed cells with various forms of stress, including heat shock, serum starvation, hypoxia, and hyperoxia, did not induce mouse or human Raet1 proteins at the cell surface. These treatments also failed to induce expression of Raet1 family ligands in cultures of untransformed mouse or human fibroblasts (79). These data prompted us to investigate the role of other pathways in the induction of Raet1 family proteins.

Genotoxic stress regulates expression of NKG2D ligands

Another stress pathway that is relevant to cancer is the DNA damage response, which is involved in maintaining the integrity of the genome. This response, also called the genotoxic stress response, is activated in normal cells subjected to DNA damage and serves to arrest the cell cycle, promote DNA repair functions, and, in highly damaged cells, induce apoptosis (80). But recent studies have shown that the DNA damage response is often constitutively activated in human cancer cells and in cells infected by certain viruses (81–83). Accordingly, it is believed that tumorigenesis and infection can damage DNA or stress the genome by other mechanisms (81, 82). Before reviewing evidence that this pathway regulates NKG2D ligands, the protein kinases that comprise the pathway will be described. This topic is reviewed in greater detail elsewhere (80).

Following DNA damage, the PI3K-related protein kinases ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3 related) initiate the DNA damage response in concert with other proteins (80). Both kinases are activated by many types of genomic insult but also show a division of labor in detecting different types of DNA damage. For example, ATM responds preferentially to double-strand breaks in the DNA, while ATR senses single-strand breaks and stalled replication forks. The principal kinases relaying the ATM/ATR-initiated checkpoint signals are Chk1 and Chk2. These two kinases phosphorylate and activate effector proteins involved in cell cycle arrest and DNA repair, such as p53, E2F1, CDC25 family members, BRCA1, and H2AX (80). p53 and other mediators induce cell cycle arrest, allowing DNA repair to take place in the absence of DNA replication. If DNA damage is too extensive to be repaired, p53 can induce apoptosis, presumably in order to rid the host of these potentially dangerous cells. Activation of DNA repair systems and apoptotic responses thus represent different strategies for maintaining genome integrity. Defects in DNA repair functions or apoptosis lead to genetic predisposition to cancer (84).

The finding that the DNA damage response is chronically activated in many tumor cells and in certain infected cells suggested that this pathway could serve as a novel sensing system for detecting the disease status of a cell and activating

components of the immune response. Support for such a role of the DNA damage response was provided by our finding that agents that damage the DNA or impart DNA replication stress induce expression of Raet1 family proteins (79) (*Fig. 1*). DNA-damaging agents induced Rae1, Mult-1, and H60 in untransformed mouse fibroblasts, whereas human RAET1 proteins (ULBPs) were induced in cultures of secondary human fibroblasts (79) (*Fig. 2*). Furthermore, upregulation of ligands in response to genotoxic stress required the function of the ATM, ATR, or Chk1 kinases, depending on how the cell's DNA was damaged (79) (*Fig. 1*).

Nearly all tumor cell lines constitutively express one or more NKG2D ligands on the cell surface (44). Recent studies demonstrated that the DNA damage response is activated in precancerous and cancerous lesions as well as in established tumor cell lines (81, 82). To test whether constitutive activity of the DNA damage response proteins is responsible for maintaining NKG2D ligand expression on tumor cell lines, we used various approaches to inhibit the function of these proteins. We observed that knocking down expression of ATM and Chk1 with short interfering RNA (siRNA) in the murine ovarian epithelial tumor cell line T2 led to a substantial decrease in Rae1 levels at the cell surface, whereas knocking down ATR had no effect (79) (*Fig. 3*). Similar results were obtained with a second ovarian carcinoma cell line, T1 (*Fig. 3*). In contrast, NKG2D ligand expression in the lymphoma c-myc#3 was reproducibly partially inhibited by knocking down expression of ATR but not ATM (*Fig. 3*). These findings support the idea that constitutive ligand expression is maintained by persistent genotoxic stress in tumor cell lines and suggest that either ATM or ATR may be predominantly responsible for maintaining NKG2D ligand expression, presumably depending on the type of genotoxic stress present in the tumor cell line. Our results suggest that the DNA damage response serves not only to maintain the integrity of a cell's genome but also to alert the innate immune response that the cell is unhealthy. In light of the hypothesis that NK cells and NKG2D play a role in tumor surveillance, these findings suggest that DNA damage in tumor cells helps induce NKG2D ligands and increases the sensitivity of the damaged cells to NK- or T-cell-mediated lysis, possibly imposing an immune-mediated barrier to tumorigenesis (85, 86).

Consequences of DNA damage-induced NKG2D ligand expression

The possible role of NKG2D and its ligands in tumor surveillance is supported by numerous studies. Ectopic expression of NKG2D ligands in rare tumor cell lines that lack endogenous NKG2D ligands rendered the cells sensitive to NK cell-mediated lysis *in vitro* and resulted in tumor rejection *in vivo* (44, 45, 87). In some cases, long-lasting T-cell responses were also induced (87, 88). Administration of a DNA vaccine encoding NKG2D ligands and a tumor antigen induced therapeutic tumor immunity, whereas little or no immunity was induced with a control vaccine encoding only the tumor antigen (89). Other studies imply an important early role for NKG2D in controlling the incidence and progression of cutaneous carcinogenesis (90, 91) and in surveillance of carcinogen-induced tumors (91). In some cases, tumors appear to evade NKG2D-mediated surveillance, as shown by the presence of shed forms of MICA and ULBP in the serum of human cancer patients (92, 93). The appearance of shed ligands was correlated with downregulated NKG2D expression and impaired activation of NK cells and T cells. Hence, many studies suggest a role for NKG2D in tumor surveillance, but more experimental evidence will be required to provide firm support for this hypothesis and to prove that the DNA damage response serves as a key sensor mechanism in this process.

Other studies suggest a role for NKG2D in responses to virus-infected cells. For example, transcripts encoding NKG2D ligands are upregulated in cells infected with herpesviruses, though some herpesviruses have evolved mechanisms to

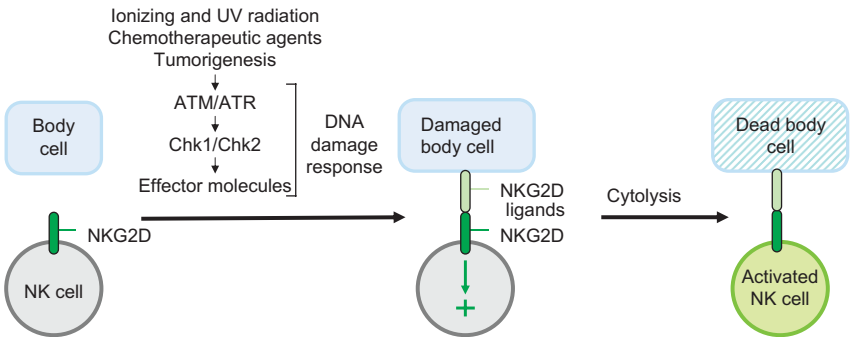

***Fig. 1.* Genotoxic stress induces expression of ligands for the stimulatory NKG2D receptor expressed by NK cells and T cells.** DNA-damaging agents and other genomic insults, some of which occur in cells undergoing tumorigenesis, activate the DNA damage response, leading to upregulation of Raet1 family ligands of the stimulatory NKG2D receptor. Expression of NKG2D ligands on diseased cells renders them sensitive to lysis by NK cells and other lymphocytes.

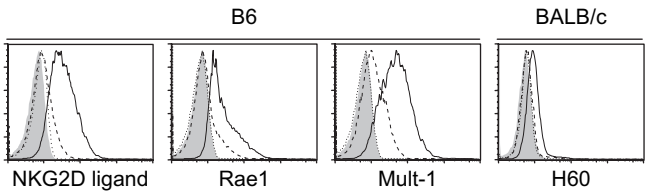

***Fig.* 2. Raet1 family NKG2D ligands Rae1, Mult-1, and H60 are all induced by an agent that induces the DNA damage response.** Adult fibroblast lines derived from C57BL/6 or BALB/c mice were incubated with 4 μM aphidicolin (16 h treatment; solid line), which activates ATR and the DNA damage response. Induced expression of NKG2D ligands was monitored by flow cytometry, gating on cells that did not stain with propidium iodide (live cells). Expression of all NKG2D ligands was assessed by staining with tetramers of soluble NKG2D, whereas expression of specific NKG2D ligands (pan-Rae1, Mult-1, and H60) was determined by staining with monoclonal antibodies [for staining we used pan-Rae1, Mult-1, and H60-specific monoclonal antibodies from R&D Systems (Minneapolis, MN, USA) and anti-rat IgG (H + L) coupled to phycoerythrin from Jackson ImmunoResearch (West Grove, PA, USA) as secondary antibody]. Solid line: treated cells stained with reagents to detect NKG2D ligands. Dashed lines: untreated cells stained with reagents to detect NKG2D ligands. Filled histograms: treated cells stained with negative control tetramers or isotype control antibodies.

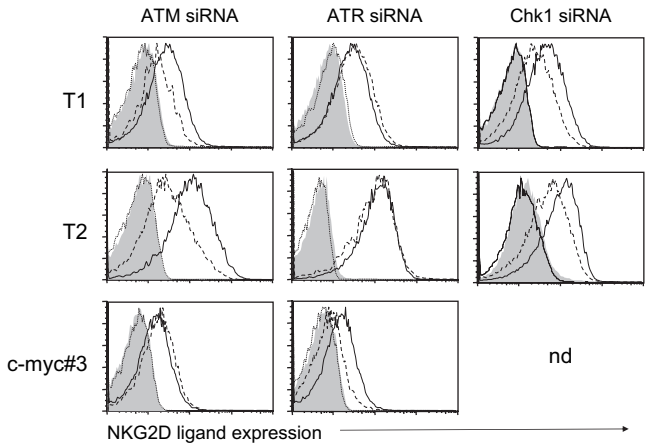

***Fig.* 3. siRNA knockdowns of ATM or ATR differentially inhibit cell surface expression of NKG2D ligands in different tumor cell lines.** T1 and T2 are independent ovarian carcinoma cell lines, and c-myc#3 is a T lymphoma induced by constitutive c-myc expression. Tumor cell lines were transduced with retroviral vectors encoding green fluorescence protein (GFP) and ATM siRNA or ATR siRNA or GFP and control siRNA, and were cultured for 7 days, splitting as necessary [using methods similar to those previously published (79)]. Cells were harvested and examined for cell surface levels of NKG2D ligands, gating on transduced (GFP^+) cells. Dashed lines: ATR or ATM siRNA-transduced cells stained with NKG2D tetramers. Solid lines: control siRNA-transduced cells stained with NKG2D tetramers. Dotted line: ATR or ATM siRNA-transduced cells stained with control tetramers. Filled histogram: control siRNA-transduced cells stained with control tetramer. As determined by real-time reverse transcriptase polymerase chain reaction using T2 cells, ATM messenger RNA (mRNA) in ATM siRNA-transduced T2 cells was reduced to 53 ± 2% of the level in untransduced (GFP^-) cells in the same culture, or 55 ± 3% of the level in control siRNA-transduced cells (mean ± SD of three replicate determinations). ATR mRNA in ATR siRNA-transduced T2 cells was reduced to 49 ± 5% of the level in untransduced (GFP^-) cells in the same culture, or 50 ± 0.4% of the level in control siRNA-transduced cells (mean ± SD of three determinations). Chk1 mRNA in Chk1 siRNA-transduced T2 cells was reduced to 36 ± 4% of the level in untransduced (GFP^-) cells in the same culture, or to 43 ± 8% the level in control siRNA-transduced cells (mean ± SD of three determinations). nd, not done.

prevent cell surface expression of these ligands, enabling the virus to evade NKG2D-mediated recognition (48, 61, 77, 78, 94, 95). The possible role of the DNA damage response in the upregulation of ligand transcripts and proteins in herpesvirus-infected cells is currently under investigation. In another example, infection of mouse preB cells by the Abelson murine leukemia virus resulted in increased expression of Rae1 proteins on the cell surface (96). Evidence was presented supporting a scenario in which viral infection induces B cells to express activation-induced cytidine deaminase (AID), which is normally expressed only by germinal center B cells in uninfected mice for the purpose of introducing somatic hypermutations in immunoglobulin (Ig) genes and inducing Ig class switching. In infected preB cells, AID expression induced the DNA damage response, presumably by damaging DNA in the infected cells, and was necessary for Rae1 upregulation (96). Hence, in this system, NKG2D ligand expression was linked to the DNA damage response by the mutation-inducing protein AID.

NK cell self-tolerance

The previous discussion addressed the pathways that sense cellular distress and induce stimulatory ligand expression so as to favor NK cell activation. Implicit in this analysis is the notion that encounters of NK cells with self-cells are normally not stimulatory, i.e. NK cells are self-tolerant. Yet, as discussed in the introduction, normal self-cells do express ligands that engage stimulatory receptors on NK cells. More generally, various normal cells express different combinations and levels of ligands that engage the corresponding stimulatory and inhibitory NK cell receptors. Furthermore, the array of stimulatory and inhibitory receptors expressed by each NK cell varies. Therefore, the combination and amount of stimulatory and inhibitory receptor occupancy that occurs when an NK cell encounters healthy cells in the body is expected to vary substantially depending on the specific NK cell and body cell involved in the encounter. Given these variations, how is self-tolerance established? This is a question that has engaged us and other groups for several years, and that some recent results are beginning to answer.

For some years, it was assumed that the explanation of NK cell self-tolerance laid solely in the nature and expression pattern of inhibitory receptors specific for MHC class I molecules. If each NK cell was endowed with at least one such receptor specific for a self-MHC class I molecule (the 'at least one' model) and if engagement of that receptor was sufficient to override the stimulation that NK cells receive when they encounter stimulatory ligands on normal cells in the body, self-tolerance would be established (31). Given the stochastic nature of the expression of KIR and Ly49 receptors by developing NK cells, the 'at least one' model implies the existence of adaptive or selective processes that ensure that mature NK cells express at least one receptor specific for self-MHC class I molecules.

A study of panels of human NK cell clones from two donors with different HLA haplotypes seemed to support the 'at least one' model. Each clone expressed one or more inhibitory receptors specific for the HLA class I molecules of the donor (32). It has not been established, however, that panels of long-term NK cell clones accurately represent the repertoire of clones present in a human or other animal.

An alternative model was suggested by studies of NK cells in MHC class I-deficient animals. As shown originally in mice with a homozygous mutation of the β2m gene, MHC class I-deficient animals contain abundant numbers of NK cells (20). Despite the absence of inhibitory MHC class I ligands in these mice, the NK cells were not demonstratively autoreactive: unlike NK cells from $\beta2m^{+/+}$ littermates, the NK cells from $\beta2m^{-/-}$ mice failed to kill Con A blasts from $\beta2m^{-/-}$ mice and failed, *in vivo*, to reject bone marrow grafts from $\beta2m^{-/-}$ mice (19–21). Furthermore, NK cell-dependent autoimmune pathology has not been associated with this mutation. These findings were later extended to other models of MHC class I deficiency, including mice and humans with mutations in subunits of the transporter associated with antigen processing and mice with a double mutation in both the K^b and D^b MHC class I genes (97–100). Collectively, these studies showed that self-tolerance of NK cells does not necessarily depend on inhibitory interactions of NK cells with MHC class I molecules.

Further analysis of the MHC class I-deficient mice showed that their NK cells exhibited some generalized functional defects, when compared with NK cells from normal animals. Among the defects observed were reduced capacity to reject allogeneic bone marrow transplants (20), reduced antibody-dependent cellular cytotoxicity (101), and reduced lysis of tumor target cells (20, 101–103), though the last defect (reduced tumor target cell lysis) was not reported by all investigators (21). In addition to defects in cytotoxicity, we also observed defects in cytokine (IFN-γ) production by NK cells from MHC class I-deficient mice, when the cells were stimulated with tumor cell targets (102). The functional defects of NK cells from MHC class I-deficient mice are reproducible and significant, but it is important to emphasize that the defects are not complete, despite the absence of detectable reactivity to MHC class I-deficient bone marrow cells. Thus, NK cells that arise in MHC class I-deficient mice are partially but not completely functionally impaired, a state we have termed 'hyporesponsiveness' (14, 102). We proposed that hyporesponsiveness is a result of alterations in signaling pathways that lead to dampened stimulatory signaling in these NK cells. We further proposed that dampened stimulatory signaling contributes to self-tolerance. This state could occur if the stimulation provided by interactions of NK cell stimulatory receptors with ligands on normal cells is below the threshold necessary to trigger hyporesponsive NK cells. The partial lysis of tumor cell targets by the same NK cells may be due to higher expression of stimulatory ligands by tumor cells than by untransformed cells.

Recent findings bearing on self-tolerance

The studies of hyporesponsive NK cells in MHC class I-deficient mice led to the hypothesis that self-tolerance of some NK cells is associated with a hyporesponsive functional phenotype, but it remained unclear whether hyporesponsive NK cells exist in normal animals. The hyporesponsive NK cells would be expected to be those that are not inhibited by self-MHC class I molecules. Therefore, it was first necessary to directly test whether some NK cells in normal mice do not express inhibitory receptors specific for self-MHC class I molecules, contrary to the predictions of the 'at least one' model. In the C57BL/6 (B6) mouse strain, the only characterized inhibitory receptors that specifically recognize H-2^b class I molecules are Ly49C, Ly49I, and CD94/NKG2A (26, 104, 105). Staining of NK cells in B6 mice showed that 10–15% of NK cells do not express any of these H-2^b class I-specific inhibitory receptors (102). These NK cells proved to be self-tolerant, as they failed to lyse autologous cells *in vitro*. Moreover, in contrast to NK cells that express H-2^b-specific inhibitory receptors, NK cells lacking these inhibitory receptors failed to lyse MHC class I-deficient lymphoblast target cells (102). Aside from demonstrating a functional defect in NK cells that lack these three receptors, the failure of these NK cells to lyse MHC class I-deficient cells argues against the possibility that their self-tolerance is due to expression of some heretofore undefined H-2^b-specific inhibitory receptors. *In vivo* experiments demonstrated that in B6 mice, these NK cells, unlike other NK cells, were also unable to reject grafts of bone marrow cells from class I-deficient mice (102).

In addition to the defect in lysing class I-deficient lymphoblasts, B6 NK cells that lack inhibitory receptors specific for self-MHC class I molecules were significantly impaired in their capacity to lyse any of several tumor target cells tested or to produce inflammatory cytokines when stimulated with those tumor cells (102). These functional defects were similar to those already documented for NK cells resident in MHC class I-deficient mice.

It was possible that self-tolerance of these NK cells and their attendant low responsiveness to tumor target cells were due to increased activity of inhibitory receptors specific for non-MHC class I ligands. Candidates for such inhibitory receptors include 2B4 (CD244) and NKR-P1B, both of which bind ligands (CD48 and Ocil/Clr-b, respectively) that may be present on the target cells studied (35–37). However, no change in the expression of 2B4 was observed in these NK cells. To test whether the impaired function of these hyporesponsive NK cells requires interactions with target cells that might potentially express inhibitory ligands, we tested the induction of cytokine production by the NK cells in a target cell-free system. The results showed that reduced functionality of these NK cells was clearly evident, even when the cells were stimulated by cross-linking any of several stimulatory receptors with immobilized receptor-specific monoclonal antibodies (102). NK cells from MHC class I-deficient mice were similarly hyporesponsive when stimulated in this manner. Thus, NK cells that lack inhibitory receptors specific for self-MHC class I molecules are hyporesponsive to stimulatory receptor engagement independent of inhibitory interactions with target cells.

The basis of hyporesponsiveness has not been determined, but some explanations appear unlikely based on the available data. The possibility that stimulatory receptors are generally downregulated at the cell surface is refuted by the finding of normal cell surface expression of all such receptors tested, including NKR-P1C (also called the NK1.1 antigen), NKG2D, Ly49D, and CD16 (102). Similarly, the intracellular levels of the DAP12 and DAP10 signaling adapter molecules were not decreased in hyporesponsive NK cells. The possibility that hyporesponsive NK cells are developmentally immature is also unlikely because the cells exhibited normal expression of markers that are associated with fully mature NK cells, including CD11b, DX5, and Ly49 receptors (i.e. those other than Ly49C and Ly49I) (102). Indeed, hyporesponsive NK cells responded almost normally in terms of cytokine production, when stimulated with pharmacological agents, such as a protein kinase C activator plus ionomycin. Collectively, these findings suggest that hyporesponsive NK cells are mature cells equipped with functional potential. Their defects are likely due to changes in signaling pathways that dampen responsiveness to stimulatory signals.

Mechanisms of NK cell self-tolerance

What process could cause the development of hyporesponsive NK cells? The dampened response is reminiscent of that of anergic T cells and B cells that arise in situations where the cells are chronically stimulated with antigen or stimulated in the absence of costimulatory signals. Analogously, we have proposed that hyporesponsive NK cells arise as a result of persistent *in vivo* stimulation (14, 16, 40, 102) (*Fig.* 4). The model is predicated on evidence, discussed in the introduction to this review, that NK cells are constantly exposed to stimulatory ligands expressed on normal self-cells in the body. Hence, we proposed that some NK cells will be persistently stimulated because the chronic stimulatory signals are not counteracted by inhibitory signals from MHC class I-specific receptors. All of the NK cells in MHC class I-deficient mice fail to receive such signals and hence are rendered hyporesponsive. In normal animals, the stochastic process of inhibitory receptor

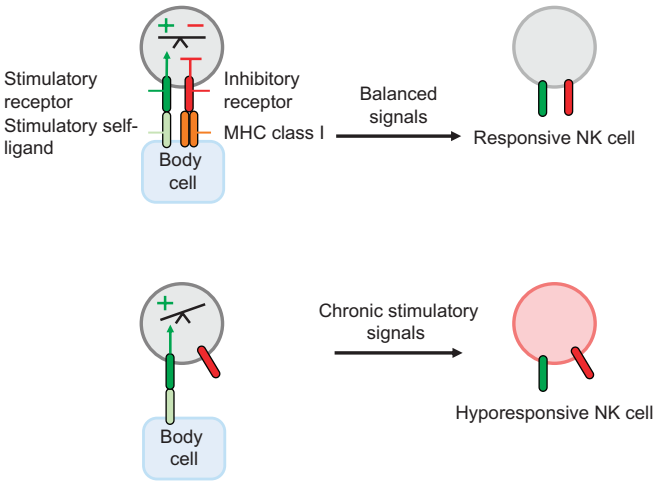

***Fig. 4.* Model for self-tolerance of NK cells.** The NK cell in the lower panel lacks self-MHC-specific inhibitory receptors to counter constitutive stimulatory signaling. The resulting continuous stimulatory signaling causes the NK cell to adopt a hyporesponsive phenotype, meaning that stimulatory receptor signals are substantially dampened. The impaired signaling helps prevent the NK cell from attacking self-cells (the 'disarming' model). The NK cell in the top panel expresses a self-MHC class I-specific inhibitory receptor, engagement of which counters persistent signaling by stimulatory receptors specific for self-ligands on normal cells. The inhibitory receptor signal counters the stimulatory receptor signal so that no hyporesponsiveness is induced and the NK cell acquires or maintains a high degree of responsiveness under steady-state condition. The cell is self-tolerant, but in response to infected or transformed cells that upregulate stimulatory ligands or downregulate MHC class I molecules the NK cell becomes activated.

expression equips some cells with inhibitory receptors specific for self-MHC class I molecules, which may account for their self-tolerance (*Fig.* 4). Other NK cells, however, happen to lack such receptors and consequently are persistently stimulated, and this stimulation renders the cells hyporesponsive. We have called this hypothetical process 'disarming' (40) to convey the sense that hyporesponsiveness is actively induced and to distinguish it from models in which high responsiveness is induced by interactions with MHC class I-expressing cells, which we have termed 'arming' (40).

Recent results by Kim *et al.* (103) are consistent with our findings in some respects. They reported that NK cells lacking Ly49C, an inhibitory receptor specific for self-MHC molecules in B6 mice, were functionally impaired, whereas Ly49C-positive cells respond well to cross-linking of stimulatory receptors. One difference from our results is the observation that NK cells that lack Ly49C are hyporesponsive in their study, despite other evidence that many of these cells express the inhibitory receptors Ly49I and/or CD94/NKG2A that also bind to MHC molecules in B6 mice. Indeed, we find that many of these NK cells respond to stimulatory receptor cross-linking (E. Treiner, N. Joncker and D.H. Raulet, unpublished data). It is not clear what might cause this discrepancy between our results and theirs. The second and more important difference from our work is the interpretation of the underlying mechanism leading to tolerance of NK cells. Kim *et al.* (103) interpreted their results to mean that maturation of highly functional NK cells requires that the cells specifically interact with host MHC class I molecules, a process they term 'licensing'. In this model, failure to receive licensing signals leaves the NK cells in a hyporesponsiveness state.

Although Kim and Yokoyama (106) have subsequently argued that the term 'licensing' is not meant to imply a specific model, the term has created considerable confusion, leading us and others to equate licensing with the 'arming' model, which states that engagement of an inhibitory MHC class I-specific receptor by developing NK cells induces a high responsive state (16, 107). Whatever the meaning of the term, it is clear that Kim and Yokoyama (106) consider hyporesponsive NK cells to be functionally immature cells that must be induced to acquire full functionality. This model may be the case, but we favor the idea discussed above that hyporesponsive NK cells are mature cells that have been actively rendered hyporesponsive as a result of persistent stimulatory signaling that is not balanced by inhibitory signaling. In comparing the virtues of the arming and disarming models in explaining the origin of hyporesponsive NK cells and self-tolerance, data bearing on three distinguishing features of the models can be considered.

First, the disarming model states that hyporesponsive NK cells arise because some developing or mature NK cells encounter neighboring self-cells that lack cognate MHC class I ligands for inhibitory receptors on the NK cells, whereas the arming model takes the opposite point of view and states that responsive NK cells arise because developing NK cells encounter cells expressing cognate MHC class I ligands that interact with inhibitory receptors on the NK cells. Studies performed several years ago attempted to test these predictions by examining NK cell development in chimeric or mosaic mice in which host cells consisted of a mixture of cells that did or did not express relevant MHC class I ligands (108, 109). The arming model predicts that encounters of developing NK cells with MHC ligand-expressing cells in the mixed chimeras should induce high functionality of the NK cells, whereas the disarming model predicts that encounters with cells lacking cognate MHC ligands should induce hyporesponsiveness and tolerance. The results showed that cells expressing MHC ligands and cells lacking them persisted in the mice despite the development of normal numbers of NK cells, suggesting that the NK cells in the chimeras had been rendered tolerant of the class I-deficient cells with which they were comingled (108–110). Functional studies showed that these NK cells were generally poorly responsive to target cells that lacked corresponding MHC class I ligands. Although additional studies will be required to achieve a definitive conclusion, the results of these studies tend to support the predictions of the disarming model.

A second distinguishing feature of the models is that the arming model suggests that hyporesponsive cells are basically a form of immature NK cell, whereas the proposed disarming process could, in theory, act on either mature or immature cells. As already discussed, hyporesponsive NK cells exhibit normal expression of all the known markers of mature NK cells, including CD11b and Ly49 receptors (102). Furthermore, they are capable of normal functional activity, such as cytokine production, under some circumstances of stimulation. Though it cannot be ruled out that the hyporesponsive NK cells are immature in some respects, the available data are consistent with hyporesponsive cells being fully mature.

A third distinguishing feature of the models is that the arming model emphasizes the importance of inhibitory receptor engagement as a key signal regulating NK cell functional maturation, whereas the disarming model emphasizes the 'net' signal the cell receives, after balancing inhibitory and stimulatory signaling. Therefore, the disarming model predicts that hyporesponsiveness would occur not only when the NK cell lacks inhibitory receptors for self-MHC but also when the developing NK cell is subject to higher levels of stimulation than

normal, even when MHC class I levels are normal. Some published work (90) suggests that disarming might occur *in vivo* in transgenic mice, whose cells constitutively express the Rae1ε ligand for the NKG2D receptor. All NK cells that developed in these transgenic mice, whether or not they expressed inhibitory receptors for self-MHC, were hyporesponsive, even when stimulated through receptors other than NKG2D. These findings are consistent with predictions of the disarming model, but it will be important to address the matter further by assessing whether the hyporesponsive NK cells that arise in these different circumstances have similar properties.

Concluding remarks

The foregoing comments suggest the existence of mechanisms that adjust the strength of signaling pathways in developing NK cells to achieve an appropriate balance of receptor signaling compatible with both self-tolerance and the capacity of NK cells to respond to diseased cells. These considerations lead to the identification of three important and related challenges for the next phase of NK cell research. One is to address mechanistically how the initial balance in signaling pathways, or 'set-point', is achieved in order to establish self-tolerance. A second challenge is to understand how the local environment and available cytokines in infected or cancerous tissue may alter the signaling capacity of NK cell receptors, favoring, or in some cases preventing, the NK cell response. A third, equally important, challenge will be to understand the principles and signaling pathways that regulate stimulatory and inhibitory ligand expression in infected or transformed cells, thus favoring NK cell (and in some cases T-cell) activation. Progress in all three areas will be necessary for a comprehensive understanding of NK cell recognition.

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