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The innate immune response to tumors and its role in the induction of T-cell immunity

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Summary: Recent genetic studies have resurrected the concept that the adaptive and innate immune systems play roles in tumor surveillance. Natural killer (NK) cells recognize many tumor cells but not normal self cells, and they are thought to aid in the elimination of nascent tumors. Two main strategies are employed by NK cells to recognize tumor targets. Many tumor cells down-regulate class I major histocompatibility complex (MHC) molecules, thus releasing the NK cell from the inhibition provided by class I MHC-specific inhibitory receptors ('missing self recognition'). More recently, it has become clear that a stimulatory receptor expressed by NK cells, T cells and macrophages (NKG2D) recognizes ligands (MHC class I chain related [MIC], H6O, retinoic acid early inducible [Rae1] and UL16 binding proteins [ULBP]) that are up-regulated on tumor cells and virally infected cells but are not expressed well by normal cells. Ectopic expression of these ligands on tumor cells leads to the potent rejection of the tumors *in vivo*. Importantly, mice that previously rejected the ligand$^+$ tumor cells develop T-cell immunity to the parental (ligand$^-$) tumor cells. The recognition of induced-self ligands as a strategy to recognize abnormal self sets a precedent for a new immune recognition strategy of the innate immune system.

Introduction: the concept of immune surveillance revisited

The immune surveillance hypothesis proposed that the immune system surveys the body for nascent malignancies, eliminating many or most tumors, and possibly slowing the growth of others (1). The hypothesis is supported by the finding that humans with congenital or acquired immunodeficiencies have a significantly higher incidence of malignancies (2–4). Many of these malignancies are, however, strongly associated with viral infections. It has been more difficult to determine whether the immune system plays a role in the surveillance of tumors that are not associated with viral infections. Such tumors are responsible for most human cancer mortality. Consistent with such a role, various components of the immune system, such as cytotoxic T lymphocytes

(CTLs), natural killer (NK) cells and antibodies, can exert potent activity against these types of tumors *in vitro*. Depletion of NK cells from mice often reduces the resistance of the mice to transplanted tumor cell lines (5–7). Furthermore, many murine tumor cell lines are at least somewhat immunogenic when irradiated and transferred to syngeneic animals, in some cases inducing long-lasting immunity to challenge with live tumor cells from the same line. The immunogenicity of even poorly immunogenic tumor cell lines can often be enhanced by employing various vaccination strategies (8, 9). In general, tumor immunity induced with cell lines is specific for the immunizing tumor cell line and is mediated by $CD8^+$ and/or $CD4^+$ T cells, in some cases with the participation of $NK1.1^+$ cells (10–12). Additional evidence favoring the existence of tumor immunity is the isolation from patients, especially melanoma patients, of T-cell clones specific for antigens on the tumor, and the identification of the corresponding major histocompatibility complex (MHC)-presented tumor antigens (13–16). The antigens generally are derived from self proteins that are overexpressed or inappropriately expressed in the tumor cells. Collectively, these data suggest that at least some tumor cells express antigens that can be targeted by the immune system, and that immunity can be generated in many cases using experimental protocols.

Despite these features suggesting a role for the immune system, the tumor surveillance hypothesis fell into disrepute after reports that the incidence of non-virus-related cancers is not increased in immunodeficient animals and humans. In mice, the most extensive and noted studies showed an unaltered tumor incidence in nude (*nu/nu*) mutant mice, which lack a thymus and consequently lack most T cells (17–19). In retrospect, the conclusions of these studies suffer from the fact that nude mice do not completely lack T cells (20, 21). Furthermore, such studies failed to address the possible role in tumor immunity of cells of the innate immune system, such as NK cells, myeloid cells or granulocytes, which are largely intact or even expanded in mutant mice lacking the adaptive immune system. Recent studies, most of which have employed gene-knockout mice lacking various components of the immune system, have led to something of a renaissance of the hypothesis.

The first observation along these lines came from analysis of mice with a disrupted gene for a component of the interferon (IFN)-γ receptor, and was later extended to mice lacking IFN-γ itself or STAT1, a transcription factor necessary for IFN signaling (22, 23). The IFN-γ deficient mice had a higher rate of both spontaneous and carcinogen-induced tumors. The tumors from IFN-γ receptor-deficient mice were less sensitive to T cells than tumors from wild-type mice, leading to the proposal that IFN-γ mediates its effects principally by upregulating MHC molecules and other proteins involved in antigen processing. A complementary finding was that many human tumor cells are selectively unresponsive to IFN-γ (22), suggesting that IFN-γ-responsive tumors are preferentially eliminated by the immune system. An important role for adaptive immunity in tumor surveillance was established by demonstrating a substantially increased rate of spontaneous tumors in aged mutant mice specifically lacking T and B cells as a result of a targeted mutation of the *Rag*-2 gene (23). Significantly, the same analysis indicated an independent role for the innate immune response in tumor immunity. When compared to *Rag*-2-mutant mice, mice deficient in both *Rag*-2 and IFN signaling (*Rag*-$2^{-/-}$*STAT*$1^{-/-}$ mice) developed carcinomas even more frequently. Moreover, the double mutant mice developed a different spectrum of tumors, showing a much higher rate of mammary tumors than *Rag*-$2^{-/-}$ mice. These results indicate that IFN-dependent but T-and B-cell-independent mechanisms contribute to tumor immunity. One such mechanism may be tumor cell elimination by NK cells, since NK cells are induced by type I interferons and produce IFN-γ.

A role for NK cells in the rejection of tumors *in vivo* had been proposed shortly after their discovery as a unique lymphocyte subset (24, 25). Subsequent studies demonstrated that mice carrying the homozygous *beige* mutation, which are defective in granule exocytosis and have a profound defect in NK cell cytolytic function, develop virus-induced and carcinogen-induced tumors at a higher frequency (26–28). However, these studies did not prove a role for NK cells in tumor immunity, because the defects caused by the *beige* mutation are not limited to NK cells.

Other studies demonstrated an increased rate of carcinogen-induced sarcomas (and of tumors induced by oncogenic viruses) in mice lacking perforin, the pore forming granule protein involved in cytotoxicity by NK cells and CTLs (29). In contrast to perforin-deficient mice, mice genetically deficient for CD8 T cells did not show significant defects in the control of carcinogen-induced tumor growth, suggesting a possible role of NK cells in tumor surveillance (29). Importantly, perforin-deficient mice also developed spontaneous disseminated lymphomas at a much higher frequency than immunocompetent control mice (30). Whether the perforin-dependence of tumor resistance in normal mice reflects the activity of NK cells or T cells, or both, was not determined in this system.

In addition to perforin, cytotoxicity can also be mediated

by tumor necrosis factor (TNF) family members interacting with TNF receptor family members on target cells. One member of the family, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), is expressed *in vivo* by activated NK cells. *In vivo* induction of TRAIL on NK cells correlated with TRAIL-mediated antimetastatic effects against various transferred tumor cell lines (31, 32). *In vivo* blockade of TRAIL resulted in an enhanced incidence of carcinogen-induced fibrosarcomas and spontaneous lymphomas in mice heterozygous for the p53 gene (33). The protective effect of TRAIL was attributable at least in part to NK cells and was dependent on the action of IFN-γ (33).

The studies cited above provide evidence that the adaptive and innate immune systems both play a role in tumor surveillance. The remaining parts of this review will focus on specific recognition systems employed by NK cells, and in some cases other immune cells, that are potentially involved in tumor surveillance. Although the available data suggest that NK cells can specifically recognize tumor cells, the molecular interactions involved in recognition are only partly understood. Two general mechanisms have been implicated in studies performed to date: (i) NK cells preferentially 'recognize' cells, such as many tumor cells, that down-regulate class I MHC molecules ('missing self recognition'), and (ii) NK cells recognize structures that are specifically up-regulated on tumor cells, including a group of proteins we and others recently identified that are distantly related to MHC class I molecules and bind to a stimulatory receptor called NKG2D. These recognition events will be described in more detail below.

Tumor cell recognition by NK cells

'Missing self recognition'

Much evidence demonstrates that NK cells preferentially attack cells in which class I MHC expression is reduced or abolished (*Fig. 1*). This phenomenon was first clearly demonstrated by comparing the activity of NK cells against wild-type and class I-low variants of MHC-positive tumor cell lines (5, 34, 35). The enhanced sensitivity of class I-low tumor cells to NK cells was reversed by transfections that restored class I expression (36, 37). Cells in advanced tumors frequently extinguish class I expression (38), suggesting one possible rationale for NK cell recognition of class I-low cells (39).

The recognition of class I-deficient cells by NK cells has been mechanistically explained by the discovery of receptors that are specific for class I molecules and inhibitory in function. Normal cells expressing high levels of class I molecules inhibit NK cells, while class I-deficient cells do not (*Fig. 1*). Three families of inhibitory class I specific receptors have been identified: Ly49 (in mice, but not humans) (40–42), KIR (in humans, but not mice) (43–46) and the CD94/NKG2A receptor (in both mice and humans) (47–50). Ly49 and CD94/NKG2A receptors are related to C-type lectins in structure, while KIR are immunoglobulin (Ig)-superfamily members. Ly49 and KIR bind directly to intact classical class I molecules. CD94/NKG2A, in contrast, binds to a peptide derived from the signal sequence of classical class I molecules that is presented in the groove of a non-classical class I molecule (Qa-1 in mice and HLA-E in humans) (51–53).

The various class I MHC-specific inhibitory receptors are expressed on overlapping subsets of NK cells, leading to a complex combinatorial repertoire of NK specificities for MHC class I molecules (54). The inhibitory receptor repertoire is shaped by an education mechanism that guarantees that NK cells are self-tolerant but are unleashed against target cells that down-regulate some or all self-class I molecules (54).

The notion that 'missing self recognition' by NK cells functions to eliminate MHC class I-loss variants is supported by studies in mice and (less directly) in humans. Studies demonstrated that cells that lack MHC class I molecules but are otherwise normal, such as bone marrow cells from class I-deficient mice, are strongly rejected when transplanted into normal mice (55). The rejection is mediated by NK cells. Subsequent studies indicated that a similar type of rejection occurs in a non-transplant scenario, in the case of variant class I-deficient cells that arise *in vivo* as a result of mutations. Total body irradiation of MHC-heterozygous mice resulted in the transient appearance of mutant lymphocytes that lacked one or the other parental MHC haplotype, but these cells rapidly disappeared after irradiation. It was shown that the rapid disappearance of these cells in normal mice is mediated by NK cells (56). In analogous cohorts of humans, such as survivors of atomic bombs or the Chernobyl nuclear plant accident, increased frequencies of mutations were documented in many different genes (57, 58), the exception being human leukocyte antigen (HLA)-loss mutations in T cells. These analyses suggest that the HLA mutant cells may be eliminated by the immune system (59). The collective results suggest that 'missing self recognition' plays an important role in the elimination of cells that have down-regulated class I MHC expression *in vivo*. With respect to tumors, many studies have clearly demonstrated that class I-deficient transplantable tumor cell lines are rejected by NK cells (5, 34, 35). Restoration of MHC class I expression on the tumor cells reversed this effect (36, 37). Although these studies, together,

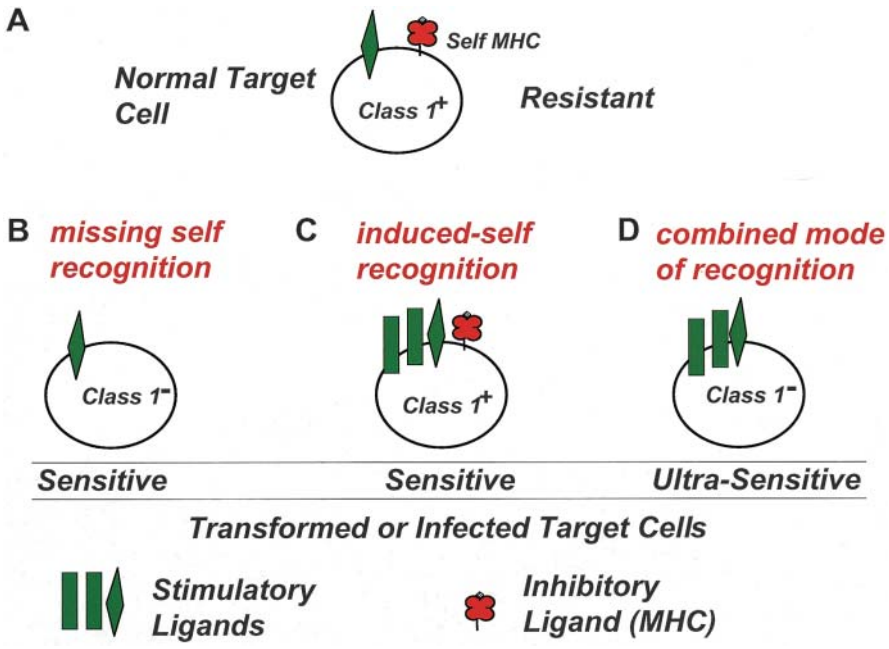

***Fig. 1.* Regulation of stimulatory and inhibitory ligands determines NK target cell lysis.** The diagram illustrates four scenarios of target cells expressing different combinations of stimulatory and inhibitory ligands. **A.** A normal target cell expressing self class I MHC molecules and low levels of stimulatory ligands. The inhibitory signal outweighs the stimulatory signal, the NK cell is inhibited and the target cell is resistant to NK cell lysis. The expression of low levels of stimulatory ligands by normal cells is consistent with the observation that some (but possibly not all) normal cells stimulate NK cells when MHC class I inhibition is prevented (55, 108, 109). **B.** 'Missing self recognition'. The target cell has down-regulated class I MHC expression. Inhibitory receptors expressed by the NK cell are no longer engaged. The signal provided by the stimulatory ligand activates the NK cell for lysis of the target cell. The tumor cell lines RMA/S and B16-BL6 may represent examples of this type of target cell recognition. **C.** 'Induced-self recognition'. Up-regulation on target cells of induced self ligands (e.g. Rae1, H60 and MIC proteins) results in engagement of a large number of stimulatory receptors, and overwhelms inhibitory signals, leading to target cell lysis. **D.** Combination of the two recognition mechanisms. A target cell that has up-regulated induced self ligands and at the same time down-regulated MHC class I expression would be extraordinarily sensitive to NK cell lysis. An example of a target cell showing this ligand expression pattern would be the YAC-1 lymphoma cell line.

suggest that NK cells should target MHC-loss variants of tumor cells as they arise *in vivo*, it has not yet been possible to design appropriate experiments to test this hypothesis directly.

Recognition of induced self ligands as markers of abnormal self

Before missing self recognition was discovered, it was assumed that NK cells would recognize target cells in much the same way as other lymphocytes – with stimulatory receptors specific for 'antigens' (albeit commonly expressed ones) expressed on target cells. Once the various families of MHC class I specific inhibitory receptors were discovered, the misapprehension grew that missing self recognition could account in general terms for tumor cell recognition by NK cells. In fact, numerous examples had been reported previously of NK-susceptible tumor cells with apparently normal class I expression (60–63), suggesting the existence of additional NK recognition systems (*Fig. 1*). Furthermore, numerous stimulatory NK receptors had been identified over the years, and continue to be identified (64, 65). Some of these receptors were previously implicated in specific tumor cell recognition (66). Nevertheless, our understanding of the role of most of these receptors in target cell recognition by NK cells and in immune surveillance has lagged because of the inability to identify their ligands. Only recently, progress has been made in cloning ligands for one of the stimulatory receptors expressed by NK cells, NKG2D (*Fig. 2*). Strikingly, ligands for NKG2D are encoded by cellular genes, some of which are not expressed in normal cells but are selectively expressed or up-regulated in tumor cells and virally infected cells (*Fig. 1*). The NKG2D receptor–ligand system represents a new theme in immune recognition in which cells in distress, such as tumor

cells, up-regulate ligands that are specifically designed to enlist an attack from immune effector cells.

The NKG2D receptor–ligand system

Human NKG2D was cloned along with NKG2A and NKG2C in the early 1990s (47). The sequence of NKG2D predicted a stimulatory C-type lectin-like receptor. NKG2A and C are very closely related to each other in sequence and associate with CD94 to create a receptor specific for MHC class I peptides bound to HLA-E in humans or Qa-1^b in mice (*Fig.* 2). In contrast, NKG2D is only distantly related to these other NKG2 proteins, does not associate with CD94 and exhibits a distinct specificity and function. Therefore, despite its name, it is inappropriate to consider NKG2D as a member of the 'NKG2 family'. *Nkg2d* genes have been identified in mice and rats (*Fig.* 2) (50, 67, 68).

A striking feature of NKG2D is its expression by distinct immune cell types. In mice, NKG2D is expressed by all NK cells, a subset of NK T cells, a subset of splenic γδ T cells and all epidermal γδ T cells, but not by intestinal epithelial γδ T cells (69, 70). Furthermore, NKG2D expression is induced on essentially all $CD8^+$ T cells that have been activated through the T-cell receptor, and essentially all macrophages that have been activated with various stimulants, including lipopolysaccharide (LPS), type I interferons, and IFN-γ (69, 70). In humans a slightly different expression pattern has been observed. In addition to NK cells, NKG2D is expressed by all unstimulated peripheral blood $CD8^+$ T cells and γδ T cells and by all intestinal intraepithelial (IEL) γδ T cells (71, 72).

NKG2D has been shown to associate with the signaling adaptor protein DAP10 (*Fig.* 2) (73–75). In contrast to most of the other adapter proteins expressed by NK cells (e.g. FcRγ, KARAP/DAP12 and CD3ζ), DAP10 does not have an immunoreceptor tyrosine-based activation motif (ITAM) in its transmembrane domain. Instead, the cytoplasmic domain of DAP10 contains a YxxM motif (73, 74). It had been previously reported that YxxM motifs are able to bind the p85 subunit of phosphatidyl inositol (PI)-3 kinase (76). Indeed, recent reports showed that DAP10 binds and functionally activates PI-3 kinase and the downstream signaling polypeptide Akt (73, 74). Cross-linking of NKG2D led to the activation of the ERK/MAP kinase pathway (77) that has been previously shown to be downstream of PI-3 kinase and Akt (78). Significantly, the YxxM motif is shared by a number of other receptors that have been attributed with costimulatory activity, like CD28, ICOS and CD19. This finding led to the proposal that NKG2D is a costimulatory receptor (73, 75, 79). This proposal was supported by evidence that NKG2D alone is not able to directly stimulate activated human $CD8^+$ T cells (which express NKG2D) but does provide a potent costimulatory signal to T cells (especially $CD28^-$ $CD8^+$ T cells) which also receive stimulation through the T-cell receptor (TCR) (8, 70, 80, 81).

Contradicting the notion that NKG2D is solely a costimulatory receptor, it was observed that cross-linking of NKG2D on NK cells and activated macrophages led to direct stimulation of cytokine production (IFN-γ in NK cells, and TNF-α in macrophages), intracellular Ca^{2+} mobilization (in NK cells) and production of nitric oxide (in macrophages) (70). Apparently, NKG2D can function in different cell types as either a costimulatory receptor (on $CD8^+$ T cells) or a primary recognition structure (on NK cells and macrophages). Recent evidence demonstrates that NKG2D accomplishes these dual tasks by associating with different adapter proteins in different cell types: the costimulatory DAP10 adapter protein in CD8 T cells and the ITAM-containing KARAP/DAP12 adapter protein in activated NK cells and macrophages (82).

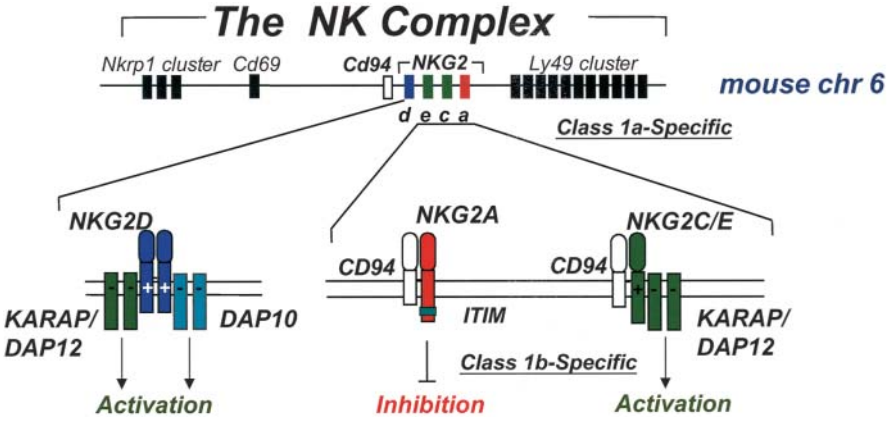

***Fig.* 2. NKG2D is encoded in the mouse 'NK complex' on chromosome 6.** NKG2A/C/E and NKG2D are encoded in the NK complex on mouse chromosome 6 that encodes various receptors expressed by NK cells. NKG2A/C/E are very closely related and associate with CD94. NKG2A is an inhibitory receptor displaying an immunoreceptor tyrosine-based innhibitory motif (ITIM) in its cytoplasmic domain. NKG2C/E are stimulatory receptors that associate with the signaling adapter protein KARAP/DAP12. NKG2D is not closely related to NKG2A/C/E and does not heterodimerize with CD94. The NKG2D homodimer associates with the signaling adapter proteins DAP10 and DAP12.

NKG2D binds to several families of ligands that are distantly related to MHC class I molecules in sequence. The first to be identified were the human MHC class I chain related proteins A and B (MICA/B) that were originally described as ligands for a subset of human γδ T cells (71, 83–85). These proteins are up-regulated by various forms of cellular stress, by viral infection and on tumor cells of epithelial origin (80, 84, 86). MIC homologs have not been identified in mice. Expression cloning experiments revealed two novel families of ligands for the mouse NKG2D receptor, the retinoic acid early inducible (Rae1) protein and H60 (69, 87). The Rae1 family of proteins are encoded by a small gene family (*Raet1a–e*) on mouse chromosome 10 that consists of four to five members (88–91). It was recognized early on that the Rae1 proteins are distantly related to class I MHC molecules (89). Most interestingly, Rae1 proteins are not expressed in most normal adult tissues but are up-regulated on many tumor cells of lymphoid and epithelial origin (*Fig.* 3) (8, 69, 90). Rae1 proteins are also expressed transiently in the embryo between days 9 and 11 of gestation, leading to the proposal that these proteins may be involved in development (88).

The other mouse ligand for NKG2D identified to date is the H60 protein, which had been initially described as a minor histocompatibility antigen in the response of BALB.B mice against C57BL/6 cells (92). H60 is expressed on some tumor cells derived from BALB mice, but it is also expressed at low levels by activated lymphoblasts and at high levels by thymocytes from BALB/c mice (*Fig.* 3) (64, 69, 92). Interestingly, all the mouse ligands for NKG2D are localized in a gene cluster on mouse chromosome 10, a different chromosome from that encoding the MHC.

Significantly, a family of related NKG2D ligands has been identified in humans (93, 94). The human family, variously called UL16 binding proteins (ULBPs), ALCAN or human RAE1 proteins, are encoded by approximately 10 related genes (six potentially functional glycoproteins and four pseudogenes) on chromosome 6q24.2-q25.3, in a region that is syntenic to the mouse Rae1 and H60 genes and well separated from the human MHC (95). The ULBPs were identified by their binding to a human cytomegalovirus protein, UL16. It is believed that, in virus-infected cells, UL16 functions in immune evasion by binding to ULBPs and interfering with ligand recognition by NKG2D$^+$ effector cells (93). Comprehensive knowledge of the expression pattern of the various human ULBPs is still lacking. Some of the family members are expressed in various normal cells as detected by reverse transcriptase polymerase chain reaction (RT-PCR). Neverthe-

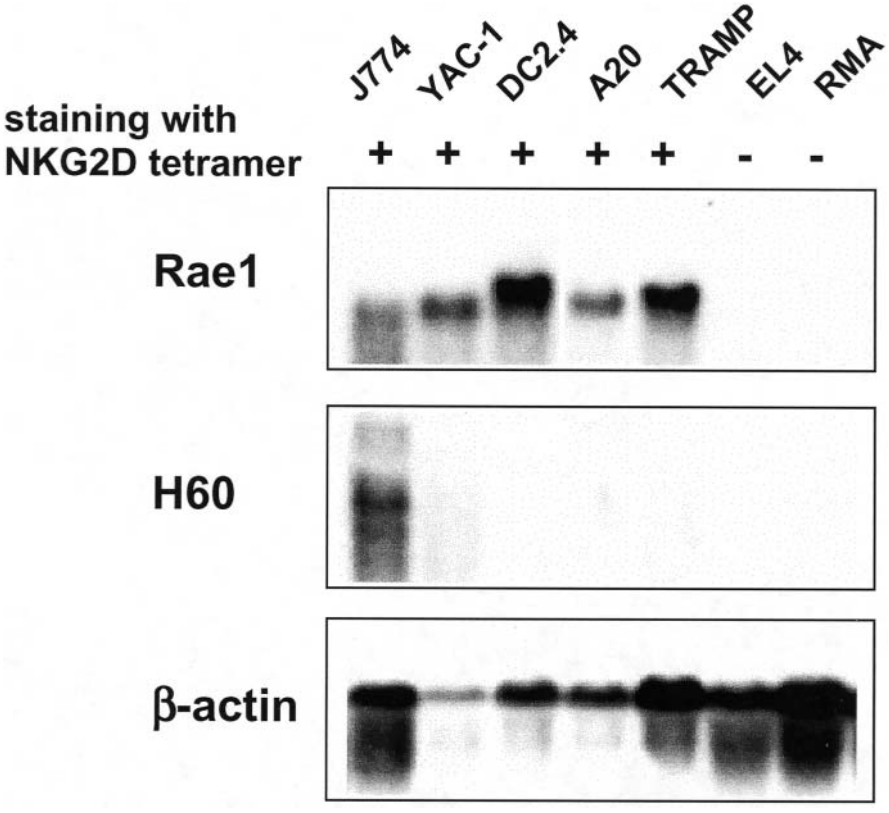

***Fig.* 3. Expression of Rae1 and H60 mRNA in various tumor cell lines.** Northern blot analysis of total cellular RNA from the indicated tumor cell lines. The membrane was consecutively hybridized with ^{32}P-labeled full-length Rae1β, H60 and β-actin probes. For comparison, the expression of NKG2D ligands at the cell surface (detected by staining with soluble NKG2D tetramers) is indicated.

less, elevated expression in tumor cells has also been reported (77, 94).

Role of NKG2D and its ligands in antitumor immune responses

Nearly all primary human tumors of epithelial origin exhibit up-regulation of MIC (86). In the mouse, NKG2D ligands, most often Rae1 family members, were expressed by approximately 75% of tumor cell lines examined (*Fig.* 3) (69, 82). The positive mouse tumor cell lines included carcinoma cell lines, lymphomas and hematopoietic tumors (*Fig.* 3). Expression of NKG2D ligands has also been observed in preliminary analyses of primary tumors (our unpublished data). Treatment of mouse skin with a carcinogen led to strong up-regulation of Rae1 and H60 in the resulting papillomas and carcinomas, whereas these molecules were not expressed by normal skin cells (90).

We and others have examined the role of the NKG2D receptor–ligand system in responses to transplantable tumor cell lines *in vivo* (8, 96). Tumor cell lines that do not naturally express NKG2D ligands were employed. Ectopic expression of Rae1 or H60 in the tumor cells resulted in uniform rejection of the cells in immunocompetent mice. The rejection was mediated by NK cells alone in the case of the B16-BL6 melanoma or the EL4 T-cell lymphoma (8). In the case of the RMA T lymphoma, by contrast, rejection was mediated cooperatively by NK cells and $CD8^+$ T cells (8). The rejection was dependent on the interaction of NKG2D with its ligands on the tumor cells, as *in vivo* blocking with a monoclonal antibody to NKG2D led to tumor cell growth (A. M. Jamieson, A. Diefenbach and D. H. Raulet, unpublished observation).

Even more striking results were obtained when mice were rechallenged with tumor cells. In the case of all three tumor cell lines tested (B16-BL6, EL4 and RMA), mice that had rejected tumors expressing NKG2D ligands were immune against a re-challenge with the parental cell lines that lacked NKG2D ligands (8). This effect was dependent on the presence of $CD8^+$ T cells. Also, vaccination with irradiated cell lines expressing NKG2D ligands, unlike the parent lines lacking NKG2D ligands, led to efficient priming of CTLs specific for the parental tumor cell line. The CTLs lyzed the parent tumor cell line but did not lyze unrelated tumor cells, suggesting specificity for 'tumor antigens' (8). These data suggested that NKG2D ligands expressed by tumor cells lead to potent priming of memory CD8 T cells.

In contrast to our results with three tumor cell lines, another group failed to observe the induction of T-cell immunity with Rae1 transfectants of one of the cell lines we also studied, RMA. The two studies differed in several respects, including the Rae1 family member employed for transfection (Rae1γ in theirs, Rae1β in ours), the inclusion of other expressed marker genes in the vector (a *neo* cassette in theirs, none in ours), the level of Rae1 expression (higher in theirs) and the route of immunization (intraperitoneally in theirs, subcutaneously in ours). We obtained their cells and compared them to ours in the same experiment. Both $Rae1^+$ cell lines were rejected in the initial challenge, but only ours induced immunity to re-challenge with the parental lines (data not shown). Similar results were obtained whether the mice were challenged subcutaneously or intraperitoneally. These experiments confirm the results of both laboratories and rule out the immunization route as a critical variable. Experiments are underway to determine whether the outcome is affected by the specific Rae1 isoform used, the level of Rae1, the inclusion of additional marker proteins in the transfectants (which may elicit competing T-cell responses), or other differences between the parent cell lines.

The mechanism of CTL priming by cell lines expressing NKG2D ligands remains to be fully established (*Fig.* 4). We demonstrated that CTL priming was unaffected by prior elimination of NK cells from the animals (8), ruling out the possibility that enhanced priming is attributable primarily to tumor cell lysis by NK cells and the consequent greater accumulation of antigenic tumor cell debris (*Fig.* 4*A*). Two other non-exclusive hypotheses are currently being tested: (i) $CD8^+$ T cells stimulated by tumor cell antigens up-regulate NKG2D, which then binds to Rae1/H60 on the tumor cell, resulting in delivery of a costimulatory signal that leads to memory cell formation (*Fig.* 4*B*); (ii) inflammatory signals (including IFNs) induce antigen-presenting cells (APCs) (e.g. macrophages) to express NKG2D; Rae1/H60 expressing tumor cells or tumor cell fragments engage NKG2D on APCs, leading to receptor-mediated uptake of tumor cell antigens by the APC and presentation to T cells by conventional mechanisms (*Fig.* 4*C*). The two models can be distinguished on several accounts, such as the requirement for direct priming (model 1) vs. cross priming (model 2), or the requirement for conventional costimulatory signals (model 2) (*Fig.* 4).

We have observed that the majority of tumor cell lines studied (approximately 75%) express NKG2D ligands (*Fig.* 3). Furthermore, in the few cases where primary tumors have been examined, up-regulation of NKG2D ligands has also been observed (90 and our unpublished data). These findings are, we believe, consistent with the expectation that such

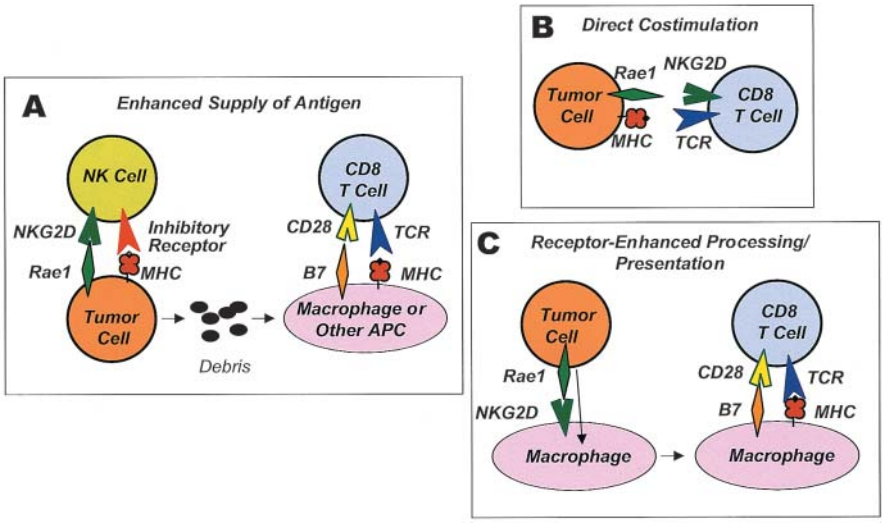

***Fig. 4.* Three models for enhanced CTL priming after vaccination with NKG2DL$^+$ tumor cells. A**. Enhanced lysis of tumor cells by interaction of NKG2D on NK cells with NKG2D ligands on tumor cells results in increased release of tumor cell debris. The enhanced supply of antigen could be passively taken up by antigen-presenting cells, leading to enhanced opportunities for CTL priming. This model is unlikely to be the most important mechanism, because we observed that increased CTL priming is not appreciably impaired in mice lacking NK cells (8). **B.** Enhanced CTL priming through direct interaction between the NKG2D$^+$ CTL and NKG2DL$^+$ tumor cells, leading to direct costimulation of CTL specific for tumor antigens. **C.** Inflammatory signals, perhaps via interferon action, lead to up-regulation of NKG2D on professional antigen-presenting cells (APCs). NKG2D on the APC interacts with the NKG2D ligand on the tumor cell resulting in more efficient antigen presentation to CTLs and enhanced CTL priming.

ligands should be commonly up-regulated by tumor cells. Although not yet examined in detail, it is interesting to note that different NKG2D ligands are often up-regulated in different tumor cell lines. The non-coordinate expression of NKG2D ligands suggests some interesting hypotheses to account for the existence of multiple distinct ligands binding to a unique NKG2D receptor. One possibility is that the various ligands are differentially regulated by events correlated with distinct cellular insults, such as activation of different oncogenes, inactivation of different tumor repressors, and/or events associated with different forms of infection. The ligands may, in turn, vary somewhat in the immune responses they activate, leading to differences in the overall quality of the immune response. Alternatively, tumors as they are formed may initially coexpress multiple ligands as a form of redundancy, with immunoselection leading to selective loss of one or more ligands as the tumor develops.

A contrary view from ours is that the common expression of NKG2D ligands by tumor cell lines may suggest that the system is irrelevant or ineffective in eliminating tumors *in vivo*. The argument assumes that all ligand-expressing tumors would be normally eliminated *in vivo*, so that only ligand-negative tumors would be represented among panels of tumor cell lines. This view fails to take into account the many variables that can influence the success or failure of antitumor immune responses. As in any immune response, an antitumor immune response presumably can be considered as a race between the tumor and the immune system. Depending on how well the tumor is otherwise adapted, the immune system can be expected to sometimes lose the race. Furthermore, tumors are expected to vary in their immunogenicity for reasons that have nothing to do with the NKG2D receptor–ligand system, such as the expression of various adhesion molecules or the expression of immunogenic T-cell epitopes. Such variations could allow a tumor to escape the immune response despite expressing NKG2D ligands. As a related possibility, immunoselection of tumor cells can lead to escape from the immune system. Although it would be predicted that immunoselection should in some cases lead to loss of NKG2D ligands by tumor cells, other types of alterations may be more common, including mutations that affect susceptibility to immune effector mechanisms (as opposed to mechanisms that initiate an immune response). Finally, it is possible that tumor cells that escape the immune response often do, in fact, express lower levels or different sets of NKG2D ligands compared to the nascent tumor from which they arose. In the case of standard laboratory tumor cells lines, the original nascent tumors are unavailable for study, precluding the necessary comparisons. Considering all these variables, it is evident that the simple pattern of NKG2D ligand expression by tumor cell lines or primary tumors is not highly informative as to the importance of the system in immune surveillance. Nor can one expect there to be a simple correlation between the levels of NKG2D ligands on unrelated tumors and their immuno-

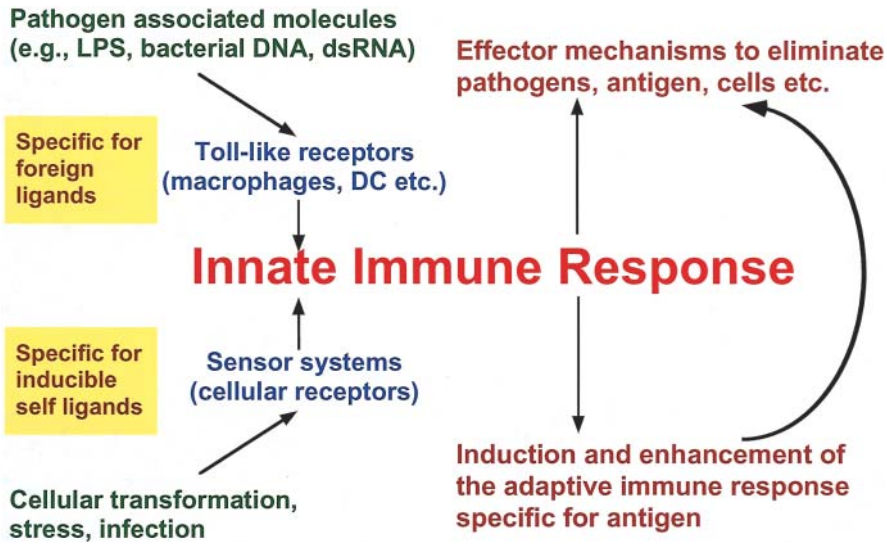

***Fig. 5.* Strategies of innate immune recognition.** Pathogen-associated molecules (e.g. LPS, bacterial DNA, and double-stranded viral RNA) are recognized by Toll-like receptors. This innate recognition mode is specific for foreign (non-self) ligands. Under conditions of cellular transformation, infection and 'stress', the abused cells up-regulate 'induced self' ligands (e.g. Rae1/H60 and MICA/B) as markers of abnormal self. Strikingly, both recognition mechanisms (induced self recognition and foreign pattern recognition) not only directly induce effector mechanisms of the innate immune system but also induce and enhance adaptive immune responses. By employing these different strategies, the innate immune system is enabled to efficiently respond to both internal and external insults.

genicity *in vivo*. Ultimately, assessing the detailed role of the NKG2D receptor–ligand system in immune surveillance will require examination of tumor formation and disease susceptibility in animals in which the receptor or its ligands are inactivated or blocked.

Other NK cell receptors involved in tumor cell recognition

Available data suggest that, in addition to the NKG2D receptor–ligand system, other receptors may also specifically recognize tumor cells. An early study implicated the NKR-P1 receptor family in recognition of a macrophage tumor cell line by rat NK cells, though the ligands recognized by these receptors have not been identified (66). More recently, the human NK cell receptors NKp46, NKp44 and NKp30 have been shown to participate in the recognition of many tumor cell lines *in vitro* (96–101). The cellular ligands recognized by these receptors are also not known.

Very recently, Smyth and colleagues investigated the contribution of the CD27 receptor and its ligand (CD70) in antitumor responses *in vivo*. CD27 is a member of the TNF receptor superfamily that is expressed on most T cells and on all NK cells (102–104). While providing a costimulatory signal to T cells (102, 105), CD27 potently induces IFN-γ production but not cytotoxicity by NK cells (104). Interestingly, the ligand for CD27, the CD70 molecule, is expressed on lymphoma cells (Hodgkin's and non-Hodgkin's lymphoma), Epstein-Barr virus-transformed lymphoblastoid cells and human immunodeficiency virus-infected T cells but not on most normal cells (106, 107). Ectopic expression of CD70 on T-cell lymphomas led to the rejection of these tumors. Rejection was mediated by NK cells and/or T cells depending on the tumor cell type (108). No rejection of $CD70^+$ tumors occurred in mice genetically deficient for either perforin or IFN-γ (108). Strikingly, immunization with CD70-expressing tumor cells, but not with the untransfected cells, led to potent induction of T-cell memory (108).

Concluding remarks

In recent years the immune surveillance hypothesis of tumor immunity, once dismissed, is on the rebound. Data from induced mutant mouse models lacking distinct immune cell populations or effector molecules clearly demonstrate an increased incidence of spontaneous tumors as well as a higher susceptibility to induced tumors and transplanted tumors. Most of these tumors are rejected when transferred into fully immunocompetent hosts, but not into the induced mutant host, clearly suggesting that the immune system is exerting selective pressure on nascent tumors.

The molecular basis for the recognition of tumor cells by the innate immune system is not fully understood. Two concepts have been advanced to understand self/non-self discrimination by NK cells. Both appear to be involved in the antitumor responses of NK cells. The missing self hypothesis predicts that NK cells recognize cells that have down-regulated class I MHC expression. Class I MHC molecules are indeed down-regulated on many tumor cells, and class I-deficient tumor cells generally exhibit a greater susceptibility to NK cells, suggesting that missing self recognition probably plays a significant role in the elimination of tumor cells by NK cells. Data employing *in vitro* and *in vivo* approaches support

the idea that NK cells are capable of efficiently rejecting cells that have lost MHC class I expression. However, direct evidence that missing self recognition plays a role in immunosurveillance of tumor cells in normal animals remains elusive.

We recently proposed another tumor cell recognition strategy used by NK cells. NK cells are endowed with receptors, such as NKG2D (and presumably others), that recognize induced self ligands. These ligands are not expressed by most normal cells but are up-regulated on transformed, infected and stressed cells to draw an attack by the immune system against these potentially dangerous cells. Indeed, we and others demonstrated that the expression of these induced self ligands on tumor cells efficiently stimulates antitumor responses by NK cells (8, 96) and/or $CD8^+$ T cells (8). Most importantly, this innate immune recognition system potently induced an adaptive immune response specific for antigens unique to the tumor cell line studied (8). The recognition of induced self ligands by the innate immune system can be considered one important strategy of innate immunity, along with recognition of conserved molecular patterns of non-self ligands (mediated by Toll-like receptors), and recognition of 'missing self' (by NK cells). Strikingly, at least two of these mechanisms (induced self recognition and foreign pattern recognition) not only directly induce effector mechanisms of the innate immune system but also induce and enhance adaptive immune responses. By employing these different strategies, the innate immune system is enabled to efficiently respond to both internal and external insults (*Fig.* 5).

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