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Current Opinion in Immunology

Immunosurveillance and immunotherapy of tumors by innate immune cells

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Increasing evidence supports a role for innate immune effector cells in tumor surveillance. Natural killer (NK) cells and myeloid cells represent the two main subsets of innate immune cells possessing efficient but quite different tumor suppressive abilities. Here, we describe the germline-encoded NK cell receptors that play a role in suppressing tumor development and describe briefly the cellular pathways leading to the upregulation of their ligands in tumor cells. We also describe mechanisms underlying the elimination of tumor cells by macrophages and a recently characterized mechanism dedicated to sensing cytosolic DNA that is implicated in antitumor immune responses.

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Current Opinion in Immunology 2016, **38**:52–58

This review comes from a themed issue on **Innate immunity**

Edited by **Eric Vivier** and **Ruslan Medzhitov**

For a complete overview see the Issue and the Editorial

Available online 11th December 2015

http://dx.doi.org/10.1016/j.coi.2015.11.001

Introduction

The innate immune system plays a significant role in recognizing and eliminating tumor cells. Innate cells and particularly NK cells express a fixed set of germline-encoded receptors, which bind tumor-specific ligands to provide tumor-suppressive functions. This review focuses on the most characterized receptor/ligand systems employed by innate immune cells to mediate innate recognition and elimination of tumor cells as well as recently discovered mechanisms of tumor sensing and immune cell activation.

NKG2D and anti-tumor immunity

NKG2D is an activating receptor expressed on NK cells, certain $CD8^+$ T cells, γδ T cells, NKT cells, and certain $CD4^+$ T cells [1]. Engagement of NKG2D upon encounters of NK cells with cells expressing ligands for NKG2D stimulates NK cell cytotoxic activity and cytokine production.

NKG2D recognizes several MHC-related ligands including three subfamilies of ligands in mice (RAE-1α-ε, MULT1, and H60a-c), and two subfamilies of ligands in humans (MICA-B and ULBP1-6) [2]. The ligands are expressed poorly by normal cells but are often induced on cancer and virus-infected cells as the result of the activation of various pathways, many associated with cell stress [2]. It is now well established that NKG2D and its ligands represent a potent and specific system that allows the recognition and elimination of unhealthy cells. NKG2D was first implicated in immune surveillance of tumors by the demonstration that many tumors, but few normal cells, express NKG2D ligands [3–5]. Subsequently, subcutaneous tumor transfer models confirmed that expression of NKG2D ligands causes tumor cell rejection [6,7] (Table 1). Further studies showed that the NKG2D receptor is important for immune surveillance of certain lymphoid and epithelial malignancies using the Eμ-Myc model of B lymphoma and the TRAMP model of prostate adenocarcinoma, respectively [8].

Understanding specific pathways that regulate NKG2D ligands has been a major effort in our laboratory for the last several years. Table 2 summarizes our current knowledge on the regulation of NKG2D ligands in mice and humans.

Many tumor cell lines release soluble NKG2D ligands through a variety of mechanisms, and ligand shedding is often considered a mechanism of immune evasion [2,9]. For instance, soluble MIC and ULBP proteins have been identified in the sera of cancer patients and their detection may in some cases serve as prognostic indicators of cancer [9]. Shedding of NKG2D ligands from tumor cells can result in dramatic reductions in the corresponding cell-surface levels, reducing the susceptibility of the tumor cells to cytolysis mediated by NK cells and T cells.

The effects of soluble NKG2D ligands likely depend on their form and specific properties. In the case of ligands cleaved from the cell surface, which are expected to be monomeric, binding to NKG2D may prevent interactions of the receptor with membrane-bound ligands [10–12]. Ligands vary in affinity, however, and some, such as MICA, may bind NKG2D with too low an affinity to have much impact in this respect. Our recent study

Table 1

NK cell activating receptors involved in tumor surveillance *in vivo*

Receptor	Ligand	Tumor type	Model	Reference
NKG2D	Transd. RAE-1/H60	Melanoma	Transferred B16	[6]
NKG2D	Transd. RAE-1/H60	T Lymphoma	Transferred RMA	[6,7]
NKG2D	Transd. MULT-1	T Lymphoma	Transferred RMA	[54]
NKG2D	RAE-1 and MULT-1	B Lymphoma	Spont. Eμ-Myc	[8]
NKG2D	RAE-1 and MULT-1	Prostate Cancer	TRAMP	[8]
DNAM-1	Transd. CD155/CD112	Melanoma	Transferred B16	[28,31]
DNAM-1	Transd. CD155/CD112	T Lymphoma	Transferred RMA	[30]
DNAM-1	CD155	Fibrosarcoma	MCA	[29]
DNAM-1	CD155	Papilloma	DMBA	[29]
DNAM-1	CD155	Multiple Myeloma	Spont. Vκ*MYC	[27]
DNAM-1	CD155/CD112	B Lymphoma	Spont. Eμ-myc	[26]
NKp30	Transd. Bat-3	B lymphoma	Transferred RPMI8226	[18]
NKp46	?	Melanoma	Transferred B16F10.9	[20]
NKp46	?	Lewis lung carcinoma	Transferred D122	[20]

Transd: transduced ligand, Spont: spontaneous model, TRAMP: transgenic adenocarcinoma mouse prostate, MCA: 3-methylcholanthrene, DMBA: 7,12-dimethylbenz(a)anthracene.

showed that in mice, a shed monomeric form of a high-affinity NKG2D ligand, MULT1, caused NK cell activation and tumor rejection [13••]. We demonstrated that soluble MULT1 inhibited the engagement of NKG2D with other membrane NKG2D ligands expressed on non-tumor cells in tumor-bearing mice, thus preventing global desensitization of NK cells. These results challenge the conventional thought that soluble NKG2D ligands generally act as inhibitory molecules.

Some forms of ligands may impair immune surveillance by modulating NKG2D expression, but this may be more likely in the case of multimeric ligands, such as ligands on exosomes, which can crosslink the receptor and modulate it from the cell surface. NKG2D ligand-containing exosomes derived from human DCs were reported to directly activate human NK cells *ex vivo* [14], but reduced levels of NKG2D on immune cells *in vivo* could also reduce tumor killing.

NCRs and anti-tumor immunity

Natural cytotoxicity receptors (NCRs) such as NKp46, NKp44, and NKp30 play roles in tumor cell recognition. NKp46 and NKp30 are expressed on both resting and activated human NK cells, whereas NKp44 is expressed only on activated human NK cells. Recognition of tumor

Table 2

Regulation of NKG2D ligands

Transcriptional regulation

- Proliferative conditions induce expression of *Raet1* family genes and the *MICA* and *ULBP2* genes. E2F transcription factors transactivate *Raet1* family genes [55].
- Heat shock and the heat shock factor 1 (HSF1) regulate the *MICA* and *MICB* genes [56,57].
- The p53 transcription factor amplifies transcription of *ULBP1* and *ULBP2* genes [58,59].
- NF-κB and Sp family transcription factors regulate the transcriptional activation of human NKG2D ligands [60,61].
- The ATF4 transcription factor induces *ULBP1* gene expression [62].

Regulation at the mRNA level

- The DNA damage response (DDR) pathway is an important mode of regulation of NKG2D ligands in both mouse and human cells and appears to act largely post-transcriptionally [32,63,64].
- AID deregulation in Abelson murine leukemia virus-infected cells induced the DDR and the expression of NKG2D ligands [65].
- The HIV Vpr protein activates the ATR kinase and the DDR leading to the expression of NKG2D ligands [66].
- The HIV Vif protein degrades the antiviral host protein APOBEC3G, preventing the deamination of cytosine residues, the DDR and the expression of NKG2D ligands [67].
- Many different microRNAs have been implicated in NKG2D ligands regulation, including miR-17-5p, miR-20a, miR-34a, miR-34c, miR-93, miR-106b, miR-373, and miR-520 [68].
- PI3K signaling was implicated in the induction of RAE-1 [69].
- The oncogene RAS induces the expression of RAE-1α and RAE-1β in mouse cells as well as ULBP1-3 in human cells [70].
- The adenovirus E1A oncogene protein induces *Raet1* mRNAs and the RAE-1 protein [71].
- The RNA-binding protein RBM4 supports ULBP1 expression by facilitating proper splicing of the first two exons of the primary transcript [62].

Regulation at the protein level

- UV irradiation or heat shock reduces the poly-ubiquitination of MULT1 protein resulting in its stabilization and induction at the cell surface. MULT1 degradation was in part mediated by two ubiquitin ligases, MARCH4 and MARCH9, which regulate turnover of the ligand cell-surface induction [72,73].

cells and infected cells through these receptors trigger NKcell-mediated killing and secretion of IFN-γ [15]. Identification of the tumor cell ligands for some of these receptors is still under investigation, though candidates for some have emerged recently. B7-H6, a molecule that is expressed on the surface of tumor cells, was identified as a novel ligand for NKp30 [16,17]. In addition, the nuclear protein BCL2-associated athanogene 6 (BAG-6), also known as BAT3, was also proposed as a cellular ligand for NKp30 and implicated in tumor recognition *in vivo* [18,19]. Tumor ligands for NKp46 remain unknown but *in vivo* evidence from NKp46 knockout mice suggest a role for the receptor in eliminating tumor metastases [20,21].

DNAM-1 and anti-tumor immunity

The activating receptor DNAM-1 (CD226) is expressed on the surface of several lymphocyte subsets, including NK cells. DNAM-1 acts synergistically with other activating receptors to induce the cytotoxic activity of NK cells [22]. Several studies showed that the interaction between DNAM-1 and its ligands is in some cases essential for optimal NK cell activation and production of inflammatory cytokines [23].

In both mice and humans, DNAM-1 binds to PVR (CD155) and Nectin-2 (CD112) [24]. These molecules are broadly expressed on healthy tissues, and are upregulated on tumor cells [24,25]. The role of DNAM-1 in NK cell-mediated recognition and killing of human tumor cells has been shown for cells originating from multiple types of cancer. Using DNAM-1 KO mice, several studies showed that lack of DNAM-1 expression accelerates the onset and lethality of carcinogen-induced tumors as well as transplantable and spontaneous tumors [26–29]. In these studies, tumor immune surveillance strongly relied on the expression of DNAM-1 and the effector functions of NK cells and CD8T cells [30]. Using mouse models of cancer, studies have shown that the successful outcome of antitumor cytokine-based immunotherapy or chemotherapy relied on DNAM-1 recognition [27•,31]. Interestingly, DNAM-1 ligands are upregulated on multiple myeloma cells treated with DNA damage response-inducing therapeutic agents or nitric oxide, increasing the susceptibility of these cells to NK cell recognition [32–34]. These studies provide a rationale for combining multiple strategies to promote the anti-tumor NK cell response through the DNAM-1 receptor.

MHC-specific NK cell inhibitory receptors and anti-tumor immunity

NK cell activation results from a complex integration of signals provided by inhibitory and activating receptors. Ly49 receptors in mice, and Killer Immunoglobulin-like Receptors (KIR) in humans, recognize polymorphic components of MHC I molecules. In both species, the CD94/NKG2A heterodimer receptor recognizes peptides from MHC I molecules presented by a nonclassical MHC I molecule. Members of these receptor families are generally inhibitory, and thus inhibit lysis of cells expressing high levels of MHC I molecules. In the mid-80s, Kärre *et al.* formulated the 'missing-self hypothesis', demonstrating that loss of MHC I expression renders target cells more susceptible to NK cell recognition and elimination [35,36]. Inhibitory receptors also play a key role in educating NK cells. Specifically, NK cells whose inhibitory receptors fail to engage MHC I molecules in their environment are driven to exhibit low functional activity [37]. The importance of NK cell education and relevance of the 'missing-self hypothesis' in tumor immune surveillance was recently demonstrated in a study using gene-targeted mice with attenuated expression of Ly49 inhibitory receptors. Mice with reduced expression of Ly49 receptors were deficient in rejecting transplanted tumors and had accelerated onset of carcinogen-induced sarcomas and spontaneous B cell lymphomas [38•]. These studies indicated that the low functional activity of NK cells associated with failure of inhibitory receptors to engage MHC I molecules is associated with defective capacity to reject tumors *in vivo*. Another study reported the surprising finding that the MHC I-specific receptor, Ly49A, binds to the nonclassical MHC I molecule pH2-M3. Furthermore, in mice with a disrupted pH2-M3 gene, Ly49A-expressing NK cells showed reduced functional activity, and this was associated with reduced control of experimental metastases and MCA-induced fibrosarcomas [39].

Partial or complete loss of MHC I expression is a common feature of cancer cells [40], perhaps because of selection for tumor loss variants resulting from killing by CD8 T cells. Loss of MHC I results in increased sensitivity of tumors to NK killing, but in many cases NK cells fail to eliminate such tumors. As a likely explanation, we recently demonstrated that MHC I-deficient tumor cells induce functional anergy of tumor-infiltrating NK cells [41••]. Interestingly, provision of cytokines IL-12 and IL-18, or of a mutant form of IL-2, reversed the anergic state of NK cells, resulting in better tumor control. These findings suggested that cytokine-based immunotherapies may represent a potential therapeutic strategy for MHC I-deficient tumor cells.

Recognition of tumor cells by myeloid cells

The role of myeloid cells in tumor development and the anti-tumor immune response is complex, involving both tumor-promoting and tumor-suppressive capabilities. Whereas CD8 T cell and NK cell functions are known to facilitate tumor elimination, the potential ability of myeloid cells to participate in tumor immunosurveillance remains poorly understood. Several recent studies have produced intriguing data suggesting that myeloid cells may be able to directly recognize cancer cells and facilitate tumor clearance.

Recognition of tumor cells by Dectin-1

Dectin-1 is a pattern recognition receptor expressed on dendritic cells and macrophages that binds β-glucan structures present mainly in fungal cell walls [42•,43]. Chiba *et al.* recently showed that a soluble Dectin-1-Ig fusion protein bound the plasma membrane of a panel of mouse and human tumor cell lines, but not primary untransformed cells [42•]. Interestingly, Dectin-1-deficient mice showed increased susceptibility to transplanted tumor cell lines, including those that generate metastases. Using co-culture experiments, the authors showed that Dectin-1 expression on myeloid cells enhanced the ability of NK cells to kill tumor cells *in vitro*. This study suggested that myeloid cells utilize Dectin-1 to recognize tumors and amplify anti-tumor NK responses, revealing an interesting cross talk between macrophages and NK cells in eliminating tumor cells.

Programmed cell removal

Macrophages are crucial mediators of programmed cell removal, in which dead and dying cells are engulfed using the macrophage's phagocytic apparatus [44]. Macrophages utilize a panel of cell surface receptors to recognize potential target cells for programmed cell removal. These receptors can either promote or inhibit phagocytosis, and the balance of these signals determines the susceptibility of target cells to elimination [45]. Translocation of the resident endoplasmic protein calreticulin to the cell surface represents an important pro-phagocytic signal [46••]. Chao *et al.* showed that cell-surface calreticulin was a common feature on human cancer cells, but that these cancer cells become resistant to programmed cell removal by upregulating cell surface ligands that inhibit phagocytosis, such as CD47 [47••]. *In vivo* administration of antibodies that block CD47 triggered programmed cell removal of cancer cells *in vitro* and *in vivo*, and interaction of calreticulin with its cognate receptor, LDL receptor-related protein(s), was necessary for programmed cell removal mediated by anti-CD47 antibodies [47••,48]. These studies have paved the way to consideration of new therapeutic strategies that amplify phagocytosis of tumor cells by myeloid cells. New strategies targeting the CD47 molecule are currently being explored as potential cancer immunotherapies in clinical trials.

Role of cytosolic DNA sensing by the cGAS/STING pathway in anti-tumor response

Upon recognition of double stranded cytosolic DNA, the cytosolic enzyme cyclic GMP-AMP synthase (cGAS) synthesizes cyclic GMP-AMP (2′3′-cCGAMP) [49]. cGAMP serves as a ligand for the adaptor STING which activates the TBK1/IRF3 and IKK/NF-κB pathways leading to type I IFN and cytokine secretion, respectively. Initially, this pathway was thought to play a role primarily in responses to intracellular pathogens [50]. However, recent studies have implicated the cGAS/STING pathway in anti-tumor immunity as well. Cytosolic DNA can be detected in mouse and human tumor cell lines as well as primary tumors. Interestingly, cytosolic DNA appears in cell lines upon activation of the DNA damage response, and is followed by the secretion of type I IFN and other cytokines, as well as by the induction of the NKG2D ligand RAE-1 on the cell surface [51••,52••]. Induction of RAE-1 was dependent on STING, TBK1 and IRF3, implicating the DNA sensing pathway in ligand induction [52••]. Moreover, *Irf3* (+/−) lymphoma-prone (Eμ-Myc) mice succumbed to lymphoma significantly faster than *Irf3* (+/+) Eμ-Myc mice, and an antibody that blocks the type I IFN receptor inhibited the rejection of Eμ-Myc tumor cells injected intravenously. These results indicated that rejection of the tumor cells depends on type I IFN signaling.

A recent study also suggested a distinct role of the cGAS/STING pathway in anti-tumor immunity, in this case in non-tumor cells [53••]. The authors showed that injection of mice with immunogenic tumor cells primes the host's T cells in a manner that depends on STING and IRF3 in host cells, as opposed to tumor cells. STING and IRF3 KO mice displayed defective accumulation of anti-tumor T cells, and defective rejection of immunogenic tumors. Tumor-derived DNA could be detected in the cytosol of tumor resident CD11c+ dendritic cells (DCs). These cells displayed nuclear localization of phosphorylated IRF3, and produced IFN-β. The authors suggested that tumor-derived DNA enters the cytosol of DCs, hereby activating the cGAS-STING pathway to initiate the anti-tumor response. Taken together these studies suggest that the cGAS/STING pathway plays an important role in orchestrating the antitumor immune response and the tumor microenvironment.

Conclusion

Studies reviewed in this article provide evidence for the role of innate immune cells in mediating tumor surveillance (Table 1). Experiments performed in mouse models of cancer support the proposal that malignant transformation is coupled to events that render cells immunogenic, such as the expression of ligands for germ-line encoded NK receptors or the sensing of cytosolic tumor DNA. Tumors evolve to escape the immune system by losing some of the immunogenic determinants presented earlier or by suppressing/desensitizing the immune response. Understanding the mechanisms involved in the recognition of tumor cells by innate immune cells and how tumors evade the immune response will lead to the development of innovative therapeutic strategies for cancer treatment.

Acknowledgements

The authors acknowledge grant support for their research from the National Institutes of Health (R01 CA093678 and R01 AI113041 to DHR). Thornton Thompson was the recipient of the Cancer Research Institute Student

Training in Tumor Immunology Fellowship. Alexandre Iannello was the recipient of the Leukemia and Lymphoma Society Special Fellow award. Michele Ardolino was the recipient of a Cancer Research Institute Irvington post-doctoral fellowship.

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