CHAPTER THREE

Recognition of Tumors by the Innate Immune System and Natural Killer Cells

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Abstract

In recent years, roles of the immune system in immune surveillance of cancer have been explored using a variety of approaches. The roles of the adaptive immune system have been a major emphasis, but increasing evidence supports a role for innate immune effector cells such as natural killer (NK) cells in tumor surveillance. Here, we discuss some of the evidence for roles in tumor surveillance of innate immune cells. In particular, we focus on NK cells and other immune cells that express germline-encoded receptors, often labeled NK receptors. The impact of these receptors and the cells that express them on tumor suppression is summarized. We discuss in detail some of the pathways and events in tumor cells that induce or upregulate cell-surface expression of the ligands for these receptors, and the logic of how those pathways serve to identify malignant, or potentially malignant cells. How tumors often evade tumor suppression mediated by innate killer cells is another major subject of the review. We end with a discussion on some of the implications of the various findings with respect to possible therapeutic approaches.

1. INTRODUCTION

Research performed over the past two decades has provided much evidence supporting a role for the immune system in controlling cancer. Seminal studies showed that important components of the immune system such as perforin (van den Broek et al., 1996), interferon-γ (Dighe, Richards, Old, & Schreiber, 1994), and lymphocytes (Shankaran et al., 2001) can limit the outgrowth of transplanted, carcinogen-induced, and spontaneous tumors. These initial studies were followed by an explosion of clinical and experimental evidence describing how immune cells and molecules can influence the development of cancer (Vesely, Kershaw, Schreiber, & Smyth, 2011). Although certain immune responses can protect the host from neoplasia, other immune processes such as chronic inflammation can promote the initiation or progression of cancer (Schreiber, Old, & Smyth, 2011). Notably, these contradictory roles of the immune system can manifest themselves in the same tumor model, illustrating the complex interaction between the immune system and the tumor (Swann et al., 2008).

Before discussing the role of the innate immune system in tumor surveillance, it is useful to briefly summarize the known role of the adaptive immune system. Many studies have sought to clarify the cellular and molecular components responsible for the immune system's antitumor activities. There is much evidence that certain adaptive immune cells, specifically $CD8^+$ T cells and Th1-polarized $CD4^+$ T cells, can exert antitumor effects

by recognizing tumor-specific antigens presented on MHC molecules (Diamond et al., 2011; van der Bruggen et al., 1991). These T-cell antigens are derived from oncogenic viral products, mutations in cellular genes, and/or host proteins that are normally absent in adult animals but aberrantly expressed by cancer cells.

Acting as cell-extrinsic tumor suppressor mechanisms, these adaptive immune responses are thought to limit the establishment of certain types of cancer, which may therefore never be detected clinically. Indeed, immunocompromised humans and mice have significantly higher rates of numerous cancers of both viral and nonviral etiology (Vesely et al., 2011). However, in some cases tumor cells can escape the selective pressure from the immune system by acquiring mutations or other changes that allow tumor progression in the face of an ongoing immune response (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002; Schreiber et al., 2011). The functional consequence of this selective pressure by the immune system, also known as "immunoediting," is demonstrated by the observation that tumors transplanted from an immune-deficient animal to a syngeneic immune-competent animal are often rejected by the recipient's immune system, whereas tumors that arise in immune-competent animals generally grow unimpeded after transplantation to either type of host (O'Sullivan et al., 2012; Shankaran et al., 2001). Observations made in advanced tumors from patients lend further support to the existence of immunosurveillance mechanisms. For example, many tumor cells contain mutations affecting the MHC I processing pathway, presumably to avoid recognition by $CD8^+$ T cells (Chen et al., 1996; Garrido, Cabrera, Lopez-Nevot, & Ruiz-Cabello, 1995; Seliger et al., 2001), while other tumors undergo selection for loss of peptide sequences that can serve as antigens for T cells (Matsushita et al., 2012). Taken together, these studies suggest that T cells exert strong selective pressure on tumors both in mice and in humans.

Although the importance of T cells in immunosurveillance is supported by considerable data, the adaptive immune system is not the sole mediator of antitumor immunity. Indeed, many innate leukocytes can differentiate normal cells from tumor cells and mediate important tumor suppressive functions. Whereas conventional T cells recognize cancer cells using a rearranged antigen receptor with a myriad of specifities for tumor antigens, innate cells express a fixed set of germline-encoded receptors, suggesting that the molecular basis of cancer surveillance by innate cells is fundamentally different from that of the adaptive immune system. Nevertheless, adaptive immune cells also express germline receptors (such as NKG2D on $CD8^+$ T cells),

and these receptors can play an important role in driving adaptive immune responses (Andre et al., 2012). Furthermore, the adaptive response is amplified by, and in some cases may be dependent on, innate recognition mechanisms. One example to consider in the purview of this review is the documented capacity of NK cells, an innate component of the immune system, to induce dendritic cell maturation, which may amplify T-cell responses (Moretta et al., 2005). Various other innate lymphoid cell types (ILCs), which are only now being characterized functionally, may also turn out to play roles in initiating adaptive responses to tumors. This review will focus on the role of innate immunity in detecting and preventing cancer, with particular emphasis on the receptors and ligands mediating innate recognition of tumor cells.

2. INNATE CELLS AND EFFECTOR MOLECULES IN TUMOR SURVEILLANCE

The role of the adaptive immune system in tumor surveillance has been well studied, but the innate immune system also plays a role. Natural killer cells are perhaps the best-studied mediators of innate immunosurveillance of cancer. The original characterization of NK cells noted their potent ability to kill tumor cells *in vitro* without prior sensitization, and numerous early studies suggested the potential for NK cells to mediate antitumor responses. Many transplanted tumor cells are rejected in an NK-cell-dependent manner (Diefenbach, Jensen, Jamieson, & Raulet, 2001; Ljunggren & Karre, 1985; Seaman, Sleisenger, Eriksson, & Koo, 1987), and early experiments suggested that perforin production by NK cells protected mice from methylcolanthrene (MCA)-induced sarcomas (van den Broek et al., 1996). Later, more direct, evidence showed that NK cells (in some cases in cooperation with NKT cells) eliminate many MCA-induced sarcomas (O'Sullivan et al., 2012; Smyth, Crowe, & Godfrey, 2001). Clinical evidence suggests that NK cell infiltrates in tumor biopsies are associated with favorable prognoses in cancer patients (Coca et al., 1997; Ishigami et al., 2000), and therapies aimed at boosting the tumoricidal activity of NK cells are the subject of much clinical interest (Ames & Murphy, 2013). Additional studies have shown a role for other innate lymphocyte populations in tumor surveillance. Gamma/delta T cells are another class of cytolytic ILCs that can lyse tumor cells. γδ T cells have been shown to limit cancer incidence in models of carcinogen-induced skin cancer (Girardi et al., 2001) and a transgenic

model of prostate adenocarcinoma (Liu et al., 2008). In addition, NKT cells can cooperate with NK cells to limit tumorigenesis in MCA-treated mice (Crowe, Smyth, & Godfrey, 2002; Smyth et al., 2001).

Innate cells can control cancer by directly interacting with tumor cells and/or augmenting the activities of other cells in the tumor microenvironment. Direct tumor cell lysis by NK cells is thought to be mediated principally by perforin, as shown *in vivo* and *in vitro* (Davis, Smyth, & Trapani, 2001; Hayakawa et al., 2002; Kagi et al., 1994; Lee, Spielman, Zhao, Olsen, & Podack, 1996; Smyth et al., 1999; van den Broek et al., 1996; van den Broek, Kagi, Zinkernagel, & Hengartner, 1995; Vermijlen et al., 2002). NK cells can also induce tumor cell elimination through death receptor-mediated pathways such as TRAIL and FasL (Johnsen et al., 1999; Lee et al., 1996; Zamai et al., 1998). A recent two-photon microscopy study showed that NK cells infiltrating tumors establish brief cytotoxic interactions with target cells, allowing NK cells to rapidly kill numerous cancer cells (Deguine, Breart, Lemaitre, Di Santo, & Bousso, 2010).

Activated NK cells and γδ T cells are also potent producers of numerous cytokines, including IFN-γ, TNF-α, growth factors such as G-CSF and GM-CSF, and numerous chemokines (Vivier et al., 2011). IFN-γ, in particular, is thought to have powerful antitumor activities, such as inducing MHC I expression and sensitizing tumor cells to $CD8^+$ T-cell killing. Gamma/delta T cells are appreciated as an important source of IFN-γ in subcutaneous tumor transplantation models (Gao et al., 2003), and NK-cell-derived IFN-γ is positively associated with patient survival in some cancers (Menard et al., 2009). TNF-α can have direct cytotoxic activity by triggering caspase 8-mediated apoptosis (Peter & Krammer, 2003). In addition, the combination of IFN-γ and TNF-α can drive tumor cells into senescence (Braumuller et al., 2013). Thus, cytokines secreted by innate cells can have direct antitumor activities. NK cells also modulate activity of other leukocytes, such as dendritic cells and T cells, through cytokine secretion or various receptor–ligand interactions (Martin-Fontecha et al., 2004; Moretta et al., 2005). Although cooperation between different leukocytes has not been deeply explored in the context of tumor immunology, a recent report suggested that NK-cell-derived IFN-γ polarizes macrophages toward a tumoricidal "M1" state that confers protection from carcinogen-induced sarcomas (O'Sullivan et al., 2012).

Initiation of the effector mechanisms described above requires innate cells to differentiate between normal cells and their transformed counterparts. However, unlike T cells, innate leukocytes cannot recognize

canonical neoantigens that arise during tumorigenesis. What then is the molecular basis for the recognition and elimination of tumor cells by innate immune cells? Some innate receptors recognize ubiquitous intracellular self-ligands, such as nucleic acids, that stimulate responses in certain cancer-associated contexts. Such receptors may be engaged in the context of the abnormal physiology of cancer cells. In contrast, other innate receptors recognize ligands that are displayed primarily by abnormal cells, so-called "induced self ligands." The remainder of this review will focus on these latter receptors that bind to self-ligands expressed or upregulated preferentially by tumor cells, enabling certain innate cells to respond to tumor cells. We will introduce and examine these germline-encoded receptors, including the activating receptors NKG2D; DNAM; the natural killing receptors NKp30, NKp44, NKp46, and NKp80; the SLAM-family receptors (the activating receptors are summarized in Table 3.1); and the inhibitory Ly49 receptors (which are expressed in mice) and KIRs (expressed in humans). Next, we will describe the biological processes that lead to tumor cell expression of the ligands for these activating receptors. Particular emphasis will be placed on how pathways intrinsic to the tumorigenesis process itself result in expression of such ligands. Finally, we will discuss immune escape strategies employed by tumors to avoid these mechanisms of elimination by innate cells. While these receptors and ligands are mostly associated with NK cells, the role of other cells involved in these processes will be addressed when relevant.

3. GERMLINE-ENCODED RECEPTORS IMPLICATED IN TUMOR SURVEILLANCE

3.1. NKG2D

NKG2D is a lectin-like type 2 transmembrane receptor encoded by gene *Klrk1* (killer cell lectin-like receptor subfamily member 1) located in the NK gene complex (Raulet, 2003). Due to its short intracellular domain, NKG2D cannot transmit activating signals alone (Houchins, Yabe, McSherry, & Bach, 1991). Instead, charged residues in the transmembrane region enable NKG2D to pair with the signaling adaptor proteins DAP10 (in humans and mice) and DAP12 (in mice) (Diefenbach et al., 2002; Gilfillan, Ho, Cella, Yokoyama, & Colonna, 2002; Rosen et al., 2004; Zompi et al., 2003), which are essential for NKG2D surface expression and downstream signaling. Pairing with DAP12 occurs only with a specific splice isoform of NKG2D that is present in activated but not resting mouse

Table 3.1 NK cell receptors with possible roles in tumor immune surveillance

Receptor	Adaptor protein(s)	Ligand(s)	Consequence of engagement
NKG2D (CD314)	DAP10/DAP12	NKG2D ligands	Cytotoxicity/IFN-γ
NKp30 (CD337)	CD3ζ–CD3ζ/CD3ζ–FcεRIγ	B7-H6, BAG-6	Cytotoxicity/IFN-γ
NKp44 (CD336)	DAP12	Viral HA, HN, MLL5	Cytotoxicity/IFN-γ
NKp46 (CD335)	FcεRIγ–CD3ζ	Viral HA, HN, ?	Cytotoxicity/IFN-γ
2B4 (CD244)	SAP/EAT2/ERT	CD48	Cytotoxicity/IFN-γ
NTB-A (Ly108)	SAP/EAT2/ERT	NTB-A	Cytotoxicity/IFN-γ
CD84	SAP/EAT2/ERT	CD84	Cytotoxicity?/ IFN-γ?
CRACC (CD319)	SAP/EAT2/ERT	CRACC	Cytotoxicity?/ IFN-γ?
DNAM-1 (CD226)	FYNT	PVR, Nectin-2	Adhesion
TACTILE (CD96)	FYNT	PVR, Nectin-2	Adhesion
NKp80 (KLRF1)	?	AICL	Cytotoxicity/IFN-γ

AICL, activation-induced C-type lectin; HA, hemagglutinin; HN, hemagglutinin-neuraminidase; NKG2D ligands: MICA, MICB, ULBP1-6, MULT-1, RAE-1α–ε, H60a–c; PVR, poliovirus receptor; ?=unknown or uncertain.

NK cells (Diefenbach et al., 2002). DAP10 recruits the p85 subunit of phosphoinositide 3-kinase (PI3K) and growth factor receptor-bound protein 2 (GRB2) (Sutherland et al., 2002; Wu et al., 1999), while DAP12 carries an immunoreceptor tyrosine-based activation motif (ITAM) for the recruitment of spleen tyrosine kinase (SYK) and ζ-chain-associated protein kinase 70 (ZAP70) (Zompi et al., 2003). NKG2D engagement leads to downstream activation of PI3K and GRB2, and in activated mouse NK cells, SYK and ZAP70. These signaling molecules stimulate killing, cytokine production, immune cell activation, and proliferation.

NKG2D is expressed on almost all NK cells, certain $CD8^+$ T cells, γδ T cells, NKT cells, and certain $CD4^+$ T cells. In humans, NKG2D is expressed by all $CD8^+$ T cells and certain activated CD4 T cells, whereas in mice, NKG2D is expressed by activated $CD8^+$ T cells and has not been documented on conventional $CD4^+$ T cells. NKG2D recognizes several MHC-related ligands that are poorly expressed by normal cells and tissues but were shown to be upregulated on various tumor cells in early studies (Bauer et al., 1999; Cerwenka et al., 2000; Diefenbach, Jamieson, Liu, Shastri, & Raulet, 2000; Guerra et al., 2008). In the mouse, NKG2D ligands include the family of retinoic acid early inducible-1 proteins (RAE-1α–ε), murine UL16-binding protein-like transcript-1 (MULT-1), and the H60 family (H60a, H60b, and H60c) of proteins. In humans, NKG2D ligands include UL16-binding proteins (ULBP1–6) and MHC class I-chain-related proteins A and B (MICA and MICB). There is ample evidence that NKG2D ligands are expressed in many human tumors, including melanoma, leukemia, myeloma, glioma, and carcinomas of the prostate, breast, lung, and colon (Friese et al., 2003; Salih et al., 2003; Vetter et al., 2002; Watson et al., 2006). Accumulating evidence shows that the expression of NKG2D is crucial for tumor cell elimination both *in vitro* and in tumor transplantation experiments *in vivo* (Bauer et al., 1999; Cerwenka, Baron, & Lanier, 2001; Diefenbach et al., 2001). Conflicting results have been obtained using the MCA-induced sarcoma model regarding the role of NKG2D. It was reported that NKG2D antibody blockade increased the incidence of MCA-induced tumors, but no increase was seen in NKG2D knockout (KO) mice (Crowe et al., 2002; Guerra et al., 2008). The best evidence for the importance of NKG2D comes from experiments using NKG2D KO mice, which were crossed to mice expressing transgenic oncogenes. In the TRAMP model of prostate cancer, the incidence of aggressive adenocarcinoma was increased in the absence of NKG2D. Similarly, in the *Eμ–Myc* model of B-cell lymphoma/leukemia, tumor development was markedly accelerated (Guerra et al., 2008).

3.2. Other natural cytotoxicity receptors

In addition to NKG2D, several other NK cell receptors are thought to play roles in tumor cell recognition. Three examples of such receptors, NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337), have been termed "natural cytotoxicity receptors (NCRs)," though their role in natural killing is no greater than the role of NKG2D.

NKp46 and NKp30 are expressed on both resting and activated human NK cells, whereas NKp44 is expressed only on activated human NK cells. In mice, NKp46 has been characterized in some detail, but NKp44 is not present in mice and *NKp30* is a *pseudogene*.

These receptors, when engaged, each trigger NK-cell-mediated killing and secretion of IFN-γ. NCRs belong to the immunoglobulin (Ig) superfamily of receptors (Cantoni et al., 1999; Pende et al., 1999; Pessino et al., 1998; Sivori et al., 1997; Vitale et al., 1998), have extracellular Ig domains, and associate noncovalently with adaptor proteins like DAP12 (NKp44), FcεRIγ–CD3ζ (NKp46), and CD3ζ–CD3ζ or CD3ζ–FcεRIγ (NKp30) (Moretta et al., 2001). These adaptor proteins contain ITAMs to recruit and activate SYK and ZAP70. These kinases phosphorylate transmembrane adaptors such as Linker for the Activation of T cells (LAT) and NTAL (Non-T cell Activation Linker), resulting in the activation of PI3K, phospholipase C (PLC), and VAV proteins (Billadeau et al., 1998; Cella et al., 2004; Spaggiari et al., 2001; Tassi et al., 2005; Upshaw, Schoon, Dick, Billadeau, & Leibson, 2005). The calcium flux resulting from these signaling events leads to cytoskeletal reorganization, cellular cytotoxicity, and cytokine secretion, mainly IFN-γ and TNF-α (Cantoni et al., 1999; Pende et al., 1999; Pessino et al., 1998).

In vitro studies have demonstrated roles for all three of these receptors in NK-mediated killing of certain tumor cells and infected cells (Arnon et al., 2004; Costello et al., 2002; Fauriat et al., 2007; Moretta et al., 2001; Pende et al., 1999; Sivori et al., 1999).

The identity of the tumor cell ligands for these receptors is a matter of ongoing research, but candidates have emerged over recent years. B7-H6, a molecule that is expressed on the surface of tumor cells and on TLR-triggered monocytes and neutrophils, was identified as a novel ligand for NKp30 (Brandt et al., 2009; Kaifu, Escaliere, Gastinel, Vivier, & Baratin, 2011). 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 *in vivo* tumor recognition (Pogge von Strandmann et al., 2007; Simhadri et al., 2008).

Despite much research, the tumor-associated ligand for NKp46 remains unknown. Nevertheless, *in vivo* evidence from NKp46 KO mice suggests that NKp46 can inhibit tumor metastasis (Glasner et al., 2012). Other ligands of both cellular and viral origins have also been reported for one or more of these three receptors, but their role in tumor immunosurveillance is unclear.

3.3. NKp80 (KLRF1)

NKp80 is a C-type-lectin-like homodimeric receptor expressed on human NK cells and $CD3^+$ $CD56^+$ T cells (Welte, Kuttruff, Waldhauer, & Steinle, 2006). The cytoplasmic tail of NKp80 contains atypical tyrosine-based motifs called hemITAMs that activate the SYK-kinase pathway and trigger NKp80-mediated cytotoxicity (Dennehy, Klimosch, & Steinle, 2011; Ruckrich & Steinle, 2013). The receptor ligates activation-induced C-type lectin (AICL), an activating receptor preferentially expressed by myeloid cells (Welte et al., 2006). The AICL–Nkp80 interaction stimulates NK cytotoxicity and plays a role in the recognition of myeloid tumor cells (Welte et al., 2006).

3.4. SLAM-related receptors

The SLAM (signaling lymphocyte activating molecule)-related receptors are expressed on a wide variety of immune cells including NK cells, monocytes/macrophages, mast cells, granulocytes, γδ T cells, DCs, and $CD8^+$ T cells (Veillette, 2006). The family includes the SLAM (CD150), 2B4 (CD244), NTB-A (Ly108), CD48, CD84, Ly9 (CD229), and CRACC (CD319) receptors (Veillette, 2006). Most SLAM receptors engage in homotypic interactions with the same protein on another cell, with exception of 2B4 and CD48, which interact with each other. SLAM receptor engagement results in activating signals via the action of several receptor-associated adaptor proteins belonging to the SAP (SLAM-associated protein) family (Veillette, 2006). In the absence of the known SAP-family adaptors, the SLAM-family receptors convey strong inhibitory signals that repress activation caused by other receptors such as NKG2D (Dong et al., 2009; Lee, McNerney, et al., 2004; McNerney, Guzior, & Kumar, 2005; Vaidya et al., 2005). The SLAM receptors play critical roles in NK-mediated killing of hematopoietic target cells, including MHC-deficient normal hematopoietic cells and lymphoma cells, but SLAM receptors are dispensable for recognition of nonhematopoietic tumor cells (Dong et al., 2009). NK cells from SAP-deficient humans and mice have a reduced ability to kill hematopoietic target cells (Benoit, Wang, Pabst, Dutz, & Tan, 2000; Bloch-Queyrat et al., 2005; Nakajima et al., 2000; Tangye, Phillips, Lanier, & Nichols, 2000).

3.5. Adhesion molecules and DNAM-1

In adhering to target cells, NK cells create an immunological synapse where activating and inhibitory receptors spatially cluster in order to increase the

efficacy of intracellular signaling. Adhesion molecules such as the integrin LFA-1 play a key role in the formation of immunological synapses (Long, Sik Kim, Liu, Peterson, & Rajagopalan, 2013; Watzl & Long, 2010). The engagement of LFA-1 with its ligands ICAM-1 or ICAM-2 on target cells results in polarization of cytotoxic granules toward the immunological synapse (Barber, Faure, & Long, 2004; Bryceson, March, Barber, Ljunggren, & Long, 2005; Mentlik, Sanborn, Holzbaur, & Orange, 2010) and is essential for NK cell and T-cell interactions with many target cells.

The Ig-superfamily receptor DNAM-1 (CD226) associates with LFA-1 on the cell surface and potentiates activating signals. LFA-1 engagement results in Fyn kinase-mediated phosphorylation of the DNAM-1 cytoplasmic domain (Shibuya et al., 1999, 2003). DNAM-1 is expressed by a variety of leukocytes such as NK cells, T cells, B cells, monocytes, and platelets (Burns, Triglia, Werkmeister, Begley, & Boyd, 1985; Scott et al., 1989; Shibuya et al., 1996). Patients with Leukocyte Adhesion Deficiency lack LFA-1 and are unresponsive to stimulation with DNAM-1 ligands (Shibuya et al., 1999).

Two ligands for DNAM-1 have been identified in humans and mice: PVR (CD155), a member of the Nectin family of proteins, and Nectin-2 (CD112), a member of the Nectin-like family (Bottino et al., 2003; Tahara-Hanaoka et al., 2004). Nectin-2 and PVR are broadly distributed on many tissues throughout the body, including neurons, epithelial cells, endothelial cells, and fibroblasts (Fuchs & Colonna, 2006). PVR can also be bound by the receptors TACTILE and TIGIT, but the roles of those receptors in immunosurveillance have not been characterized (Fuchs, Cella, Giurisato, Shaw, & Colonna, 2004; Stanietsky et al., 2013). DNAM-1 ligands are frequently expressed by tumor cells (Bottino et al., 2003; Masson et al., 2001; Sloan et al., 2004; Tahara-Hanaoka et al., 2004), and the interaction is in some cases essential for normal triggering of NK cell-mediated cytotoxicity and cytokine production (Bottino et al., 2003; Burns et al., 1985; Lakshmikanth et al., 2009; Shibuya et al., 1996). NK cell killing of neuroblastoma cells from different patients was well correlated with the expression of DNAM-1 ligands on the tumor cells (Castriconi et al., 2004). However, DNAM-1 engagement is not sufficient to induce target cell killing, but synergistically activates NK cells that are also stimulated via 2B4, NKp46, and possibly other receptors (Bryceson et al., 2005; Bryceson, March, Ljunggren, & Long, 2006).

Notably, *Dnam1*$^{-/-}$ mice had accelerated growth of fibrosarcomas and papillomas in response to chemical carcinogens as well as reduced rejection

of transplanted tumor cells expressing DNAM-1 ligands (Gilfillan et al., 2008; Iguchi-Manaka et al., 2008). These findings suggest a significant role of DNAM-1 in tumor immunosurveillance.

3.6. MHC-specific NK cell inhibitory receptors

NK cells express a variety of receptors that transmit inhibitory signals when engaged by MHC I molecules expressed by target cells. These include the Ly49 and KIR families of receptors that recognize MHC class Ia molecules directly. Mice express Ly49 receptors but not KIRs, whereas humans express KIRs but not Ly49 receptors. A third, shared, inhibitory receptor is the CD94/NKG2A heterodimeric receptor, which recognizes a peptide derived from the class Ia leader sequence presented by a specific nonclassical MHC I molecule (HLA-E in humans and Qa-1 in mice) (Braud et al., 1998; Lee et al., 1998; Vance, Kraft, Altman, Jensen, & Raulet, 1998). Inhibitory receptors signal though immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. Upon receptor engagement, ITIMs are tyrosine-phosphorylated and recruit the protein tyrosine phosphatases SHP-1 and SHP-2 (Lanier, 1998; Long, 1999; Ravetch & Lanier, 2000). The phosphatases are believed to inhibit activation by dephosphorylating one or more critical signaling molecules, especially VAV-1 (Binstadt et al., 1996; Burshtyn et al., 1996; Campbell, Dessing, Lopez-Botet, Cella, & Colonna, 1996; Olcese et al., 1996).

Each KIR, Ly49, or CD94/NKG2A receptor is expressed on only a fraction of NK cells, such that the resulting NK repertoire allows for recognition of host cells that have lost just a single class Ia allele (Moretta et al., 1996; Raulet, Vance, & McMahon, 2001; Shilling et al., 2002). Because selective loss of class I MHC expression is a common feature of many tumor cells (Garrido et al., 1997), the diverse KIR expression by NK cells allows for recognition and elimination of tumor cells or other cells that have such alterations in MHC I expression. The recognition and destruction of cells lacking MHC I is called "missing self-recognition" (Ljunggren & Karre, 1990).

Missing self-recognition was discovered in studies showing that RMA-S, a lymphoma cell line selected for loss of MHC I expression, was sensitive to NK cells while RMA cells, an MHC^+ counterpart to RMA-S, was not (Karre, Ljunggren, Piontek, & Kiessling, 1986). Later studies showed that even normal cells lacking MHC I are destroyed by NK cells (Bix et al., 1991). The absence of MHC I molecules by RMA-S cells or MHC I-deficient normal cells is not a sufficient condition for destruction of the

cells, as the susceptible cells must also express ligands that activate NK cells, albeit weakly. Recent evidence suggests that such killing does require engagement of specific activating receptors on NK cells, namely, the SLAM-family receptors (Dong et al., 2009). This conclusion is based in part on the finding that NK cells lacking the SAP-family adapters for SLAM receptors were unable to kill MHC-deficient normal cells or RMA-S cells *in vivo* or *in vitro*. Notably, the SLAM proteins, which also serve as ligands for SLAM receptors, are restricted in expression to hematopoietic cells. Signals provided by SLAM receptor engagement are insufficient to override inhibition imparted by MHC I-specific receptors, explaining why normal MHC^+ cells are not susceptible to NK-mediated killing.

4. THE IMMUNOGENICITY OF CANCER: HOW ALTERATIONS COMMON TO CANCER CAN RESULT IN DETECTION BY INNATE IMMUNE CELLS

4.1. Proliferation

Dysregulated proliferation is a hallmark of cancer. Mutations of proto-oncogenes that promote cell-cycle progression, coupled with insensitivity to growth inhibition, can cause transformed cells to proliferate in an uncontrolled manner (Hanahan & Weinberg, 2000).

Many cell-intrinsic mechanisms can sense excessive proliferation. For example, oncogenic mutations that constitutively activate the RAS, PI3K, and/or MAPK pathways, central drivers of cell growth and proliferation, can activate tumor suppressor proteins such as p53. Activation of p53 can mediate temporary growth arrest, permanent inhibition of cell-cycle progression (senescence), or programmed cell death (Vousden & Prives, 2009). Activation of the RAS pathway has recently been shown to upregulate expression of the RAE-1 proteins in mice and ULBP1–3 in human cells (Liu, Ho, Tan, Kamran, & Gasser, 2012). In that study, induction of NKG2D ligands depended on RAF, MAPK/MEK, and PI3K, but not ATM or ATR. Interestingly, activation of the PI3K pathway has also been linked to induction of RAE-1 ligands in MCMV infection and in various tumor cell lines (Tokuyama et al., 2011).

Recent evidence suggests that proliferation is also coupled to cell-extrinsic tumor suppressive pathways mediated by immune cells that express NKG2D. Primary mouse fibroblasts cultured *ex vivo* were shown to spontaneously upregulate the NKG2D ligand RAE-1 and acquire sensitivity to NKG2D-dependent killing by NK cells (Jung, Hsiung, Pestal, Procyk, &

Raulet, 2012). RAE-1 induction was blocked by inhibition of cyclin-dependent kinases (Roscovitine), the PI3K-mTOR pathway (LY294002, Rapamycin), and the MAPK pathway (SB202190). Cells cultured in serum-limiting conditions showed a delayed and reduced induction of RAE-1, and cells cultured in high-serum concentrations (and thus displaying high RAE-1 levels) rapidly downregulated RAE-1 expression upon serum starvation. Furthermore, when cells were serum-starved and subsequently cultured in the presence of serum, only the cells that reentered the cell cycle (as demonstrated by BrdU incorporation) upregulated RAE-1. Addition of recombinant epidermal growth factor to serum-free media was sufficient to rescue proliferation and RAE-1 expression.

RAE-1 expression on a variety of mouse tumor cell lines (and MICA or ULBP2 expression on human cell lines) was dependent on proliferation, as serum starvation or CDK inhibition reduced cell-surface RAE-1 levels (Jung et al., 2012). Interestingly, certain untransformed cells undergoing rapid proliferation, such as early embryonic brain cells and cells proliferating in healing skin wounds, were found to express RAE-1 *in vivo* (Jung et al., 2012). Earlier studies showed that proliferating human T cells upregulate NKG2D ligands (Zingoni, Ardolino, Santoni, & Cerboni, 2012), and several reports have described constitutive NKG2D ligand expression in subpopulations of cells in rapidly proliferating tissues such as the bone marrow (Ogasawara, Benjamin, Takaki, Phillips, & Lanier, 2005) and intestinal epithelium (Groh et al., 1996). On the other hand, proliferation is frequently insufficient to result in substantial upregulation of NKG2D ligands. For example, little or no upregulation was observed in mouse T and B cells stimulated with mitogens *in vitro* (Diefenbach et al., 2000), or in brain cells in the late embryonic period, where cells are proliferating at a slower rate than at earlier stages of development (Jung et al., 2012). In those cases, expression of NKG2D ligands may require a faster rate of cell proliferation or cooperation with other signals.

In primary fibroblasts, the molecular link between proliferation and RAE-1 expression was found to depend on the E2F family of transcription factors (Jung et al., 2012). E2F proteins transactivate expression of numerous cell-cycle-associated genes, and E2F activity is tightly regulated by a variety of transcriptional and posttranslational mechanisms. E2F proteins bind to sites in the *Raet1e* promoter in proliferating cells but much less so in serum-starved cells. E2F overexpression was sufficient to activate endogenous *Raet1* genes in cells as well as a luciferase reporter construct driven by the *Raet1e* promoter (Jung et al., 2012). Neither the DNA-damage

response (DDR) nor p53 were found to be involved in RAE-1 expression in proliferating primary cultures of fibroblasts (Jung et al., 2012).

This connection between proliferation and NKG2D ligand expression provides evidence of a compelling link between an inherent characteristic of cancer and cell-extrinsic tumor suppressor activities mediated by immune cells. It will be important to further investigate tumor cell proliferation and NKG2D ligand expression in primary cancer models, to understand the role of NKG2D ligand expression on normal proliferating cells, and to uncover why some proliferating cells but not others express these ligands. As discussed elsewhere in this review, other mechanisms associated with aberrant cell-cycle progression, such as p53 activation, may enhance tumor suppression by immune cells by independent mechanisms.

4.2. Role of the DNA-damage response (DDR)

Replication stress (the result of stalled replication forks) and DNA double-strand breaks activate the DDR in tumor cells and precancerous lesions (Bartkova et al., 2005; Gorgoulis et al., 2005). DNA damage and replication stress activate the protein kinases ATR and/or ATM, which initiate the DDR kinase cascade (Cimprich & Cortez, 2008; Shiloh & Ziv, 2013) by phosphorylating the checkpoint kinases CHK1 and CHK2, respectively, in addition to many other target proteins. Activation of the DDR arrests cell-cycle progression and induces DNA repair functions. If DNA damage is extensive or irreparable, the DDR activates the p53 tumor suppressor, which can induce apoptosis or senescence depending on the cellular context. The DDR is often activated in developing tumors, but is also induced by many common cancer therapies, including irradiation and many chemotherapeutic drugs (Lord & Ashworth, 2012).

In response to DNA damage, the mouse NKG2D ligands RAE-1 and MULT-1 (Gasser, Orsulic, Brown, & Raulet, 2005; Gasser & Raulet, 2006) and the human NKG2D ligands ULBP1–3 and MICA/B (Gasser et al., 2005; Soriani et al., 2009) are upregulated, although which ligand(s) and the degree of upregulation varies between different cell types and patient samples. Expression of DNAM-1 ligands also increases in response to DDR activation (Ardolino et al., 2011; Soriani et al., 2009). In the studies cited above, upregulation of ligands for both NKG2D and DNAM-1 in response to DNA damage occurred in an ATR/ATM- and CHK1/CHK2-dependent manner. The *in vivo* relevance of these findings is suggested by recent evidence that spontaneous tumor regression in the *Eμ-Myc* transgenic

model of lymphoma/leukemia is partially ATM and DNAM-1 dependent (Croxford et al., 2013).

Although NKG2D ligand mRNA levels increase in response to DNA damage, the rate of transcription of these genes did not increase in nuclear run-on experiments, whereas the rate of degradation of the *Raet1* mRNA was decreased, suggesting that the DDR drives stabilization of NKG2D ligand mRNAs rather than transcription of the ligand genes (B. Hsiung & D. H. Raulet, unpublished data). The mechanism of transcript stabilization remains unclear. DDR-mediated increases in DNAM-1 ligand expression also occur at the mRNA level, but the specific mechanism has not been investigated to date. It is also unknown whether NKG2D and DNAM-1 ligands are controlled by the same or different DDR effectors.

Activation of the tumor suppressor p53 is a major downstream effect of the DDR, but NKG2D ligand upregulation in response to DNA damage was p53-independent in the mouse studies performed to date (Gasser et al., 2005; Iannello, Thompson, Ardolino, Lowe, & Raulet, 2013). In human cells, however, two different studies suggested that p53 stimulates transcription of certain NKG2D ligands. It was reported that preventing proteasomal degradation of p53 by the MDM2 inhibitors RITA and Nutlin allows p53 to bind the promoter region of the *ULBP1* and *ULBP2* genes, resulting in enhanced *ULBP1* expression (Li et al., 2011; Textor et al., 2011). Surprisingly, another study concluded that p53 activation negatively regulates human NKG2D ligand expression (Heinemann et al., 2012). Notably, neither of these effects of p53 was studied in the context of the DDR.

4.3. Role of oncogene-induced senescence in innate responses against tumor cells

p53 plays a central role in regulating various cell-intrinsic biological processes including apoptosis, DNA repair, and the induction of cellular senescence. By limiting cell proliferation, cellular senescence represents an intrinsic barrier to tumorigenesis (Braig & Schmitt, 2006; Collado & Serrano, 2010). The two major types of cellular senescence are replicative senescence, which is linked to telomere shortening occurring in normally dividing cells, and oncogene-induced senescence, which is associated with oncogene activation (Collado & Serrano, 2010; Stewart & Weinberg, 2006). Activation of p53/p21 and p16/Rb tumor suppressor pathways during oncogenic stress can trigger senescence. Interestingly, a recent study suggested that adaptive $CD4^+$ T-cell-mediated immune responses against

tumors can also induce a senescent state through the secretion of TNF-α and IFN-γ, expanding the array of effector mechanisms that lymphocytes employ to inhibit tumor progression (Braumuller et al., 2013).

The link between cellular senescence and innate immune responses has been investigated in several contexts. One study showed that elimination of tumor cells, rendered senescent as a result of induction of p53, was mediated by NK cells, macrophages, and neutrophils (Xue et al., 2007), whereas another study determined that senescence was associated with adaptive ($CD4^+$ T cell-mediated) responses against tumor cells (Kang et al., 2011). Yet another study concluded that immune-mediated removal of senescent cells resulting from fibrotic injury was essential for resolution of the injury (Krizhanovsky et al., 2008).

A recent study investigated how p53 activation and the accompanying senescence enable NK cells to eliminate senescent tumor cells (Iannello et al., 2013). First, it was shown that NK-mediated elimination of mouse hepatocellular carcinoma cells, rendered senescent by induction of p53 in growing tumors, was nearly entirely dependent on NKG2D-mediated recognition of RAE-1 ligands displayed on the senescent tumor cells. Interestingly, however, RAE-1 expression was not dependent on p53 expression, as it was already high on growing tumor cells. In seeking an explanation for why the NK cells failed to eliminate RAE-1-expressing nonsenescent tumor cells, it was noted that very few NK cells infiltrated such growing tumors, whereas many NK cells infiltrated senescent tumor cells in which p53-expression had been induced. NK cell infiltration occurred in NKG2D knockout mice and so was independent of NKG2D signaling. Analysis revealed that p53 restoration substantially increased tumor cell production of numerous chemokines known to recruit NK cells and other immune cells, including CCL2, CCL3, CCL4, CCL5, CXCL1, and CXCL2. Indeed, it has been previously established that senescent cells exhibit the "senescence-associated secretory phenotype," which is characterized by secretion of numerous soluble factors including chemokines (Campisi, 2012; Tchkonia, Zhu, van Deursen, Campisi, & Kirkland, 2013). Neutralization of CCL2 *in vivo* with antibodies caused a dramatic reduction in NK cell accumulation in tumor cells, whereas neutralization of CCL3, CCL4, and CCL5 had no effect (Iannello et al., 2013). Furthermore, CCL2 neutralization, but not neutralization of the other chemokines, resulted in delayed elimination of the senescent tumor cells, providing direct evidence that p53-induced CCL2 expression was responsible for NK cell recruitment and senescent tumor elimination. An interesting implication of these

findings was that tumor elimination depended on the cooperation of two independently regulated features of the tumor cells: chemokine production, which was p53-dependent, and RAE-1 expression, which was independent of p53 and likely regulated by the pathways discussed in detail earlier in this review.

5. INTERPLAY BETWEEN TUMORS AND INNATE LYMPHOCYTES

The accumulation of studies showing roles in tumor surveillance for the immune system raise important questions, such as why tumors progress despite such surveillance. In the following sections, some findings pertinent to this question will be reviewed.

5.1. Selective loss of NK-activating ligands associated with evasion of innate immune surveillance

In studies of T cell-mediated antitumor responses, it has become clear that tumors evolve under the selective influences of the immune response. One line of evidence for such selection is that tumors that arise in immune-deficient animals grow progressively if transferred to a similarly immune-deficient host, but tend to be rejected if transferred to wild-type mice (Shankaran et al., 2001). In some cases, specific mutations accumulate in tumor cells that eliminate epitopes that T cells would otherwise recognize (Matsushita et al., 2012). This phenomenon has been termed "cancer immunoediting" (Dunn, Old, & Schreiber, 2004; Schreiber et al., 2011) and provides a distinct line of evidence for immune surveillance of tumors and, at the same time, delineates one mechanism of how immune surveillance is overcome.

Evidence for immunoediting has also been provided in the case of innate responses against cancer. In the TRAMP transgenic model of prostate adenocarcinoma, aggressive tumors that arose in wild-type mice lacked NKG2D ligands on the cell surface, whereas similar tumors that arose in NKG2D-deficient littermates uniformly expressed one or more NKG2D ligand (Guerra et al., 2008). These data suggested that tumors detected in mice that express NKG2D had been selected for loss of NKG2D ligands. Considering the reduced number of aggressive tumors in these NKG2D-expressing mice, and the absence of ligands on such tumors, it was proposed that NKG2D-dependent immune selection eliminates a majority of newly arising aggressive tumors, but that another fraction of tumors persists because

variant cells within those tumors extinguish ligand expression and are therefore able to evade immune detection (Guerra et al., 2008).

Selection for loss of NKG2D ligands does not occur in all cancer models. In the *Eμ–Myc* model of B lymphoma, for example, tumors that arose in mice expressing NKG2D did not lack NKG2D ligands, despite the fact that tumorigenesis was delayed in those mice (Guerra et al., 2008). It is not known why such tumors are refractory to selection by NKG2D whereas others are not, but one speculation is that certain types of tumors evolve mechanisms to dominantly inactivate NK cells or other relevant effector cells and therefore can evade the response without extinguishing NKG2D ligands.

Demonstrating immunoediting resulting from other modes of NK cell recognition is more difficult because the relevant ligands have not been identified for most of the other NK receptors. One study attempted to detect a role for the NKp46 activating receptor in surveillance of MCA-induced fibrosarcomas by comparing tumor incidence and phenotype in NKp46 mutant versus wild-type mice. The incidence of tumors was not different, but tumors arising in NKp46-deficient mice stained with an NKp46-Ig fusion protein, suggesting they expressed NKp46 ligands, whereas tumors arising in wild-type mice did not (Elboim et al., 2010). Furthermore, when cell lines derived from these tumors were injected into mice, it was observed that tumors that arose in the absence of NKp46 grew more slowly than tumors that originated from wild-type mice. On the basis of these findings, it was concluded that tumors readily undergo selection for loss of NKp46 ligands.

5.2. Ligand shedding as a mechanism of evasion

Many tumor cell lines release soluble NKG2D ligands through a variety of mechanisms, including alternative splicing, PI-PLC-mediated cleavage, proteolytic shedding, or exosome secretion (reviewed in more detail in Chitadze, Bhat, Lettau, Janssen, & Kabelitz, 2013; Raulet, Gasser, Gowen, Deng, & Jung, 2013). Indeed, soluble MIC and ULBP proteins have been identified in the sera of patients with various tumor types including breast, lung, colon, and ovarian carcinoma, glioma, neuroblastoma, leukemia, and melanoma (Boissel et al., 2006; Doubrovina et al., 2003; Groh, Wu, Yee, & Spies, 2002; Jinushi et al., 2005; Marten, von Lilienfeld-Toal, Buchler, & Schmidt, 2006; Salih, Goehlsdorf, & Steinle, 2006; Salih, Rammensee, & Steinle, 2002). In the mouse, both RAE-1

(Champsaur & Lanier, 2010) and MULT-1 (W. Deng & D. H. Raulet, unpublished data) ligands have been detected in soluble form in cell culture supernatants. The presence of soluble ligands in the sera of cancer patients may in some cases serve as prognostic indicators of cancer. For example, the level of soluble ULBP2 was shown to discriminate patients at an early stage of pancreatic adenocarcinoma from healthy donors (Chang et al., 2011) and to identify melanoma patients at risk for disease progression (Paschen et al., 2009). Furthermore, increased serum concentrations of soluble ULBP2 were associated with a poorer prognosis in patients with early-stage B-cell chronic lymphocytic leukemia (Nuckel et al., 2010).

Depending on the specific setting and the nature of the excreted ligands, the various forms of soluble NKG2D ligands can potentially exert distinct effects on NKG2D/NKG2D ligand interactions. Shedding of NKG2D ligands from tumor cells can result in dramatically lower cell-surface levels, reducing their susceptibility to cytolysis by NK cells and T cells. At the same time, the accumulated shed ligands may interact with NKG2D on the surface of NK cells and T cells, even those at a distance from the primary tumor (Chauveau, Aucher, Eissmann, Vivier, & Davis, 2010). Binding of the soluble ligands may prevent interactions of NKG2D with membrane-bound ligands. Alternatively, if the soluble ligands can transmit signals through NKG2D, these interactions have the potential to either activate or desensitize the NK cells or T cells. Indeed, rather than inhibiting NK activity, NKG2D ligand-containing exosomes derived from human DCs were reported to directly activate human NK cells *ex vivo* (Viaud et al., 2009). Presumably, the capacity of ligand-containing exosomes to cross-link NKG2D can explain the activating effect of the exosomes.

Soluble NKG2D ligands are also thought to impair immune surveillance by modulating NKG2D expression. In some cases, for example, cancer patients with elevated soluble MICA in their serum exhibited strongly reduced NKG2D staining of their peripheral blood $CD8^+$ T cells (Groh et al., 2002). Similarly, soluble ULBP1–3 was found to downregulate NKG2D on NK cells (Fernandez-Messina et al., 2010; Song, Kim, Cosman, & Choi, 2006). Notably, however, a functional impact of soluble NKG2D ligands was not always observed (von Lilienfeld-Toal et al., 2010). For example, the sera from MICA transgenic mice, which contained high levels of soluble MICA, had only a marginal effect on NKG2D surface expression on nontransgenic NK cells (Wiemann et al., 2005). In addition, no inhibitory effects on NKG2D expression were observed with supernatants containing soluble MULT-1, a mouse NKG2D ligand (W. Deng &

D. H. Raulet, unpublished data). Also of concern is that in most studies, the form of the ligands (exosomes vs. enzymatically shed molecules) was not determined.

In a few studies, the role of soluble NKG2D ligands was examined by attempting to neutralize the soluble ligands with anti-MIC antibody (Wang et al., 2008) or NKG2D-Fc fusion proteins (Hilpert et al., 2012). Those studies suggested a correlation between elevated soluble NKG2D ligand levels in specific tumor patients and reduced NKG2D-dependent immune responses, but the generality of these findings and the specific mechanisms responsible remain unclear. Serum from tumor patients contains many additional immunosuppressive factors (e.g., TGF-β) which reportedly downregulate NKG2D. For example, despite the presence of soluble NKG2D ligands in the sera of glioblastoma patients, NKG2D downregulation was primarily caused by tumor-derived TGF-β (Lee, Lee, Kim, & Heo, 2004). Another point of concern is that exosomes may "bundle" a variety of tumor-derived ligands of other molecules, which may have to act together to impact NK and T-cell immune responses.

5.3. Evasion of NK-cell-mediated immunosurveillance as a result of anergy of NK cells

As already mentioned, in some cases, tumors develop without losing expression of immune-activating ligands. While the underlying mechanisms for this outcome remain unclear, it is known that chronic engagement of activating receptors can lead to immune dysfunction. *In vitro* experiments showed that chronic engagement of NK cells with cells expressing NKG2D ligands substantially diminishes the function of the NK cells, even affecting responses mediated through receptors other than NKG2D (Coudert, Scarpellino, Gros, Vivier, & Held, 2008; Coudert et al., 2005). In the case of a transgenic mouse strain expressing RAE-1 constitutively, the NK cells not only exhibited lower activity against cells with NKG2D ligands but also were less effective at rejecting MHC-deficient cells that lack NKG2D ligands, suggesting a general dysfunction of the cells (Oppenheim et al., 2005). Although this finding was not confirmed with a distinct RAE-1 transgenic line, which may express lower levels of RAE-1 (Champsaur et al., 2010), a similar general functional defect was observed in mice that constitutively expressed the viral protein m157, which binds the Ly49H-activating receptor (Sun & Lanier, 2008). It has not been investigated directly, but these findings raise the possibility that in some cases tumors expressing activating ligands, such as NKG2D ligands, may induce anergy

or hyporesponsiveness of NK cells, enabling the tumors to evade immune surveillance.

A recent study (M. Ardolino and D. Raulet, in preparation) addressed whether there are conditions in which NK cells within MHC-deficient lymphoma cells are rendered anergic to the tumor cells, a pertinent question since many tumor cells lack MHC I (Garrido & Algarra, 2001), and NK cells in cancer patients often display functional defects (Costello et al., 2002; Epling-Burnette et al., 2007; Fauriat, Moretta, Olive, & Costello, 2005). It was shown that when the capacity of NK cells to reject MHC I-deficient tumor cells was overwhelmed by the inoculation of a large dose of MHC I-deficient lymphoma cells (RMA-S cells), NK cells were recruited to the tumor but were rendered hyporesponsive. The potential significance of these findings is that they suggest a likely mechanism of immune evasion. When the capacity of NK cells to mediate tumor rejection is overwhelmed, perhaps because the tumor is well advanced at the time that it is infiltrated, the persistent stimulation of the NK cells drives them into a hyporesponsive state.

6. CONCLUDING REMARKS

As discussed in the preceding pages, evidence from knockout mice and antibody depletion studies suggest a role for innate components, including NK cells and various germline receptors, in immune surveillance in both carcinogen-induced and genetic models of cancer. Table 3.2 summarizes the various ways NK cells can be activated during tumor development. Complementary data show that tumors that arise in wild-type mice often contain alterations that are absent in tumors that arise in mice lacking innate components, suggesting that the innate response plays an active role in selecting variant, resistant tumors, a process that has been termed immunoediting.

NKG2D and DNAM-1 ligands can be induced by proliferation (Ardolino et al., 2011; Cerboni, Zingoni, Cippitelli, Frati & Santoni, 2007; Jung et al., 2012) and by the DDR (Ardolino et al., 2011; Gasser et al., 2005; Soriani et al., 2009), and it has been suggested that DNAM-1 ligands play roles in cancer invasion and metastasis (Sloan et al., 2004). In addition to the expression of cell-surface ligands, soluble factors such as cytokines and chemokines may also play a role in activating the immune system (Iannello et al., 2013; O'Sullivan et al., 2012). Some of these processes are considered "hallmarks of cancer" (Hanahan & Weinberg,

Table 3.2 How NK cells become activated during cancer development
NK cells may become activated and undergo expansion to eliminate cancer cells in several ways:

1. Recognizing tumor-induced immune-activating ligands on the host cells via activating receptors.
2. Responding to tumor cells that have lost expression of MHC or other immune-inhibitory ligands.
3. Reacting to activating cytokines (IFN-α/β, IL-12, IL-15, IL-18, IL-21) produced by tumor cells or by other immune cells stimulated by tumor cells.
4. By interaction with tumor infiltrating and tumor-associated immune cells, for example, DCs or macrophages.

2011), supporting the proposal that malignant transformation is coupled to events that render cells immunogenic. In the future, it will be of interest to explore the role of other aspects of tumorigenesis in the immunogenicity of cancer cells.

Some of the pathways in tumor cells that control NK ligands and other aspects of immunogenicity are active in normal cells as well. This consideration prompts the question: can tumor cells be reliably distinguished from normal cells by these mechanisms? Are the pathways that support induction of NK-activating ligands sufficiently specific to prevent the destruction of normal cells? As an obvious example, cellular proliferation is presumably insufficiently specific as a basis for immunogenicity of cancer cells.

Multiple mechanisms and processes are likely to explain the specificity of the NK response in different contexts. First, cellular proliferation is not sufficient for ligand induction in all cell types, such as activated mouse T cells (Diefenbach et al., 2000), possibly due to a specific genetic repression of the ligand genes; this would ensure that those cells are not inadvertently destroyed. In some cell types, it is likely that multiple pathways must cooperate to support high-level expression of the ligands. As discussed, the regulation of ligands at distinct levels of biogenesis (transcription, translation, protein and mRNA stabilization) by different dysregulated pathways, may explain why cells sustain high expression of activating ligands only in unhealthy cells. In other cases, efficient activation of NK cells, especially resting NK cells, is thought to depend on simultaneous engagement of multiple activating or accessory receptors. Hence, induction of ligands for one NK receptor may not always be sufficient to stimulate an active NK response. Yet another important consideration is that induction of NK-activating ligands on certain cells will have little effect if NK cells are not recruited to the vicinity of

those cells, as previously discussed in the case of senescent versus non-senescent tumors. In that instance, mobilization of an independent pathway is necessary to cause chemokine production and NK-cell recruitment. A requirement for immune cell recruitment is likely to provide an added level of specificity to innate responses in other contexts as well.

It is interesting to speculate on the evolutionary basis of innate antitumor responses and their relationship to antipathogen responses. In light of the fact that these cells, receptors, and ligands participate in both antipathogen and antitumor responses, a relevant but difficult question is whether one or the other form of selection (infection versus cancer) played the greater role in the initial appearance of a cell type or receptor–ligand system. It is commonly asserted that the predominance of cancer late in life means that selective pressures for antitumor immune mechanisms would come at a post-reproductive age and therefore be ineffective. Assumptions as to the timing in the life cycle, or source, of selective pressures that acted on organisms when these mechanisms evolved are full of uncertainties, however. Moreover, a counter argument is that cancer is delayed in life because of tumor suppressor mechanisms, including immune-mediated mechanisms, which would act in concert with cell-intrinsic tumor suppressive mechanisms such as p53 and PTEN. Regardless of the types of selection present during the early evolution of these cells and their recognition systems, selective pressures are likely to have adapted the cells or their receptor systems for additional purposes. The fact that common stress pathways that regulate expression of NK-cell-activating ligands are activated in both infected and transformed cells is consistent with this notion.

There are several potential benefits of using the immune system to control tumors. In some cases immune mechanisms may have advantages compared to cell-intrinsic mechanisms. First, cell-intrinsic mechanisms, like any other mechanism, are prone to failure, necessitating the existence of redundant systems. A second, unique, benefit of immune mechanisms of tumor suppression is that they can act in a paracrine manner. Secretion of cytokines such as IFN-γ, for example, can suppress the growth of tumor cells that do or do not upregulate immune-activating ligands. The importance of this concept is suggested by the documented prevalence of cellular heterogeneity in tumors (Navin et al., 2011). Third, activation of innate immunity, including NK cells, can promote tumor-specific adaptive immune responses that can provide dominant and systemic protection and long-lasting memory. It must be emphasized that separately assessing the benefits of intrinsic and immune-mediated tumor suppression mechanisms is complicated by the fact that in

some cases they act cooperatively. An example is the induction by p53 of immune-mediated antitumor mechanisms, such as chemokine production that attracts NK cells to tumors. Notably, the mobilization of immune responses by intrinsic tumor suppressors confers the same advantage just mentioned with respect to IFN-γ production: even if only a fraction of tumor cells express p53, the attracted NK cells may nevertheless kill the p53-deficient tumor cells.

The two main pathways that allow tumors to escape the immune system are loss of immunogenic determinants and the tumor-driven suppression or desensitization of the immune response. Loss of immunogenic determinants can occur at the genetic level (deletion or mutation of a gene), epigenetic level (silencing of a gene), or at the posttranslational level. Active suppression of the immune response can occur through expression of immune-inhibiting molecules at the cell surface (such as PD-L1/2; Hirano et al., 2005) or secretion of immunosuppressive cytokines (such as TGF-β; Eisele et al., 2006). An example of desensitization of the immune response is the chronic stimulation of the immune system by immunogenic tumors, which can eventually lead to anergy and immune dysfunction, and which may occur in NK cells in the tumor microenvironment (Coudert et al., 2008; Oppenheim et al., 2005). Some of the ways tumors can escape elimination by NK cells are summarized in Table 3.3.

Reversing these defects in innate immunosurveillance is an attractive approach for cancer therapy that is receiving much recent attention. Interestingly, conventional chemotherapeutic agents may already have such effects, since they have been reported to induce NKG2D ligands on tumors cells. The relevant drugs can be divided into three broad categories: DNA damaging agents (Soriani et al., 2009; Gasser et al., 2005), proteasome inhibitors (Hallett et al., 2008), and histone deacetylase inhibitors (Diermayr et al., 2008). Hence, it is possible that some of the therapeutic benefit seen with chemotherapy stems from immune activation rather than a direct cytotoxic effect. Given that tumor cells are already experiencing various stresses, induction of immune-activating ligands may be more likely to occur in these cells as opposed to normal healthy cells.

The scenario in which tumors are not rejected despite expression of immune-activating ligands (such as NKG2D ligands) requires a different therapeutic approach. In these cases, the ineffectiveness of the response may be due to shedding of ligands, failure to recruit the appropriate immune cells, or inactivation of the cells once they infiltrate the tumor. In the case of shed ligands, drugs that inhibit shedding or neutralize shed ligands may be effective.

Table 3.3 Possible mechanisms of tumor evasion of the NK cell response

1.	Loss of expression of activating ligands for NK receptors such as NKG2D, NKp46, or DNAM-1.
2.	Secreting/shedding soluble ligands for activating NK receptors, for example, NKG2D, thereby reducing ligand expression on the tumor surface, and in some cases, inhibiting NK cell recognition and function.
3.	Persistent stimulation of NK cells in the absence of inflammatory cytokines, which may induce a state of NK cell anergy.
4.	Loss of tumor suppressors that induce secretion of chemokines that recruit NK cells.
5.	Modulation of the tumor microenvironment resulting in secretion of immunosuppressive cytokines, for example, IL-10 and TGF-β.

In consideration of this possibility, an interesting study showed that the presence of naturally arising antibodies to MICA/B is correlated with an improved outcome in multiple myeloma patients (Jinushi et al., 2008). Inhibitory cytokines may be neutralized by injections of appropriate monoclonal antibodies. The molecular mechanisms of anergy in innate immune cells are not yet known, so it is not yet possible to specify the best approaches to reverse anergy of these cells. Nevertheless, the recent dramatic clinical success of CTLA4 and PD1 antibodies in cancer patients (Hodi et al., 2010; Topalian et al., 2012) make it tempting to speculate that reversing anergy of innate immune cells could also provide significant therapeutic benefit.

Substantial progress has been made over the past decade in elucidating mechanisms underlying the innate immune response to cancer. A big-picture understanding of how tumors progress in the presence of the immune system is still elusive. Cancer genome-sequencing studies have identified recurring mutational signatures in various cancers, but the corresponding immunological signatures of tumors have not been extensively studied. Remedying this knowledge gap is likely to be important, given that infiltration of immune cells into tumors is correlated with positive prognoses (Coca et al., 1997; Galon et al., 2006). Increased understanding of the complex interactions between cancer and the immune system are likely to lead to improvements in current therapeutic approaches and to spur the development of novel ones.

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