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REVIEW

# NK cells and cancer immunosurveillance

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**Natural killer (NK) cells are lymphocytes of the innate immune system that monitor cell surfaces of autologous cells for an aberrant expression of MHC class I molecules and cell stress markers. Since their first description more than 30 years ago, NK cells have been implicated in the immune defence against tumours. Here, we review the broadly accumulating evidence for a crucial contribution of NK cells to the immunosurveillance of tumours and the molecular mechanisms that allow NK cells to distinguish malignant from healthy cells. Particular emphasis is placed on the activating NK receptor NKG2D, which recognizes a variety of MHC class I-related molecules believed to act as 'immuno-alerters' on malignant cells, and on tumour-mediated counterstrategies promoting escape from NKG2D-mediated recognition.**
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## NK cells: a historical precis

In 1975, NK cells were first identified in mice as a distinct sub-population of lymphocytes endowed with the capacity to kill tumour cells without prior sensitization (Herberman *et al*., 1975a, b; Kiessling *et al*., 1975a, b). Since then, NK cells underwent a fascinating metamorphosis in scientists' minds from dumb, unspecific killer machines to highly sophisticated and well-educated detectives of harmful changes in cellular self and pivotal catalysers of adaptive T-cell responses. Major advances in the current understanding of NK cell biology originated from the models of 'missing-self' and 'induced-self' recognition. These models propose that NK cells, in contrast to their sister lymphocytes, T and B cells, do not recognize foreign antigens, but rather are 'self-centered' by detecting changes in self-molecules displayed at the surface of autologous cells. The model of 'missing-self' recognition is based on the original observations by Karre and colleagues describing that syngeneic tumour cells with a deficient expression of major histocompatibility complex (MHC) class I molecules are selectively rejected by NK cells (Ljunggren and Karre, 1985, 1990; Karre *et al*., 1986). This MHC class I-dependent recognition mode explains why virally infected or malignant cells with an impaired MHC class I expression are attacked by NK cells, whereas 'healthy' autologous cells are protected from NK cytotoxicity (Algarra *et al*., 2000; French and Yokoyama, 2003). NK-mediated cytolysis of MHC class I-deficient cells provides an important safeguard for the MHC class I-restricted elimination of 'dangerous' cells by CD8 T cells. NK cells detect a state of 'missing-self' by MHC class I-specific inhibitory receptors (see Section Inhibitory NK receptors). However, the 'missing-self' hypothesis failed to explain why NK cells spare autologous cells with absent MHC class I expression (such as human erythrocytes) or kill certain MHC class I-sufficient tumour cells. The ensuing characterization of several activating NK receptors, foremost of the NKG2D receptor that detects cell stress-induced self-ligands on 'dangerous' cells, led to the proposition of the 'induced-self' recognition model (Bauer *et al*., 1999; Diefenbach and Raulet, 2001; Raulet, 2004): the latter complements the 'missing-self' recognition model by stating that NK cell triggering requires the expression of inducible ligands of activating NK receptors. Altogether, it is now common sense that the activation of NK cells depends on an intricate balance between activating and inhibitory signals (Lanier, 2005): 'healthy' cells express NK-inhibiting MHC class I molecules and no or few activating NK ligands leaving NK cells quiescent. In contrast, 'dangerous' cells stimulate NK cell responses by an increased expression of activating NK ligands and a reduced MHC class I outfit. However, a full appreciation of these concepts and, in particular, of NK-mediated tumour surveillance needs to await the thorough molecular characterization of the ligands of activating NK receptors (see Section Activating NK receptors).

## NK cell subsets and effector mechanisms

NK cells are primarily viewed as cytotoxic lymphocytes; upon activation, NK cells exocytose cytotoxic granules containing perforin and various granzymes, leading to the perforation of target cells and subsequent apoptotic death induced by the permeated granzymes (Lieberman, 2003; Voskoboinik *et al*., 2006). In addition to the perforin/granzyme pathway, the engagement of tumour

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necrosis factor (TNF) receptor superfamily (TNFRSF) members, such as Fas/CD95, TRAIL receptors and TNFR1, on tumour cells by the corresponding ligands (FasL, TRAIL and TNF) expressed on or secreted by NK cells contributes to NK cytotoxicity under certain circumstances (Zamai *et al*., 1998, 2007; Voskoboinik *et al*., 2006). A second, often underrated effector mechanism of NK cells is their capacity to secrete a variety of cytokines and chemokines, including interferon-$\gamma$ (IFN-$\gamma$), TNF, GM-CSF (granulocyte-macrophage colony stimulating factor), MIP-1$\alpha$ (macrophage inflammatory protein-1$\alpha$) and RANTES (regulated upon activation, normal T cell expressed and secreted) (Biron *et al*., 1999; Dorner *et al*., 2004). In fact, NK cells are considered as the major source of IFN-$\gamma$ *in vivo*, and recent studies demonstrated that NK-derived IFN-$\gamma$ is crucial in priming T helper 1 (Th1)-biased T-cell responses (Mocikat *et al*., 2003; Martin-Fontecha *et al*., 2004).

In humans, NK cells usually are defined as $CD3^{-}CD56^{+}$ lymphocytes and comprise about 5–20% of peripheral blood lymphocytes. They can be divided into two major sub-populations, namely $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$. The $CD56^{dim}$ population predominates in the blood ($\sim$95% of NK cells) and at sites of inflammation, exhibits a high cytotoxic potential and broadly expresses MHC class I-specific inhibitory receptors. In contrast, the $CD56^{bright}$ subset predominates in lymph nodes ($\sim$75% of NK cells), mainly produces cytokines upon activation, displays little cytotoxicity and is considered to represent a precursor stage of terminally differentiated $CD56^{dim}$ NK cells (Lanier *et al*., 1983; Ferlazzo and Munz, 2004; Freud and Caligiuri, 2006; Chan *et al*., 2007). Mouse NK cells do not express CD56, but recent work by Hayakawa and Smyth categorized mouse NK subsets according to their expression levels of the TNFRSF member CD27. Similar to human $CD56^{bright}$ NK cells, mouse $CD27^{high}$ NK cells produce large amounts of cytokines in response to monokines and predominate in lymph nodes (Hayakawa *et al*., 2006; Hayakawa and Smyth, 2006a). However, at difference to human $CD56^{bright}$ NK cells, they also are potent cytolytic effectors. Recently, it has been suggested to define NK cells in humans and mice unifyingly by the expression of the activating NK receptor NKp46, which, in contrast to other markers like CD56, CD16 (human), NK1.1 or DX5 (mouse), is almost exclusively expressed by all NK cells in both species (Walzer *et al*., 2007).

## NK cell-mediated immunosurveillance of cancer: evidence from mouse models

There are countless studies in mice supporting the notion that NK cells are engaged in the eradication of tumour cells. Most of these studies were performed by implanting syngeneic tumour cells in mice that either were genetically deficient in NK cell function or depleted of NK cells by the administration of antibodies (Kim *et al*., 2000; Smyth *et al*., 2002; Wu and Lanier, 2003; Hayakawa and Smyth, 2006b). Eliminating NK cells in such models often led to a more aggressive tumour growth and metastasis. Evidently, the outcome was strongly determined by the tumour cells' expression of surface molecules relating to the principles of missing-self and induced-self recognition. Unfortunately, most of these studies suffered from the deficit that genetic defects or administered antibodies did not exclusively target NK cells. Cytokines, such as interleukin (IL)-2, IL-12, IL-15, IL-21 and IFN-$\alpha/\beta$, enhance the activation state of NK cells or promote NK maturation, thereby augmenting NK cytolytic activity against tumour cells (Biron *et al*., 1999; Smyth *et al*., 2002, 2004; Nutt *et al*., 2004). Administration of these cytokines resulted in an enhanced elimination of implanted tumours further supporting a beneficial role of NK cells in anti-tumour responses (Wu and Lanier, 2003; Hayakawa and Smyth, 2006b).

More informative with regard to a physiological tumour immunosurveillance are studies addressing the control of newly arising tumours. In this regard, an interesting series of experiments was published by Schreiber and co-workers showing that frequencies of spontaneously arising tumours or tumours induced by the chemical carcinogen methylcholanthrene (MCA) were higher in mice that were genetically deficient for key effector molecules of NK cells or the respective receptors (Kaplan *et al*., 1998; Shankaran *et al*., 2001). This includes mice deficient for perforin, IFN-$\gamma$, IFN-$\gamma$R or STAT1, an important signal transducer of type I and type II IFN receptors. As NK cells share employment of these key effectors with cytotoxic T cells, mice combining these deficiencies with deficiencies specifically abrogating T cells, for example in recombinase activating genes (RAG), are required to properly assess the contribution of NK cell-mediated tumour surveillance. Interestingly, mice deficient for both RAG2 and STAT1 have been reported to spontaneously develop adenocarcinoma at higher rates as compared with mice deficient only for RAG2, implicating NK cells in tumour immunosurveillance (Shankaran *et al*., 2001; Dunn *et al*., 2004; Swann and Smyth, 2007). In a recent study by the Schreiber group, it was shown that although NK cells are important in the early elimination of MCA-induced tumours, control of the 'dormant' tumour state depends on adaptive immunity and not on NK cells (Koebel *et al*., 2007). Another study provided evidence that NK cells together with $\gamma\delta$ T cells control spontaneously arising MHC class I-deficient B-cell lymphomas via perforin-mediated cytotoxicity (Street *et al*., 2004). Anti-tumour activities of NK cells may directly lead to tumour eradication by means of cytolysis or IFN-$\gamma$ secretion, but may also indirectly contribute to tumour control by inducing an efficient T-cell-mediated anti-tumour response. Recent *in vivo* studies provided insights into the course of such events: after the implantation of MHC class I-low tumour cells, the release of IFN-$\gamma$ by NK cells was instrumental in the stimulation and maturation of dendritic cell (DC) to a IL-12-producing DC1

phenotype that promoted a strong and protective anti-tumour $CD8^+$ T-cell response (Mocikat *et al*., 2003; Adam *et al*., 2005).

## NK cells and cancer in humans

In humans, most evidence for a role of NK cells in tumour surveillance is derived from correlative studies. In an 11-year follow-up study, it was found that a low NK-like cytotoxicity of peripheral blood lymphocytes correlates with an increased risk for cancer (Imai *et al*., 2000). Furthermore, infiltration of tumours with NK cells has been shown to represent a positive prognostic marker in different carcinomas (Coca *et al*., 1997; Ishigami *et al*., 2000; Villegas *et al*., 2002). However, in established human tumours, there are often only a few infiltrating NK cells that are unlikely to greatly contribute to the elimination of tumour cells (Albertsson *et al*., 2003; Esendagli *et al*., 2008). It has been suggested that low NK cell numbers in tumours are due to their inefficient homing into malignant tissues, which may be overcome by cytokine-mediated activation in immunotherapeutical regimens (Hokland *et al*., 1999; Albertsson *et al*., 2003). Convincing evidence for a beneficial role of NK cells in control of human malignancies comes from clinical studies of leukaemia patients who received alloreactive NK cells in the course of allogeneic haematopoietic stem cell transplantation. Here, a remarkable increase in survival and protection from relapse in myeloid leukaemia were reported for patients lacking HLA class I ligands for donor-inhibitory killer cell Ig-like receptors (KIR) (Hsu *et al*., 2005; Ruggeri *et al*., 2007).

## NK cell receptors: sensors of 'altered-self'

A prerequisite for the intelligent implementation of NK cells in anti-tumour regimen is a thorough molecular understanding of how NK cells recognize malignant cells and how established malignancies manage to subvert NK cell-mediated recognition and elimination. NK cells resort to a large repertoire of germ line encoded inhibitory and activating receptors to sense 'danger' in the form of 'altered self' cell surfaces. Structurally, these NK receptors mostly belong either to the immunoglobulin superfamily (IgSF) or to the C-type lectin superfamily (CLSF). Many immunoglobulin-like NK cell receptors, such as the KIR, the Ig-like transcripts (ILT) or the natural cytotoxicity receptor (NCR) NKp46, are encoded in the leukocyte receptor complex (LRC), whereas C-type lectin-like receptors (CTLR) of NK cells are encoded in the natural killer gene complex (NKC) (Lanier, 1998; Yokoyama and Plougastel, 2003; Kelley *et al*., 2005). Both groups of NK receptors comprise activating and inhibitory receptors. Interestingly, recent studies revealed that a group of C-type lectin-like NK receptors encoded in the telomeric subregion of the NKC of both man and mouse engages genetically linked CTLR of the C-type lectin domain family 2 (CLEC2) (Iizuka *et al*., 2003; Carlyle *et al*., 2004; Aldemir *et al*., 2005; Welte *et al*., 2006).

## Inhibitory NK receptors: mediators of 'missing-self' recognition

Important inhibitory NK receptors are members of the KIR family in humans and of the C-type lectin-like Ly49 receptors in mice, both sensing the expression of various allelic variants of classical MHC class I molecules (Yokoyama, 1993; Moretta *et al*., 1996; Long *et al*., 1997; Lanier, 2005). The human KIR family consists of ~15 functional genes in the LRC, which are highly polymorphic and not only expressed on overlapping NK cell subsets but also on a subset of memory T cells (Moretta *et al*., 1996; McMahon and Raulet, 2001; Vivier and Anfossi, 2004). There are marked interindividual differences in the number and type of KIR genes encoding for both activating or inhibitory receptors with two or three Ig-like domains and specific for subgroups of HLA-A, HLA-B and HLA-C molecules, respectively (Parham, 2005).

Mouse Ly49 receptors were the first MHC class I-specific inhibitory receptors identified (Karlhofer *et al*., 1992; Yokoyama and Seaman, 1993). Like KIR genes, Ly49 genes are polymorphic, expressed on overlapping NK cell subsets and memory T cells, and specific for allelic subsets of MHC class I molecules (Karlhofer *et al*., 1992; Vivier and Anfossi, 2004). The variegated expression of individual KIR and Ly49 receptors allows NK cells to detect and eliminate cells having lost single MHC class I molecules. As mentioned above, mismatching KIR of adoptively transferred, allogeneic NK cells to the recipient's MHC class I is already clinically exploited to achieve a graft-versus-leukaemia effect (Ruggeri *et al*., 2007).

In contrast to KIR and Ly49 receptors, CD94/NKG2A, a heterodimeric CTLR shared by human and mice, is a broad detector of MHC class I expression, as NKG2A/CD94 binds to the non-classical MHC class I molecules HLA-E in humans and Qa-$1^b$ in mice, presenting signal peptides from many classical MHC class I molecules (Braud *et al*., 1998; Lee *et al*., 1998). CD94/NKG2A is expressed on about ~50% of NK cells and some memory $CD8^+$ T cells. Inhibitory NK receptors contain cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM), which, upon phosphorylation, recruit and activate SH2-containing protein tyrosine phosphatase (SHP)-1 and SHP-2, eventually causing NK cell inhibition (Long *et al*., 1997). Most NK cells express at least one inhibitory receptor that is believed to be randomly expressed from a pool of inhibitory receptor genes (Raulet and Vance, 2006; Yokoyama and Kim, 2006). NK cells that do not express at least one inhibitory receptor matching the own MHC class I outfit are hyporesponsive (Kim *et al*., 2005; Anfossi *et al*., 2006).

In addition to the KIR and Ly49 families of NK receptors, there are other inhibitory NK receptors that

engage non-MHC-encoded self-surface molecules. These include the NKC-encoded CTLR KLRG1 (human and mouse), NKR-P1A (human) and Nkrp1d (mouse) that bind to cadherins, LLT1 and Clr-b, respectively, and are proposed to mediate an alternative mode of 'missing-self' recognition (Iizuka *et al*., 2003; Aldemir *et al*., 2005; Rosen *et al*., 2005; Grundemann *et al*., 2006; Ito *et al*., 2006). For example, KLRG1 is suggested to make NK cells 'aware' of cadherin downregulation, an event frequently occurring during the progression of epithelial tumours (Ito *et al*., 2006).

## Activating NK receptors: NK cells' alarm system

In humans, major activating NK receptors are the NCR NKp30, NKp44 and NKp46, and the CTLR NKG2D (Figure 1) (Moretta *et al*., 2001; Raulet, 2003). Blocking studies with anti-NCR antibodies demonstrate that these receptors in concert with NKG2D determine NK activation towards tumour cells (Moretta *et al*., 2001; Pende *et al*., 2001). Strikingly, tumour-associated NCR ligands, which reportedly also comprise heparan sulphate structures (Bloushtain *et al*., 2004), have withstood their molecular characterization in spite of a decade of intense research efforts. In contrast, several viral structures binding to NCR were described, including hemagglutinins of influenza and sendai viruses (to NKp46 and NKp44) and pp65 tegument protein of human cytomegalovirus (to NKp30) (Mandelboim *et al*., 2001; Arnon *et al*., 2005, 2006). The latter was proposed to engage NKp30 following cell destruction, a mechanism that may also apply for the recently reported intracellular NKp30 ligand BAT3 (Pogge von Strandmann *et al*., 2007).

NK cells are also strongly activated upon cross-linking of the low-affinity IgG receptor FcγRIII (CD16) by opsonizing antibodies mediating the so-called antibody-dependent cellular cytotoxicity (Lanier *et al*., 1983). All these activating NK receptors lack signalling motifs in their cytoplasmic sequences. Instead, they associate with adaptor molecules via charged amino acids in the transmembrane domain. CD16, NKp30 and NKp46 signals are transduced by the associated adaptors CD3ζ and FcεRIγ, whereas NKp44 couples to DAP12 (Lanier, 1998; Moretta *et al*., 2001). Upon receptor ligation, immunoreceptor tyrosine-based activation motifs (ITAMs) in CD3ζ, FcεRIγ, and DAP12 are phosphorylated and allow recruitment and activation of tyrosine kinases ZAP70 and Syk, eventually resulting in NK activation, if activating signals outweigh inhibitory signals. NCRs are almost exclusively expressed by NK cells, with NKp44 being restricted to activated NK cells (Vitale *et al*., 1998). There are several other stimulatory NK receptors, but their immunological function and signalling pathways mostly are insufficiently understood. Examples are the IgSF member DNAX accessory molecule-1 (DNAM-1)/CD226 and the CTLR NKp80 (Pende *et al*., 2006; Welte *et al*., 2006). DNAM-1 binds to adhesion molecules poliovirus receptor (PVR/CD155) and Nectin-2 (CD112) which also are IgSF members and broadly are expressed both by 'healthy' and malignant cells of epithelial and hematopoetic origin (Bottino *et al*., 2003; Pende *et al*., 2005; Carlsten *et al*., 2007; El Sherbiny *et al*., 2007). Engagement of DNAM-1 by these ligands was shown to substantially contribute to anti-tumour NK cytotoxicity (Pende *et al*., 2005, 2006; Tahara-Hanaoka *et al*., 2006; Carlsten *et al*., 2007; El Sherbiny *et al*., 2007). NKp80 interacts with the genetically linked, myeloid-specific CTLR AICL and promotes lysis of a malignant myeloid

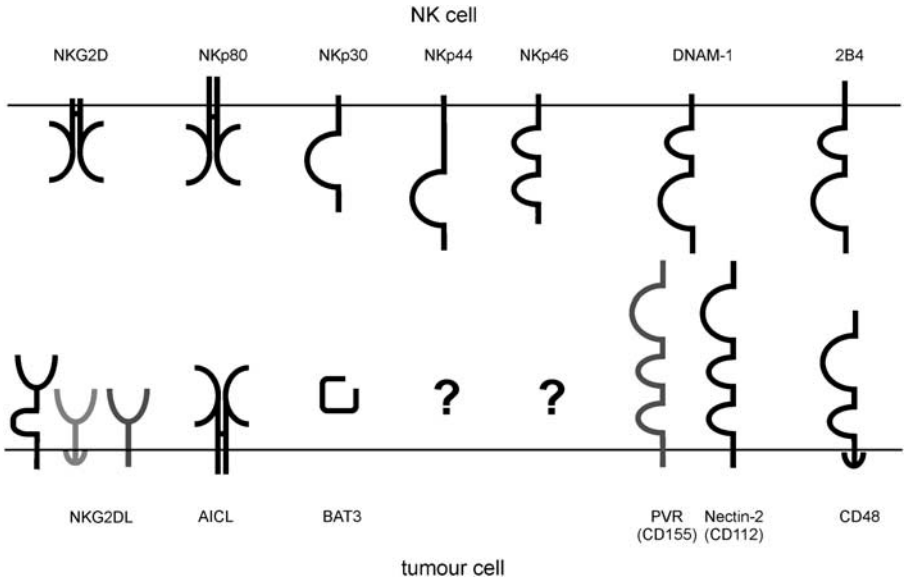


**Figure 1** Human activating natural killer (NK) receptors and their cellular ligands. Tumour-associated ligands of natural cytotoxicity receptor (NCR) NKp46 and NKp44 are unknown. NKp30 binds BAT3 released from tumour cells. NKG2D engages multiple major histocompatibility complex (MHC) class I-like ligands (see Figure 2).

cell line (Welte *et al*., 2006), but a relevance in NK cytotoxicity towards patients' leukaemic cells remains to be shown. The stimulatory IgSF-receptor 2B4 (CD244) is ligated by CD48 which is broadly expressed by cells of the haematological lineage and upregulated on Epstein-Barr virus-infected B cells (Brown *et al*., 1998; Ma *et al*., 2007). 2B4 signals via the adaptor protein SAP which is deficient in X-linked immunoproliferative disease suggesting a role of 2B4 in the immune control of EBV-infected B cells (Nakajima *et al*., 2000; Parolini *et al*., 2000; Ma *et al*., 2007).

The KIR, Ly49 and NKG2 families of NK receptors also contain several members which do not contain ITIMs, but rather associate with the ITAM-bearing adaptor DAP12 (Moretta *et al*., 2001; Lanier, 2005). Examples are KIR2DS and KIR3DS in humans, Ly49D and Ly49H in mice, and CD94/NKG2C in human and mice. Some of these receptors exhibit similar specificities for MHC class I molecules as their sequence-related inhibitory counterparts, albeit these interactions are often of lower affinity (Lanier, 2005; Parham, 2005). The immunological relevance of these activating MHC class I-specific receptors which seem to counter the principles of 'missing-self' recognition remains to be elucidated. In this context, it is of interest that recent *in vivo* studies unexpectedly revealed an immunosuppressive function of DAP12 (Takaki *et al*., 2006).

In the last years, intensive work on the NKG2D receptor revived the 'tumour immunosurveillance hypothesis' (Lanier, 2001) and shifted NKG2D into the focus of NK-mediated anti-tumour reactivity. Therefore, in the following, we focus on this unique activating NK receptor with its large family of stress-inducible ligands.

## NKG2D: a unique activating receptor of cytotoxic lymphocytes

NKG2D is a homodimeric, C-type lectin-like activating receptor expressed by cytotoxic lymphocytes and encoded in the NKC (Bauer *et al*., 1999; Raulet, 2003). Although genetic linkage and naming suggest a close relationship of NKG2D to other Natural Killer group 2 (NKG2) receptors such as NKG2A and NKG2C (Houchins *et al*., 1991), the low sequence homology between NKG2D and the other NKG2 receptors as well as the unique biology of NKG2D do not support this view. Consequently, NKG2D has been considered a misnomer (Lanier, 2005).

In humans, NKG2D is expressed on almost all NK cells, $CD8^+$ αβ T cells and γδ T cells, whereas there are only a few NKG2D-bearing CD4 αβ T cells (Bauer *et al*., 1999). This is different for specific pathological situations like rheumatoid arthritis or tumours, where increased frequencies of NKG2D-positive CD4 αβ T cells have been reported (Groh *et al*., 2003, 2006). In mice, NKG2D expression significantly differs from the human situation, since only a fraction of γδ T cells and activated or memory $CD8^+$ αβ T cells, but not naive $CD8^+$ αβ T cells express NKG2D (Ho *et al*., 2002; Jamieson *et al*., 2002). NKG2D expression is variably regulated by cytokines: whereas IL-15 enhances NKG2D surface expression (Kubin *et al*., 2001; Roberts *et al*., 2001), transforming growth factor-β (TGF-β) downmodulates NKG2D (Castriconi *et al*., 2003; Friese *et al*., 2004). Also IL-21 mediated down-regulation of the human NKG2D–DAP10 complex (Burgess *et al*., 2006), and a moderate NKG2D up-regulation by IL-10, IL-12 and IFN-α was described (Sutherland *et al*., 2002).

NKG2D uniquely associates with the adapter protein DNAX-activating protein of 10 kDa (DAP10) facilitated by charged interactions in the transmembrane domains of both proteins (Wu *et al*., 1999; Garrity *et al*., 2005). NKG2D ligation causes phosphorylation of DAP10 which eventually leads to activation of NK cells and costimulation of T cells. DAP10 contains a cytoplasmic YINM motif which upon phosphorylation allows recruitment and activation of the p85 subunit of phosphatidylinositol-3-kinase (PI-3K) (Wu *et al*., 1999). In addition, DAP10 also binds a Grb2-Vav1 intermediate which, together with PI-3K activation, triggers NK cytotoxicity (Upshaw *et al*., 2006). In mice, but not in humans, there is an alternate splice variant of NKG2D, NKG2D-S, which additionally associates with the ITAM-bearing DAP12 adaptor and, hence, triggers both, cytotoxicity and cytokine release (Diefenbach *et al*., 2002; Gilfillan *et al*., 2002; Rosen *et al*., 2004).

## NKG2D ligands

A hallmark of NKG2D is the multitude of NKG2D ligands (NKG2DL) (Figure 2). In humans, there are two families of NKG2DL, the MHC class I chain-related molecules A and B (MICA/MICB) and the UL16-binding proteins (ULBP). MICA and MICB are closely related molecules encoded by tandem genes in the MHC near the *HLA-B* locus (Bahram *et al*., 1994). MICA/B ectodomains are built out of MHC class I-like α1, α2, and α3 domains, but in contrast to MHC class I molecules, MICA/B do not associate with $\beta_2$-microglobulin or present antigenic peptides (Groh *et al*., 1996; Li *et al*., 2001). Members of the ULBP family of proteins were identified due to their binding to the HCMV protein UL16 and their similarity to mouse retinoic acid early inducible-1 (Rae-1) proteins, respectively (Cosman *et al*., 2001; Steinle *et al*., 2001). The ULBP gene cluster on the long arm of chromosome 6 comprises six functional genes encoding for ULBP1-4, RAET1G and RAET1L (Radosavljevic *et al*., 2002). All ULBP have a MHC class I-like α1α2 platform domain, but lack an α3 domain, and are anchored in the plasma membrane either by a GPI structure (ULBP1-3, RAET1L) or by a transmembrane domain (ULBP4 and RAET1G) (Cosman *et al*., 2001; Radaev *et al*., 2001; Bahram *et al*., 2005).

In mice, all NKG2DL share an MHC class I-like ectodomain structure similar to ULBP (α1α2 platform domain, no α3 domain), but markedly differ at the

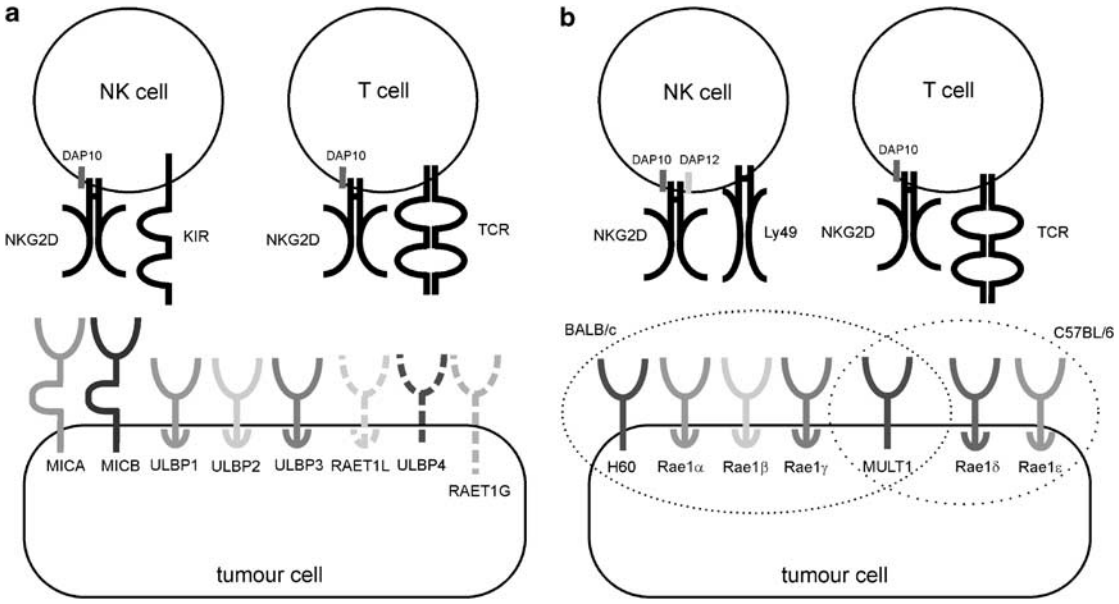


**Figure 2** NKG2D and NKG2DL in human and mouse. (**a**) In humans, NKG2D is expressed by virtually all cytotoxic lymphocytes, including natural killer (NK) cells, $CD8^+$ αβ T cells, and γδ T cells. NKG2D monitors NKG2DL surface expression by stressed, infected or malignant cells. Human NKG2DLs are MIC molecules MICA and MICB, and ULBP molecules ULBP1, -2 and -3, RAET1L, ULBP4 and RAET1G. Tumour-associated surface expression of RAET1L, ULBP4 and RAET1G remains to be shown. (**b**) In mice, NKG2D is expressed by virtually all NK cells, activated $CD8^+$ αβ T cells and a subset of γδ T cells. Mouse NKG2DLs are Mult-1, H60 and members of the Rae1 family. Rae1α,-β,-γ and H60 are expressed by BALB/c; Rae1δ and Rae1ε by C57BL/6 mice; and Mult-1 by both.

sequence level. Mouse NKG2DL comprise members of the GPI-anchored Rae-1 family of proteins (Rae1α, −β, −γ, −δ, and −ε) as well as the transmembranous ligands H60 (Cerwenka *et al*., 2000; Diefenbach *et al*., 2000) and murine UL16-binding protein–like transcript 1 (Mult1) (Carayannopoulos *et al*., 2002a; Diefenbach *et al*., 2003). No MIC homologues have been identified in mice so far. In spite of their pronounced sequence diversity, crystallographic analyses suggest that all NKG2DL form a similar tertiary structure which is engaged by NKG2D homodimers in a comparable mode of binding (Li *et al*., 2001, 2002; Radaev *et al*., 2001). However, affinities of NKG2DL to NKG2D vary over a broad range (O'Callaghan *et al*., 2001; Steinle *et al*., 2001; Carayannopoulos *et al*., 2002b) which may contribute to the fine tuning of NKG2D-mediated NK cell function. Presently, it is not clear why there are so many NKG2DL (Bahram *et al*., 2005; Eagle and Trowsdale, 2007). Redundancy of NKG2DL markedly differing at the sequence level may help to counter attempts of viral immunoevasion, but could also be explained by diversification in tissue expression or stress responses (Eagle and Trowsdale, 2007).

## NKG2DL expression

In general, NKG2DL are thought not to be expressed on healthy tissue. Rather, NKG2DL expression is induced by various forms of cellular stress such as heat shock, viral infection, DNA damage or UV radiation (Groh *et al*., 1996, 2001). Interestingly, expression of NKG2DL was shown to be a consequence of genotoxic stress and the resulting DNA damage response which has been reported to occur in precancerous lesions and many tumours in humans. The DNA damage response involves activation of protein kinases ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) kinases which are sensors of DNA double-strand breaks and stalled DNA replication, respectively (Gasser *et al*., 2005). Silencing of ATM resulted in a suppression of NKG2DL expression by tumour cell lines suggesting that NKG2DL expression by malignant cells is due to a persistent DNA damage response (Gasser *et al*., 2005). In fact, a broad expression of MICA and MICB, the best-studied NKG2DL, on epithelial tumours has been reported (Groh *et al*., 1996, 1999), and also for melanoma, hepatic carcinoma, and some haematopoetic malignancies (Vetter *et al*., 2002; Jinushi *et al*., 2003; Salih *et al*., 2003), but not for the corresponding 'healthy' tissues. From these data, one might speculate that expression of NKG2DL induced by genotoxic stress during processes of malignant transformation represents a measure of the immune system to counter tumour development.

But MICA and/or MICB was also detected on gastrointestinal epithelium, LPS-stimulated macrophages, activated T cells, HCMV-infected fibroblasts, mycobacteria-infected DCs, and tissues exposed to autoimmune attack demonstrating that MICA/B expression is not strictly tumour-associated, but can be induced by a broad variety of stimuli on many cell types under various circumstances (Groh *et al*., 1996, 2001,

2003; Das *et al*., 2001; Molinero *et al*., 2002; Nedvetzki *et al*., 2007).

In contrast to MIC proteins, *in vivo* expression of ULBP and mouse NKG2DL is ill-defined. ULBP transcripts appear to be broadly expressed (Cosman *et al*., 2001; Radosavljevic *et al*., 2002), but comprehensive immunohistochemical studies on the expression of the various ULBP are not yet available. Marked expression of ULBP1, ULBP2, and ULBP3 on B cells and, donor-dependently, on monocytes and granulocytes has been reported, but the functional implications remain unclear (Nowbakht *et al*., 2005). Knowledge on other ULBP such as ULBP4 and RAET1G is scarce due to the lack of specific antibodies which impedes further characterization of their expression and function.

In the original description of murine Rae1 genes, Rae1 transcripts were detected in brains of mouse embryos (Nomura *et al*., 1996). Early studies also documented NKG2DL expression on thymocytes and Concanavalin A-activated splenocytes of BALB/c mice (Diefenbach *et al*., 2000). Later, selective induction of Rae-1 on macrophages by TLR-ligands was shown (Hamerman *et al*., 2004). Rae1 and H60 transcripts were also detected in skin following treatment with carcinogens and in papillomas and carcinomas of tumour-bearing mice also linking mouse NKG2DL with tumourigenesis (Girardi *et al*., 2001). Altogether it appears as a common theme that induced expression of NKG2DL alerts the immune system to various forms of 'danger' including malignancy with a subsequent activation of cytotoxic lymphocytes.

## NKG2D in tumour immunosurveillance

Seminal studies by Spies and colleagues revealed expression of MICA/B on many tumour cell lines and tumour tissues (Groh *et al*., 1996, 1999). *In vivo* experiments assessing a functional role of NKG2DL for tumour surveillance demonstrated that ectopic expression of Rae1β and H60 conferred a strong immunostimulatory capacity to otherwise tumour-forming malignant cell lines (Cerwenka *et al*., 2001; Diefenbach *et al*., 2001). Rejection of NKG2DL-expressing tumour cells was due to the activity of NK cells and $CD8^+$ T cells and depending on functional NKG2D (Diefenbach *et al*., 2001; Oppenheim *et al*., 2005; Wiemann *et al*., 2005). One of these studies also showed that implantation of NKG2DL-expressing tumour cells generated memory T cells which protected the host from rechallenge with parental tumour cells (Diefenbach *et al*., 2001). Transgenic mice constitutively and ubiquitously expressing NKG2DL MICA or Rae1ε exhibit a systemic down-regulation of NKG2D and deficiencies in NKG2D-mediated NK cell function (Oppenheim *et al*., 2005; Wiemann *et al*., 2005). NKG2D dysfunction caused by persistent NKG2DL exposure also affected tumour immunity and resulted not only in the failure to reject NKG2DL-expressing RMA cells, but also in a higher incidence of chemically induced tumours (Oppenheim *et al*., 2005; Wiemann *et al*., 2005). Similarly, mice treated with NKG2D-neutralising antibodies exhibited a higher incidence of spontaneous tumours caused by application of sarcoma-inducing MCA (Smyth *et al*., 2004, 2005). Collectively, these studies demonstrated that the NKG2D receptor plays a crucial role in the surveillance of tumourigenesis. In the latter tumour model, NKG2D-mediated tumour protection was mainly operated through perforin-based cytotoxicity, because perforin-deficient mice, in contrast to IFN-γ- and TRAIL-deficient mice, showed no increased tumour incidence when also deficient for NKG2D (Smyth *et al*., 2004, 2005).

In studies addressing the impact of cytokines on NK cell-mediated tumour surveillance, IL-2 and IL-12 promoted perforin-mediated anti-tumour activity of NK cells mainly via the NKG2D pathway. Accordingly, this treatment was more efficiently against tumours expressing higher levels of NKG2DL. In contrast, IL-18 supported anti-tumour activity dependent on FasL expression by tumour cells, independently of NKG2D (Smyth *et al*., 2004).

## Tumour evasion from NK surveillance

Tumour cells employ many different strategies to evade immunosurveillance by NK cells (Zitvogel *et al*., 2006). In some leukaemia cell lines up-regulation of MHC class I expression delivers stronger inhibitory signals to NK cells (Classen *et al*., 2003). Further, leukaemia patients often exhibit abnormal NK cell numbers, phenotypes and function (Pierson and Miller, 1996; Costello *et al*., 2004). For example, NCR expression by NK cells of patients with acute myeloid leukaemia (AML) is often impaired (Costello *et al*., 2002, 2004). A broad spectrum of tumours also reportedly often up-regulates expression of the non-classical MHC class I molecule HLA-G which dampens NK cell responses by engaging inhibitory receptors ILT-2 and KIR2DL4 (Rouas-Freiss *et al*., 2005; Urosevic and Dummer, 2008).

Mouse studies demonstrating that NKG2DL-bearing tumours are readily rejected raise the question why tumours are persisting in humans in spite of NKG2DL expression. One explanation is based on the counterbalance of NK activating signals by strong inhibitory signals, e.g. up-regulation of KIR-engaging MHC class I molecules (Pardoll, 2001). Another rationale comes from the observation that tumour cells release NKG2DL in a soluble form (Groh *et al*., 2002; Salih *et al*., 2002). Many sera of patients with epithelial and hematopoetic malignancies contain elevated levels of soluble MICA and MICB (Groh *et al*., 2002; Salih *et al*., 2002; Wu *et al*., 2004; Holdenrieder *et al*., 2006a, b). Shedding of MIC molecules reduces cell surface levels of NKG2DL which critically affects tumour immunogenicity by abating activating signals for NK cells (Figure 3). Further, soluble MICA (sMICA) has been shown to systemically down-regulate NKG2D, and reduced NKG2D expression on tumour-infiltrating and peripheral blood T and NK cells of tumour patients correlates

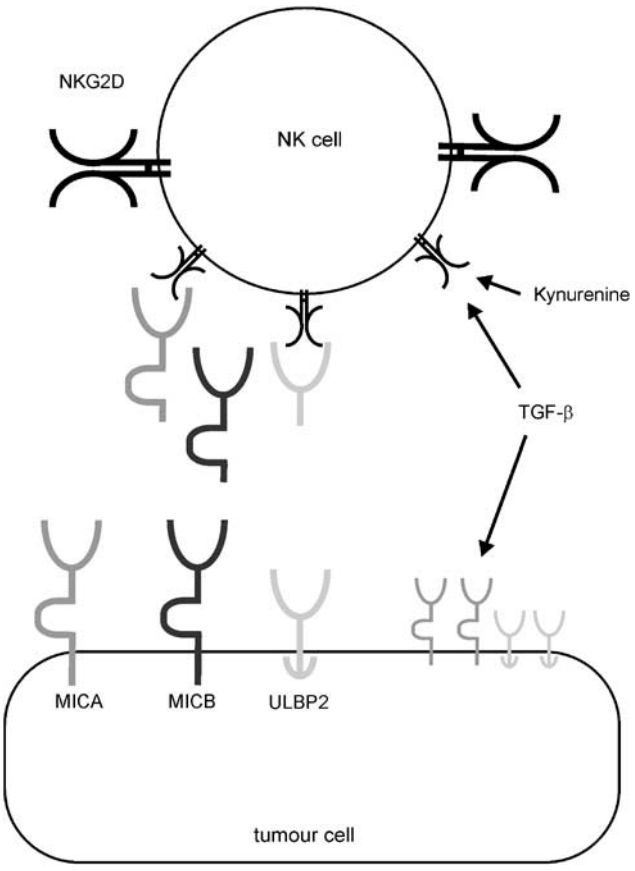


**Figure 3** Subversion of NKG2D-mediated immunosurveillance by tumours. NKG2D on natural killer (NK) cells is down-modulated by tumour-derived transforming growth factor-β (TGF-β), L-kynurenine, and soluble MICA. Surface expression of MICA and ULBP2 is also negatively affected by TGF-β. Overall NKG2DL density on tumour cells is diminished by metalloproteolytic shedding.

with elevated sMICA sera levels (Groh *et al*., 2002; Wu *et al*., 2004). Recently, sMICA was also reported to drive expansion of an immunosuppressive subset of tumour-infiltrating $CD4^+NKG2D^+$ T cells (Groh *et al*., 2006). MICA and MICB are shed from tumour cells by metalloproteases and shedding is dependent on the disulfide-isomerase Erp5 which catalyses a large conformational change in MICA enabling proteolytic cleavage (Salih *et al*., 2002; Kaiser *et al*., 2007). Soluble ULBP2 is also released by tumour cells and has been detected in sera of some patients with haematopoietic malignancies (Waldhauer and Steinle, 2006). Shedding of other ULBP and mouse NKG2DL remains to be addressed. Down-regulation of NKG2D is also mediated by TGF-β, a potent immunosuppressive cytokine (Castriconi *et al*., 2003; Friese *et al*., 2004; Lee *et al*., 2004), and L-kynurenine, a tryptophane catabolite generated by indoleamine 2,3-dioxygenase (IDO) (Della Chiesa *et al*., 2006). In addition, TGF-β also strongly affects expression of NKp30 and of the NKG2DL MICA, ULBP2 and ULBP4 by down-regulation of the respective transcript levels (Castriconi *et al*., 2003; Friese *et al*., 2004; Eisele *et al*., 2006). Interestingly, both TGF-β and L-kynurenine are not only produced by myeloid cells, but also by many tumours and, thus may substantially contribute to suppression of NKG2D-mediated immunosurveillance. Taken together, soluble MIC molecules, IDO and TGF-β appear to represent important players in tumour-mediated immunosuppression and, hence, are attractive targets for future immunotherapeutic strategies.

## Concluding remarks

Intense research efforts in the last 15 years unravelled major molecular mechanisms governing NK cell-mediated anti-tumour reactivity. Tumour-associated expression of ligands of activating receptors, in particular of the NKG2D receptor, opens new avenues for NK-based immunotherapeutical approaches. Characterization of the molecular nature of the tumour-associated NCR ligands and uncovering of major immunosuppressive pathways pursued by tumours to evade NK cell recognition will further aid in this endeavour.

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