Oncogenic stress sensed by the immune system: role of natural killer cell receptors

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Abstract | A growing body of research is addressing how pathways that are dysregulated during tumorigenesis are linked to innate immune responses, which can contribute to immune surveillance of cancer. Components of the innate immune system that are localized in tissues are thought to eliminate early neoplastic cells, thereby preventing or delaying the establishment of advanced tumours. This Review addresses our current understanding of the mechanisms that detect cellular stresses that are associated with tumorigenesis and that culminate in the recognition and, in some cases, the elimination of the tumour cells by natural killer cells and other lymphocytes that express natural killer cell receptors.

p53
A tumour suppressor that is mutated in ∼50% or more of all human cancers. p53 is a transcription factor that is activated by DNA damage, anoxia, expression of certain oncogenes and several other stress stimuli. Target genes activated by p53 regulate cell cycle arrest, apoptosis, cell senescence and DNA repair.

Kaposi's sarcoma
A tumour of endothelial cell origin that is found most frequently in immunosuppressed patients, particularly individuals with HIV. Kaposi's sarcoma-associated herpesvirus has been implicated as a cofactor in the development of Kaposi's sarcoma.

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Cancers arise in a multi-step process that involves the dysregulation of oncogenes, tumour suppressors and pro-apoptotic signals[1]. Genetic and epigenetic alterations lead to changes in cell growth cycles, cell differentiation programmes and cell death pathways[1]. Studies of colon cancer have served as a paradigm for considering the stepwise deviations from normal processes that culminate in metastatic cancer[2] (FIG. 1). Initial genetic changes result in hyperproliferation of previously normal cells, and subsequent oncogene mutations and epigenetic changes lead to the development of increasingly dysplastic pre-cancerous adenomas or other pre-cancerous lesions[2]. These initial changes activate key tumour suppressors, such as p53, which suppress cell cycle progression and may induce cell senescence or apoptosis[2]. Mutations of these tumour suppressor genes, or other genes in the relevant pathways, remove this defence against tumour development and correlate with the appearance of cancer. The accumulation of other mutations can influence angiogenesis, migration and metastasis. Although the order of occurrence of these mutations may vary in different types of cancer or even different instances of the same type of cancer, evidence suggests that homozygous mutations in p53 usually become predominant late in tumorigenesis[2]. This highlights the fact that early events in tumorigenesis activate p53 and create selective pressure for loss of this key tumour suppressor.

In addition to these largely cell-intrinsic defences against tumorigenesis, evidence suggests there are cell-extrinsic barriers to tumour development, some of which are mediated by the immune system. Several cancers are linked to infectious agents, and in some of these cases transformation depends on direct infection of the pre-malignant cell[3]. Examples that are relevant to human disease include cervical carcinoma, some lymphomas and Kaposi's sarcoma. In these instances, the transformed cell may express non-self antigens encoded by the pathogen that can be targeted by B and T cells, in the same way that they might respond during an infection. Other cancers arise by spontaneous genetic and/or epigenetic changes. In these tumours, self antigens are sometimes overexpressed and can activate the adaptive immune system owing to their unnaturally high abundance, which can overcome self tolerance. However, in other cases adaptive immune responses are not readily detected and may not have a major role in tumour suppression[4]. In these instances, tumour suppression may be carried out by the innate immune system. In general, adaptive immune responses are initiated by signals that are associated with inflammation that is caused by innate immune responses[4], and this is probably also true for responses to tumours. Therefore, the initial innate immune response to tumours may be decisive in determining whether immune surveillance is effective. It must be emphasized that many immune responses to tumours are not only non-protective, but paradoxically promote cancer. Important research in this area has been reviewed elsewhere[4,5] and will not be addressed in detail here.

Natural killer (NK) cells are an integral component of the innate immune response to tumours. NK cells are lymphocytes that differ from B and T cells in that they use numerous receptors, none of which is encoded by genes
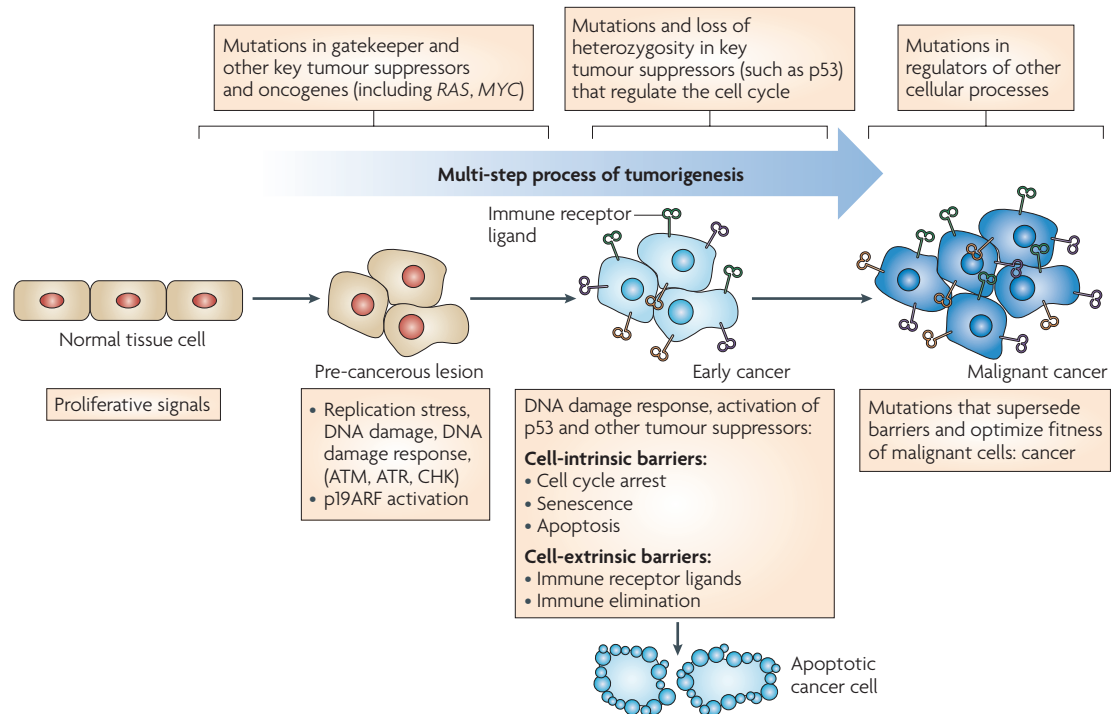
Figure 1 | Stepwise cancer progression and intrinsic and extrinsic barriers to cancer. On the basis of histopathological, clinical and molecular data generated from the analysis of colon carcinomas, it was proposed that cancer generally develops in a stepwise manner as depicted in a "Vogelgram" diagram which is the basis of the figure[2,145]. Although the order of certain specific events probably varies in different instances of cancer, certain early events, including oncogene activation, result in DNA replication stress and DNA damage and therefore activation of the DNA damage response. Oncogene activation leads to the induction of p19ARF expression by a distinct mechanism. Independently of each other, the DNA damage response and activated p19ARF activate key tumour suppressors such as p53. Depending on numerous factors, activated p53 results in cell cycle arrest, cell senescence or apoptosis, all of which are intrinsic barriers to tumorigenesis. The DNA damage response also induces the expression of ligands for the receptor natural killer group 2, member D (NKG2D), and probably other immune receptors, which can activate extrinsic antitumour immune responses. Similarly, cell senescence induced by p53 triggers an immune response that eliminates the senescent cells, although the specific receptors involved in these mechanisms have not yet been defined. Because these barriers typically arise downstream of oncogene activation, selection for homozygous p53 mutations is usually delayed relative to oncogene activation and often correlates with a transition to malignancy. Subsequently, the tumour undergoes additional evolution that optimizes its fitness and capacity to metastasize. ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3 related; CHK, checkpoint kinases.

that undergo rearrangement. Each NK cell expresses several stimulatory and inhibitory NK cell receptors that can function with some independence, enabling NK cells to separately target cells that increase or decrease their expression of various ligands[6]. Many of the NK cell inhibitory receptors are specific for MHC class I molecules, which are expressed by normal cells but are often lost from infected cells or tumour cells[7]. Ligands for NK cell stimulatory receptors are usually poorly expressed by healthy cells but are upregulated by 'unhealthy' cells, such as transformed, infected or stressed cells[6,7]. NK cell activation is controlled by the balance of stimulatory and inhibitory signalling incurred when target cell engagement occurs and the various receptors engage their ligands[6–8]. Hence, some normal cells display stimulatory ligands but fail to be killed by NK cells because their MHC class I molecules engage inhibitory receptors that counteract stimulatory signalling. Increased expression of stimulatory ligands by a target cell can overcome inhibitory signalling in the NK cell, resulting in target cell lysis. Loss of inhibitory MHC

ligands can also result in target cell lysis. Both occur in the context of disease, sometimes in the same cell[8]. In addition to their role in NK cells, some stimulatory NK cell receptors are also commonly expressed by subsets of T cells, in which they are thought to provide an innate signal that enhances T cell activation[9].

Recent evidence suggests that the host immune response to tumour cells is in some cases linked to specific events that are associated with cellular transformation and tumorigenesis. In this Review, we focus on the mechanisms that link oncogenic stress to innate immune stimuli, in particular the stimuli that act through NK cell receptors and the cells that express them.

Immune surveillance of primary tumours
Immune surveillance of cancer is a concept that was discredited for some time, but has received strong experimental support in the past 10 years (TABLE 1). Before reviewing the evidence, it is useful to summarize the experimental systems used in such studies.

Table 1 | **Immune deficiencies associated with greater tumour incidence or severity in mice**

Mouse strain	System to promote tumorigenesis	Type of tumour	Defective immune component	Refs
Spontaneous tumours				
129/Sv	None	Colon and lung	RAG2	10
129/Sv	None	Colon and mammary	RAG2 and STAT1	10
C57BL/6 and BALB/c	None	B cell lymphoma	β_2-microglobulin and perforin	11,14
C57BL/6	None	Lymphoma	TRAIL	13
C57BL/6	None	Lymphoma	Perforin	12
Transgenic and knockout cancer models				
129/Sv	$Tp53^{-/-}$	Lymphoid and other	STAT1	22
129/Sv	$Tp53^{-/-}$	Lymphoid and other	IFNγR	22
C57BL/6	$Tp53^{+/-}$	Lymphoid and other	TRAIL	13
C57BL/6	$Tp53^{+/-}$	Lymphoid	Perforin*	12
C57BL/6	$Tp53^{+/-}$	Lymphoid and other	TCRJα28 and CD1d	23
C57BL/6	TRAMP	Prostate	NKG2D	18
C57BL/6	TRAMP	Prostate	TCRδ	17
C57BL/6	Eμ–*Myc*	B cell lymphoma	NKG2D	18
C57BL/6	Eμ–*Myc*	B cell lymphoma	TRAILR	28
C57BL/6	Eμ–*Myc*	B cell lymphoma	RAG1	19
Carcinogen-induced tumours				
129/Sv	MCA	Fibrosarcoma	RAG2‡	10
129/Sv	MCA	Fibrosarcoma	IFNγR	10,22
129/Sv	MCA	Fibrosarcoma	IFNγR	27
129/Sv	MCA	Fibrosarcoma	STAT1	10,22
129/Sv	MCA	Fibrosarcoma	RAG2 and STAT1	10
C57BL/6	MCA	Fibrosarcoma	IFNγ	26,31
C57BL/6	MCA	Fibrosarcoma	Perforin§	25,26
C57BL/6	MCA	Fibrosarcoma	TRAIL	29
C57BL/6	DEN	Hepatocarcinoma	TRAILR	28
C57BL/6	MCA	Fibrosarcoma	TCRJα28	26
FVB	MCA	Fibrosarcoma	TCRβ	30
FVB	MCA	Fibrosarcoma	TCRδ	30
FVB	DMBA and TPA	Cutaneous	TCRδ	30,35
C57BL/6	MCA	Fibrosarcoma	TCRδ	31

*Contrary results have been observed in a mixed 129/Sv x C57BL/6 genetic background[27]. ‡Contrary results have been observed in RAG1-deficient C57BL/6 mice[24]. §Contrary results have been observed[24]. DEN, diethylnitrosamine; DMBA, dimethylbenz(a)anthracene; IFNγR, interferon-γ receptor; MCA, methylcolanthrene; NKG2D, natural killer group 2, member D; RAG, recombination-activating gene; STAT1, signal transducer and activator of transcription 1; TCR, T cell receptor; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; TRAILR, TNF-related apoptosis-inducing ligand receptor; TRAMP, transgenic adenocarcinoma of the mouse prostate.

Many past studies of the immune system's role in cancer have relied on tumour transplant models, typically using tumour cell lines that are implanted subcutaneously in recipient animals. Although this approach is useful, it is flawed in that implanted tumours develop from cell lines that clearly escaped immune surveillance in the animal from which they were derived and can also be aberrant owing to prolonged culturing. Furthermore, they are usually implanted in ectopic sites in large numbers and fail to recapitulate the earliest stages of transformation and tumorigenesis. As a result, the physiological relevance of observations showing immune destruction of implanted tumours is often questionable. Alternative approaches include studies of fully spontaneous cancer, cancer induced by transgenic expression of oncogenes and carcinogen-induced cancer (TABLE 1). These approaches are considered more reliable for identifying the role of the immune system because the tumours arise in the normal tissue site, typically from a single cell, and proceed through the various

stages of tumour development. Nevertheless, they vary in their ease of use and have other shortcomings, as summarized below.

Fully spontaneous tumorigenesis. Cancer that develops in normal untreated mice is presumably the most reliable mouse model of human cancer and has been studied in some analyses of immune surveillance. However, these studies have limitations because of the low incidence of fully spontaneous cancers in most mouse strains and the heterogeneity of cancers that arise.

Despite these difficulties, a key report showed increased incidence of spontaneous lung and intestinal carcinomas in 129/Sv mice that lacked all B and T cells owing to mutations in recombination-activating gene 2 (*Rag2*)[10]. Interestingly, mammary carcinomas were not detected in RAG2-deficient mice, but arose in mice deficient in both RAG2 and signal transducer and activator of transcription 1 (STAT1), a component of the interferon (IFN) receptor signalling complex, suggesting a role for innate immune responses in the control of mammary tumours. A general and robust role for perforin in immune surveillance of cancer was shown by the deletion of the gene encoding perforin in two mouse strains[11,12,14]. TNF-related apoptosis-inducing ligand (TRAIL; also known as TNFRSF10), another mediator of cytolysis, has also been implicated in responses against cancer[13]. These studies suggest a role for both the innate and adaptive immune responses in the control of fully spontaneous tumours.

Transgenic models of spontaneous cancer. Genetically engineered mice that overexpress oncogenes or have altered tumour suppressor function develop spontaneous cancers that mimic the development of natural malignancies in most respects. The tumours in these mice arise and progress autochtonously (at their natural sites), including during the earliest stages, and are likely to reflect the natural interactions between the tumour and the immune system in initially normal tissues. In most of these models, most or all animals develop tumours of the same type, and in some cases tumours develop with predictable kinetics, allowing systematic analysis of the various stages of cancer. A drawback of some of these models is their high penetrance, such that tumours are repeatedly initiated in the same host and can overwhelm the immune response. A few relevant models will be summarized here.

In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, the rat probasin promoter directs the expression of the early genes (small t and large T antigens) of simian virus 40 (SV40) by the prostate epithelium of adult mice. Prostate tumours developing in these mice are infiltrated by leukocytes, including CD8+ T cells that are specific for tumour-associated antigens[15,16]. Other cell types that infiltrate the tumours include NK cells, γδ T cells and natural killer T (NKT) cells (P. Savage, personal communication). One study recently showed that γδ T cells could suppress high-grade prostate tumours in the TRAMP model[17], and another recent study showed that the immunoreceptor NK group 2, member D (NKG2D)

contributed to the control of the high-grade, aggressive form of carcinomas that develop in some of these mice[18]. As discussed later, NKG2D is a stimulatory receptor expressed by NK cells and some T cells, suggesting that one or both of these cell types is involved in the control of aggressive prostate adenocarcinomas.

The Eµ–*Myc* transgene model consists of the *Myc* oncogene expressed under the control of the Eµ immunoglobulin heavy chain enhancer and the *Myc* promoter. Eµ–*Myc* transgenic mice that also lack B and T cells owing to a mutation in *Rag1* succumb to pre-B cell lymphomas a few weeks earlier than their *Rag1*-sufficient littermates, suggesting that mature B or T cells may limit the development of these tumours[19]. Furthermore, lymphomagenesis was also accelerated in Eµ–*Myc* mice that lacked the gene encoding NKG2D, which suggests that NKG2D has a role in promoting either NK cell-dependent or T cell-dependent elimination of lymphomas[18]. All Eµ–*Myc* transgenic mice eventually develop lymphomas, perhaps because the initiation of new tumours finally overwhelms immune system control[18,20].

Mutations of the gene encoding the tumour suppressor p53 (*Tp53*) occur spontaneously in most naturally arising cancers. Deficiency in p53 removes an important barrier to tumorigenesis and contributes to genomic instability[21]. Mice with homozygous germline mutations in *Tp53* (*Tp53*−/− mice) develop mainly lymphomas of thymic origin, whereas *Tp53*+/− mice develop both disseminated lymphomas and non-lymphoid tumours, mainly sarcomas. Studies show that tumour incidence is higher in p53-deficient mice that are also deficient in IFNγ, the IFNγ receptor and/or STAT1 (REF. 22). IFNγ-insensitive *Tp53*−/− mice developed a broader range of tumours compared with mice lacking p53 alone[22]. In addition, invariant NKT cells, the classical subset of NKT cells, have been shown to contribute to the control of tumour development in *Tp53*+/− mice[23]; both perforin- and TRAIL-mediated apoptotic pathways participate in this response[12,13].

Carcinogen models. Tumours arising as a result of chemical carcinogenesis develop autochtonously and proceed through the stages of spontaneous tumorigenesis. Carcinogen-based models, although useful, have been criticized because the potent chemicals may induce local tissue inflammation that could alter experimental outcomes[24]. However, an advantage of these models compared with some transgenic models is that the carcinogen can be titrated so that tumour incidence is limiting.

Among the most commonly studied models of mouse tumorigenesis is the induction of fibrosarcomas in mice treated subcutaneously or intradermally with the carcinogen methylcholanthrene (MCA). Genetic studies showed an increased incidence of MCA-induced fibrosarcomas in mice with defects in perforin[25,26], the IFNγ signalling pathway[10,22,27] or the TRAIL-mediated cytotoxicity pathway[28,29]. Both αβ and γδ T cells were implicated in the killing of MCA-induced fibrosarcomas[30,31]. Among cells with an αβ T cell receptor, NKT cells[32] and possibly CD4+ T cells[10] were found to be most important. In addition, NK cells have a crucial role as direct effectors

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of tumour cell lysis and possibly as collaborators with invariant NKT cells in the control of these tumours[33,34]. By contrast, CD8-deficient mice exhibited no defect in the control of MCA-induced tumours[25].

Another widely used carcinogen model is the induction of skin cancer involving the tumour initiator and promoter combination dimethylbenz(a)anthracene (DMBA) and 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). Mice deficient in skin-associated γδ T cells had a higher incidence of DMBA–TPA-induced tumours[30]. By contrast, CD8+ T cells could promote tumorigenesis in this model under some conditions[35].

Receptors mediating immune surveillance

As reviewed elsewhere[36], evidence supports a role for the adaptive immune system in immune surveillance, indicating a role for B or T cell receptors. Here we focus on receptors used by NK cells and in some cases shared by T cells that have defined antigen specificities and have been implicated in tumour surveillance. Among these receptors are NKG2D, NKp30, NKp44, NKp46, NKp80, 2B4 and DNAX accessory molecule 1 (DNAM1; also known as CD226). Several of the receptors seem to mediate 'induced self recognition'; that is, the ligands are encoded by the host's genome, are poorly expressed by normal cells and are upregulated by stressed or diseased cells[8]. This phenomenon is best characterized in the case of the NKG2D receptor. For this reason, and because its role in tumour surveillance has been examined extensively, we focus our discussion on NKG2D and its ligands, after introducing some of the other key NK cell receptors.

NKp30, NKp44, NKp46 and NKp80. These four stimulatory receptors are collectively called natural cytotoxicity receptors (NCRs)[37–40], although NKG2D and other receptors also have important roles in the recognition and cytotoxicity of tumour cells by NK cells, as discussed below. The four receptors have been well studied in human NK cells, but only one of them, NKp46, has been characterized in mice. In humans, NKp30, NKp46 and NKp80 are expressed by all NK cells, whereas NKp44 is only expressed by activated NK cells[41,42]. Blocking one or more of these receptors with antibodies often inhibits killing of tumour cell lines by human NK cells *in vitro*[37–39].

Furthermore, mice with a targeted mutation in the gene encoding NKp46 were impaired in their ability to reject a transferred lymphoma cell line that expressed ligands for that receptor[43].

A surprising diversity of unrelated ligands has been reported for NCRs, including viral haemagglutinins (for NKp46 and NKp44)[44,45], heparan sulphate proteoglycans (for NKp30 and NKp46)[46], the nuclear factor HLA-B-associated transcript 3 (for NKp30)[47] and activation-induced C-type lectin (for NKp80)[48]. Additional studies will be needed to determine the importance of these various ligands for NCRs.

2B4. 2B4 is a member of the signalling lymphocyte activation molecule (SLAM)-related family of receptors and is expressed by all NK cells, γδ T cells, a subset of CD8+ T cells and all human CD14+ monocytes[49]. The unique ligand for 2B4 is CD48 (also a SLAM-related receptor), which is expressed by all haematopoietic cells. 2B4 can function as either a stimulatory or inhibitory receptor depending on the splice isoform that is expressed, the identity of the signalling adaptor molecule it associates with and the extent of cross-linking of the receptor[50–52]. 2B4 signalling has been shown to have a role in rejecting tumours that express CD48 (REF. 53).

DNAM1. DNAM1 is an adhesion molecule that is constitutively expressed by most NK cells, T cells, macrophages and dendritic cells[54,55]. Ligands for DNAM1 include CD112 (also known as nectin 2 and PVRL2) and CD155 (also known as PVR)[56,57]. These ligands are often expressed by tumour cells and can activate or enhance tumour cell lysis *in vitro*[58,59]. Recent studies showed that DNAM1-deficient mice have reduced capacity to reject certain tumour cells and to limit the formation of carcinogen-induced tumours *in vivo*[34,60].

NKG2D. NKG2D is a lectin-like type II transmembrane homodimer that has received considerable attention owing to evidence of its role in immune responses in the context of cancer, infection and autoimmunity. NKG2D is expressed by virtually all NK cells and activated CD8+ T cells, and subsets of γδ T cells and NKT cells[61,62]. In certain conditions, NKG2D is also expressed by human CD4+ T cells[63–67].

Numerous different NKG2D ligands have been identified, all of which are related self proteins that are similar to MHC class I molecules (BOX 1). However, normal cells typically do not express the ligands at substantial levels, whereas they are often specifically upregulated in cancerous or stressed tissues (TABLE 2).

Evidence has accumulated showing that NKG2D has an important role in the immune surveillance of tumours (FIG. 2). NKG2D-dependent elimination of tumour cells that express NKG2D ligands has been well documented *in vitro*[39,62,68,69] and *in vivo* in tumour transplant experiments[70,71]. In humans, specific NKG2D gene polymorphisms have been associated with susceptibility to cancer[72].

The most direct evidence supporting a role for NKG2D in tumour surveillance came from analysis

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Box 1 | The ligands for NKG2D

Natural killer group 2, member D (NKG2D) ligands are self proteins that are related to MHC class I molecules, although they differ in that they do not present molecular cargo and fail to bind β2-microglobulin[133]. The ligands include MHC class I polypeptide-related sequence A (MICA) and MICB in humans[62], which have no mouse homologues, and the cytomegalovirus UL16-binding protein (ULBP) or retinoic acid early transcript 1 (RAET1) ligand families, which exist in both humans and mice[134–137]. Each human or mouse strain can express approximately 5–10 different NKG2D ligands. Most normal cells, however, do not express substantial levels of NKG2D ligands on the cell surface. By contrast, most tumour cell lines express one or more NKG2D ligand[39,62,135,136]. Furthermore, many primary human tumours express NKG2D ligands[117,138,139]. Similarly, expression of the NKG2D ligands RAET1 or murine ULBP-like transcript 1 (MULT1) was observed on primary lymphomas generated in Eμ–*Myc* mice[18,140] and on primary adenocarcinomas generated in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice[18]. Ligand expression is also induced in cells that are infected with certain pathogens[141].

Table 2 | **Induction of NKG2D ligands by stress pathways**

Level of regulation	Underlying pathway	Ligands regulated	Regulatory components	Refs
mRNA (transcription or mRNA stabilization)	Heat shock response	MICA and MICB (in humans)	Heat shock transcription factors	96
mRNA (transcription or mRNA stabilization)	DNA damage response	ULBP, MICA (in humans); RAET1, MULT1 and H60a (in mice)	ATR-, ATM- and CHK-dependent; p53 not required	90
mRNA (transcription or mRNA stabilization)	Cell senescence	MICA, MICB and ULBP2 (in humans)	ATR- and ATM-dependent in some cases	57,93
mRNA (transcription or mRNA stabilization)	Wounding	H60c (in mice)	ND	105
Protein stabilization	Heat shock response; UV irradiation	MULT1 (in mice)	Independent of DNA damage response	97

ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3 related; CHK, checkpoint kinase; H60, histocompatibility 60; MICA, MHC class I polypeptide-related sequence A; MULT1, murine ULBP-like transcript 1; ND, not determined; RAET1, retinoic acid early transcript 1; ULBP, cytomegalovirus UL16-binding protein; UV, ultraviolet.

of tumour incidence in gene-targeted mice that lack NKG2D and carry transgenes that increase the incidence of specific cancers[18]. In the TRAMP mouse model, the incidence of a highly aggressive form of prostate adenocarcinoma was markedly increased when the mice were also deficient for NKG2D[18]. Similarly, in mice carrying the Eµ–*Myc* transgene that causes B cell lymphoma, onset of lymphoma was accelerated by 7 weeks if the mice were also deficient for NKG2D[18]. It has not yet been established whether NKG2D-dependent control of tumours in these models is mediated by NK cells or one or more type of NKG2D-expressing T cell, or by both NK cells and T cells.

The involvement of NKG2D in tumour surveillance in the TRAMP mouse model was also suggested by the finding that many of the aggressive adenocarcinomas that arose in NKG2D-deficient mice expressed one or more of the NKG2D ligands, whereas similar tumours that arose in NKG2D-sufficient mice generally lacked expression of NKG2D ligands. A probable explanation is that aggressive prostate adenocarcinoma tumours that arose in NKG2D-sufficient mice were subjected to NKG2D-mediated immune surveillance, resulting in selection for variant tumour cells that had lost expression of the ligands (FIG. 2). In contrast to this pattern in TRAMP mice, the B cell lymphomas that arose in Eµ–*Myc* mice commonly expressed NKG2D ligands regardless of whether the mice were NKG2D deficient. These data suggested that some tumours can escape cytotoxicity mediated by NKG2D-expressing cells despite continued expression of NKG2D ligands. This is consistent with the observation that many primary tumours in normal animals or humans express NKG2D ligands.

A role for NKG2D in the surveillance of skin cancer was suggested by the increased expression of transcripts for NKG2D ligands in carcinogen-treated skin samples[30]. Interestingly, transgenic mice that constitutively expressed high levels of NKG2D ligands, which results in dampened NKG2D function, showed an increased incidence of carcinogen-induced cutaneous malignancies[73]. However, these data did not definitely implicate NKG2D in cutaneous immune surveillance because the NK cells in these transgenic mice were defective at

eliminating tumour cells that lacked NKG2D ligands in addition to those cells that expressed NKG2D ligands.

NKG2D deficiency did not result in the increased incidence or severity of some types of cancer, including a late arising, less malignant form of adenocarcinoma in TRAMP mice or fibrosarcomas induced by MCA[18]. The observation that NKG2D deficiency did not result in more MCA-induced fibrosarcomas was surprising in light of the contrasting findings of an earlier study in which MCA-treated mice were repeatedly injected with an NKG2D-specific antibody to block the receptor[74]. It is possible that sustained engagement of NKG2D by antibodies impairs both NKG2D-dependent and NKG2D-independent NK cell functions, as has been reported for sustained engagement of NKG2D by its ligands[73,75–77]. In any case, the observation that the incidence or severity of tumours was unaffected by NKG2D deficiency in some models suggests that these types of tumour readily evade NKG2D-dependent immune surveillance, fail to express NKG2D ligands at a sufficiently early stage or are otherwise sequestered or insensitive to immune destruction. Possible mechanisms of immune evasion include upregulation of inhibitory MHC class I molecules to counteract the higher levels of stimulatory ligands and/or loss of distinct stimulatory or adhesion ligands by the tumour cells. By contrast, the receptor DNAM1 has been implicated in immune surveillance of MCA-induced sarcomas, suggesting that its action is not readily evaded in this system[34].

Considering the capacity for cancers to evolve in the host and the heterogeneity of oncogenic mechanisms that operate in different cancer models, it is not surprising that the NKG2D system, or indeed any other system, is ineffective at impeding disease in some cancer models. A detailed explanation of why NKG2D is ineffective in these specific models has not yet been documented, but it is notable that in some systems NKG2D ligands are expressed on the surface of tumour cells but fail to promote tumour rejection, whereas in other cases NKG2D ligands are lost from the tumour cells. In the first case it is probable that the response of NKG2D+ lymphocytes is suppressed or avoided, whereas in the second case tumour variants that lack expression of ligands may arise
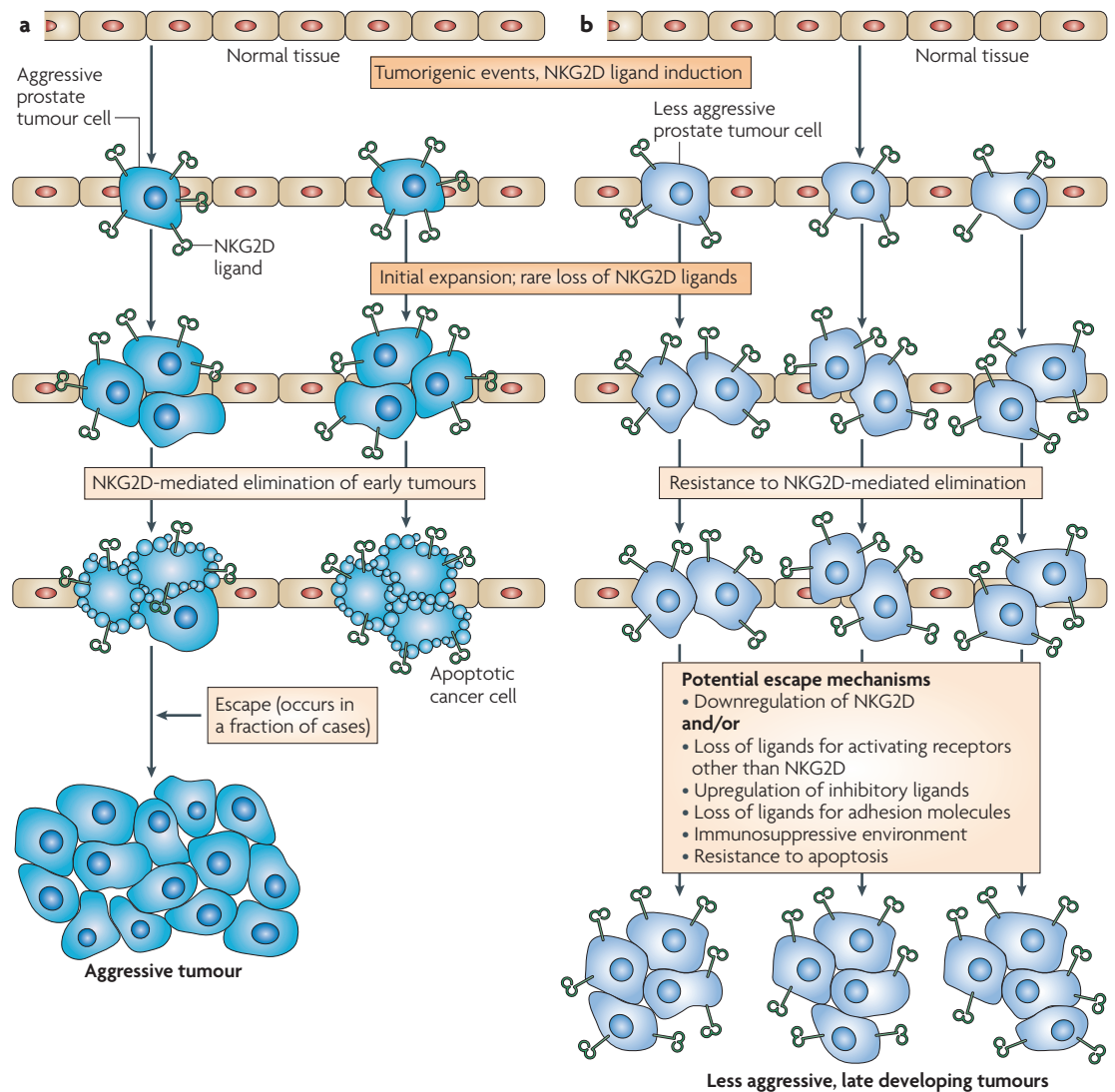
Figure 2 | **Model of NKG2D-mediated tumour surveillance of prostate adenocarcinoma.** In C57BL/6 transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, immune responses depending on natural killer group 2, member D (NKG2D) limit the development of an early highly malignant form of prostate adenocarcinoma, but not a late developing, less malignant form. **a** | The highly malignant tumours in NKG2D-deficient TRAMP mice generally express NKG2D ligands, whereas the rarer tumours of this type in NKG2D-sufficient mice generally lack NKG2D ligands, suggesting that the immune response modifies these tumours by selecting for variant tumour cells that fail to express NKG2D ligands. **b** | By contrast, the less aggressive, late developing tumours express NKG2D ligands (albeit heterogeneously) regardless of whether NKG2D is expressed, suggesting that these tumours evade NKG2D-dependent elimination by a distinct mechanism.

readily. Why some tumours may lose ligand expression more readily than others is unknown, but this could be related to the fact that the milieu of activated oncogenes, mutated tumour suppressors and other mediators varies in tumours of different origins.

Cancer-associated immune activation

Tumorigenesis is a complex process involving the dysregulation of many cellular pathways, in many cases resulting from mutations in oncogenes and tumour suppressor genes. Recognition of cancer cells by innate immune cells depends on their ability to distinguish these dysregulated cells from normal cells. Many of the distinctive features of

cancer cells, such as proliferation, invasiveness and repression of cell death pathways are features that can be exhibited by normal cells in other contexts. Other features are more specific to diseased cells, although untransformed stressed cells may also be similarly affected. For a host defence system to be effective against cancer, the mechanism in the cancer cell that alerts the immune system most probably involves signalling pathways that process several types of information that collectively, but not individually, identify the cell as a cancer cell.

There are numerous features that distinguish cancer cells from most normal cells. To mention only a few, mutations that activate oncogenes probably occur early

Box 2 | DNA damage response

The 'DNA damage response' protects the genome by facilitating the repair of minor DNA damage in cells and functions as a key barrier to tumorigenesis (see also FIG. 1). Rapid DNA replication in the context of oncogene activation is thought to result in the disruption of DNA replication forks (replication stress) and accompanying DNA breaks, both of which can trigger the DNA damage response by activating the protein kinases ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR), which are key sensors in the DNA damage response pathway[142]. ATM and ATR initiate a cascade that ultimately induces cell cycle arrest and DNA repair functions. Depending on the cell type and other factors, prolonged or severe activation of the DNA damage response results in the activation of apoptotic or cell senescence programmes. The DNA damage response therefore helps to preserve the integrity of the genome and eliminate severely damaged cells.

Unfolded protein response
A response that increases the ability of the endoplasmic reticulum to fold and translocate proteins, decreases the synthesis of proteins and causes cell cycle arrest and apoptosis.

Ataxia telangiectasia
(also known as Louis–Bar syndrome). A familial recessive disease that is characterized by progressive cerebellar ataxia, oculocutaneous telangiectases and susceptibility to pulmonary infections. It is caused by germline mutations in ataxia telangiectasia mutated (ATM), which encodes a sensor that activates the DNA damage response.

in the development of most tumours and provide persistent proliferative signals. Oncogene-induced signals activate tumour suppressors by at least two mechanisms, one involving p19ARF (encoded by cyclin-dependent kinase inhibitor 2A (CDKN2A)) and the other involving the 'DNA damage response', which is activated after DNA damage is sensed by the protein kinases ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR) (BOX 2). Activated p19ARF and the DNA damage response can each independently induce the expression of p53 and other mediators that arrest the cell cycle and can lead to cell senescence (BOX 3) or apoptosis when persistently activated[78,79].

Mutations in tumour suppressor genes, or in components of the pathways that activate tumour suppressors, enable continued cell proliferation of nascent tumour cells but dysregulate DNA replication and repair in a manner that ultimately results in instability of the genome and the accumulation of chromosomal abnormalities[80,81]. Hence, at early stages of tumorigenesis, cells may have early warning signs such as activation of p19ARF, the DNA damage response and tumour suppressors, and activation of the gene programme that is associated with cell senescence. At late stages the cells have other defects such as genomic instability. Furthermore, certain other stress pathways are commonly activated in cancer cells, including the heat shock response[82] and the unfolded protein response[83]. The roles of some of these pathways as warning signals that trigger anticancer immune responses are discussed below.

DNA damage response. The role of the DNA damage response as an important barrier to tumorigenesis was highlighted by studies of human tissues showing that early pre-neoplastic lesions in the breasts, lungs, bladder and colon of patients have chronic activation of the DNA damage response, manifested by the phosphorylation of ATM, checkpoint kinase 2 homologue (CHK2) and histone γ-H2AX, another marker of DNA damage response activation[84,85] (FIG. 1). Other studies showed that transgenic expression of the proto-oncogene *Myc* in mice leads to DNA damage, consequent activation of ATM and p53 and apoptosis of affected cells *in vivo*[86,87]. DNA damage induced by MYC may result from the production of increased reactive oxygen species, independently of cell cycle entry, and from the induction of rapid

DNA replication[88]. ATM activation under these conditions obstructs tumorigenesis, as deficiency of ATM increases MYC-induced tumorigenesis in mice[86,87]. These data are consistent with the fact that mutations in *ATM* in humans, which cause the syndrome ataxia telangiectasia when present in the homozygous state, increase the incidence of lymphoma, leukaemia and breast cancer[89].

A link between the DNA damage response and antitumour immune responses was initially established in studies of NKG2D ligands[90] (TABLE 2). Genotoxic stress that activates ATM or ATR can induce the expression of NKG2D ligands (retinoic acid early transcript 1 (RAET1), murine ULBP-like transcript 1 (MULT1), histocompatibility 60a (H60a), MHC class I polypeptide-related sequence A (MICA) and cytomegalovirus UL16-binding proteins (ULBPs)) on the surface of relatively normal cultured cells, including fibroblast cell cultures[8,90]. Induction of NKG2D ligand expression was prevented by inhibiting or knocking down the expression of ATR or ATM, depending on the nature of the genotoxic stress[8,90]. Most established tumour cell lines express NKG2D ligands constitutively, and knockdown studies established that constitutive ATM or ATR activation had an important role in maintaining constitutive ligand expression by those cells[8,90]. These studies indicated that the display of NKG2D ligands on tumour cells is mediated in part by an activated DNA damage response. This upregulation of NKG2D ligand expression is accompanied by increased levels of the corresponding mRNA transcripts, but it has not been determined whether this is due to increased transcription or alterations in mRNA processing.

The DNA damage response also has a role in inducing the expression of ligands for a distinct NK cell receptor, as indicated by a study showing that the DNAM1 ligand CD155 was induced by DNA-damaging drugs on the surface of multiple myeloma cells in an ATM- and ATR-dependent manner[57]. Finally, the DNA damage response directly regulates the expression of death receptor 5 (DR5), a ligand for TRAIL[91]. Engagement of DR5 by TRAIL induces apoptosis, and evidence indicates that TRAIL is expressed by NK cells and T cells and functions as an important effector molecule in tumour surveillance by these cells[28,92].

Box 3 | Cell senescence

Cell senescence, in which tumour cells can survive for a time in an irreversibly senescent state, is associated with a blockade in cell proliferation and an induction of a specific programme of gene expression that results in the secretion of several pro-inflammatory cytokines and chemokines. Paradoxically, many of these modulators promote tumour cell growth, and studies suggest that the senescent state can, in some cases, promote tumorigenesis[143]. However, abundant evidence has accumulated that suggests senescence restricts tumorigenesis[78,79,144] by inhibiting tumour cell growth, inducing cell death and activating immune responses that help to eliminate cancer cells.

Cell senescence. Cell senescence has been linked to immune-mediated tumour elimination mechanisms by a study of liver tumours generated from transformed cells that initially lacked p53 expression. When p53 expression was subsequently switched on, the tumours underwent growth arrest, exhibited features of cell senescence and were gradually eliminated by NK cells and other infiltrating cells[78]. These studies suggested that cell senescence associated with p53 activation in tumour cells is in some cases connected with the activation of immune responses that destroy cancer cells or their pre-malignant counterparts.

The details of the way the senescence programme is linked to anticancer immune responses have not yet been determined, but studies suggest that numerous ligands that stimulate immune responses are upregulated in senescent cells. These include intercellular adhesion molecule 1 (ICAM1), a ligand for lymphocyte function-associated antigen 1 (LFA1), which has an important role in NK cell activation, NKG2D ligands such as MICA and ULBP2, and CD155 (REFS 78,93) (TABLE 2). As already noted above, the expression of some of these ligands is induced by the DNA damage response. So, it remains unclear whether the induction of these immune-stimulating ligands is related to the senescence programme itself, which normally takes several days to establish, or the DNA damage response, which may be involved in establishing the senescence programme. Notably, the induction of NKG2D ligand expression in cultured cells by DNA-damaging agents did not require p53 (REF. 90), whereas tumour senescence and NK cell-dependent elimination of senescent tumour cells was induced by p53 reactivation *in vivo*[78]. Therefore, it seems probable that both p53-dependent and p53-independent processes linked to tumorigenesis might regulate the sensitivity of tumour cells to NK cell-mediated tumour elimination *in vivo*.

The heat shock response. It has long been known that the heat shock response is activated in many forms of cancer[82]. Mice deficient in heat shock transcription factor 1 (HSF1), which functions as a key inducer of the heat shock response, were less susceptible to cancer than wild-type mice in experimental models, suggesting that the heat shock response is 'hijacked' by tumours to enhance their survival[94]. Mounting an immune response against cells expressing heat shock proteins could therefore target a response that is otherwise beneficial to tumours, thus limiting tumorigenesis.

Studies in cultured human epithelial cells implicated the heat shock response in transcriptional activation of the human *MICA* and *MICB* genes[95] (TABLE 2). The promoter regions of both genes contain HSF1 binding elements, which were necessary for transcriptional activation[96]. Distinct elements in the promoters were required to support transcription in virus-infected cells or proliferating cells, suggesting that there was some independence in the control of *MICA* and *MICB* transcription under different conditions.

So far, heat shock-induced transcriptional activation has not been observed in the case of other NKG2D ligand families, including human ULBP and RAET1 proteins

and mouse RAET1, MULT1 and H60 proteins. In the case of MULT1, however, the heat shock response has an important role in regulating cell surface expression of the protein at a post-transcriptional step (TABLE 2). In fibroblasts and other cells, the cytoplasmic tail of MULT1 is subject to ubiquitylation, which targets the protein for destruction in lysosomes[97]. Exposure of these cells to heat shock reversed the ubiquitin-dependent destruction of MULT1, resulting in a marked increase in protein levels at the cell surface. Interestingly, ultraviolet (UV) irradiation of fibroblasts had the same effect[97]. Although UV irradiation induces the DNA damage response, the stabilization of MULT1 protein by UV irradiation was independent of the DNA damage response. UV irradiation alters several other pathways in cells, and it is possible that its effects on MULT1 stabilization are exerted through a pathway that overlaps with the heat shock response.

MULT1 expression provides an example of combinatorial regulation by distinct cancer-associated stress pathways, the DNA damage response[90] and the heat shock response[97]. Coupling MULT1 expression with multiple stress pathways, in this case operating at different stages of biogenesis of the molecule, may represent a paradigm for mechanisms that restrict the display of immune-activating ligands to seriously diseased cells.

Other mechanisms that regulate NKG2D ligands. Evidence suggests there are additional modes of regulation of stimulatory ligands that activate NK cells. In F9 embryocarcinoma cells, the transcription of *Raet1* genes was induced by retinoic acid[98], which was the basis for the first identification of these genes. Retinoids exert growth suppressive effects on normal cells and tumour cells and have been considered promising agents for cancer therapy[99]. However, the role of retinoids in regulating NKG2D ligands remains unclear. The transcription of *Raet1e* was inhibited by the transcription factor JUNB in cell lines and mouse tissues[100]. This finding is of particular interest in light of the evidence that Jun family transcription factors show complex regulation in conditions of stress and injury[101].

NKG2D is expressed by all γδ T cells that reside in the mouse epidermis[68]. These T cells participate in the destruction of cutaneous malignancies and in wound healing[102,103]. The NKG2D ligand H60c is expressed selectively in the epidermis[104,105]. Engagement of H60c displayed on keratinocytes by NKG2D on epidermal γδ T cells is essential for triggering γδ T cell activation and lysis of the keratinocytes, suggesting that NKG2D functions as a key co-stimulatory receptor in the activation of epidermal γδ T cells[105]. Interestingly, expression of H60c is upregulated in wounded skin, and cultured keratinocytes upregulate H60c but not other NKG2D ligands, suggesting that H60c may be the main NKG2D ligand that is involved in cutaneous immune surveillance of tumours[105] (TABLE 2). The mechanisms that upregulate H60c expression in diseased and stressed tissues are not yet known. Although human and mouse cutaneous T cells differ in many respects, it is interesting that one of the human NKG2D ligands, ULBP4, is also selectively expressed in human skin[106].

Regulation of NKG2D ligands by microRNAs was reported for MICA and MICB. In human cell lines, microRNAs that target the 3′ untranslated regions of the *MICA* and *MICB* mRNA transcripts inhibited steady state MICA and MICB expression[107]. Exposure of the cells to stress amplified MICA and MICB expression to an extent that could overcome the inhibition that was imposed by the microRNAs. Whether these microRNAs are themselves regulated in normal tissues by stress remains to be established. However, it was of interest that certain tumours overexpressed the microRNAs, which may serve as a mechanism to evade immunosurveillance[107].

An additional, important mechanism of regulation of NKG2D ligands is through shedding from the cell surface, which has been reported for human MICA, MICB, ULBP2 and ULBP4 (REFS 108–111) but has not been documented for the mouse NKG2D ligands. Shedding of NKG2D ligands is thought to be mediated by a disintegrin and metalloproteinase (ADAM) family metalloproteinases[112–114] and is assisted by endoplasmic reticulum protein 5 (ERP5; also known as PDIA6)[115]. The presence of free NKG2D ligands in the serum of patients with late-stage cancer[109,110] correlated with reduced levels of NKG2D at the surface of NK cells and T cells and decreased function of these cells[108,116,117]; therefore, this could be a mechanism that enables tumours to escape immune surveillance[118]. Shedding of NKG2D ligands is also seen in cases of autoimmune disease in which there is no evasion process[66], suggesting that shedding may occur spontaneously in the case of cells that express the ligands. However, it is also possible that the extent of shedding increases as tumours progress, perhaps reflecting selection for immune-evading variants.

Influences of the tumour microenvironment. In the tumour microenvironment, numerous events are thought to have an impact on the expression and function of immunoreceptors, including NKG2D and its ligands. In principle, these influences may increase or decrease NKG2D-dependent immune surveillance. For example, as noted earlier, in some cases inflammation associated with tumorigenesis can increase tumour growth and suppress protective antitumour immune responses[146]; it is possible that inhibition of NKG2D function is one example of how such inhibition occurs. It is also conceivable that in some tumour microenvironments NKG2D signalling induces a pro-tumour gene activation programme. Although the roles of these regulatory events in promoting or inhibiting antitumour immune responses remain uncertain, they must be accounted for when considering the role of NKG2D in tumour immunity.

Cytokines that are present in the tumour microenvironment are an important potential determinant of NKG2D ligand expression. RAET1 expression was downregulated following exposure to transforming growth factor-β (TGFβ)[114], which might therefore be one of the mechanisms of immunosuppression mediated by this cytokine. Surprisingly, exposure to IFNα and IFNγ downregulated H60a but not RAET1 or MULT1 expression by sarcoma cell lines[119]. In addition, the expression of MICA and, in some cases, ULBP2 by melanoma and glioma cell lines was downregulated by IFNγ treatment[120,121]. These findings were surprising because IFNs generally promote antitumour immune responses.

The expression and function of NKG2D itself is also influenced by the tumour microenvironment. Proinflammatory cytokines that are involved in proliferation and survival of NK cells and T cells, such as interleukin-2 (IL-2) and IL-15, stimulate NKG2D expression and potentiate the cytotoxic and IFNγ secretory function of NK cells and T cells[122,123]. Conversely, the immunosuppressive cytokine TGFβ, which is secreted by several tumours and is found in the serum of cancer patients[124], can directly induce NKG2D downregulation when secreted in the tumour microenvironment, as a membrane-bound cytokine on regulatory immune cells or when present in tumour-derived exosomes[125,126]. Downregulation of NKG2D expression also occurs in response to macrophage migration inhibitory factor, which is another cytokine that favours tumour growth[127].

In addition to cytokines, it is probable that NKG2D ligands expressed in the tumour microenvironment can adversely affect NKG2D function. Sustained engagement of NKG2D by its ligands *in vivo* compromises the capacity of NK cells to attack tumours. Persistent engagement by RAET1 (REF. 73), H60a[128] and MICA[75], or by soluble forms of MICA and MICB[108], is known to induce NKG2D internalization and subsequent degradation. In some cases, such persistent NKG2D engagement impairs NK cell functions more broadly, even inhibiting responses to NK cell-sensitive target cells that lack NKG2D ligands[73,76]. These findings raise the possibility that when tumour growth overwhelms protective responses, NKG2D and other NK cell functions are ultimately inhibited as a result of persistent stimulation.

Concluding remarks

As a result of the signalling associated with tumorigenesis, many developing tumour cells display cell surface ligands that engage activating receptors expressed by NK cells and in some cases T cells. Some of the same signalling pathways that activate this extrinsic response are responsible for activating intrinsic tumour suppressor mechanisms such as p53-induced apoptosis and cell senescence. However, there are probably some differences in the specific mediators of the two types of response, as suggested by the finding that induction of NKG2D ligands by the DNA damage response occurs in cells lacking p53 (REF. 90).

As exemplified by studies of the NKG2D ligand MULT1, antitumour responses that depend on NK cell receptors are in some cases regulated by cooperation of distinct stress pathways that act at different levels of biogenesis of the immune-activating ligands. Combinatorial regulation by distinct stress pathways presumably helps to prevent inappropriate responses to normal cells. On the downside, however, these features may provide multiple targets for mutational inactivation of immune recognition of tumor cells.

An interesting question is whether the ligand-induction mechanisms that are linked to tumour suppressor pathways evolved specifically for antitumour

responses or whether they have a more general role in disease responses, such as in responses to infections. In considering this question, it is often argued that because cancer is predominantly a disease of older individuals who are presumed to be post-reproductive, natural selection cannot directly select for anticancer mechanisms. It should be kept in mind, however, that this argument applies equally to intrinsic tumour suppressor mechanisms and immune-based tumour suppressor mechanisms. In light of this, some of the assumptions behind this argument may be questioned as the various barriers to tumorigenesis presumably work together to ensure that cancer is usually delayed to a later stage of life.

Immune-based antitumour mechanisms have the potential to complement, rather than simply supplement, tumour cell-intrinsic barriers in at least two respects. First, the triggers of the immune-based mechanisms may operate in cases in which expression of intrinsic tumour suppressors is lost from tumour cells, such as in the induction of NKG2D ligands by the DNA damage response that occurs in cells that have lost the expression of the p53 tumour suppressor[90]. Secondly, the innate immune system responses that are induced by these mechanisms may in some cases promote strong adaptive immune responses that have the capacity for the induction of immunological memory and sustained systemic protection.

Although NK cell receptor-dependent responses to tumours can be protective to the host, some tumours may avoid detection by downregulating or shedding ligands, as suggested by studies of NKG2D ligands. The mechanisms by which ligand expression is lost are not completely understood and must be addressed in future studies. In some cases, it is probable that loss of ligand expression results from mutations in developing tumour cells that inactivate the pathways which induce ligand expression, whereas in other cases the cells may acquire mutations in the ligand genes themselves. It is also probable that although expression of ligands for NK cell receptors is often protective against cancer, in some cases the tumour can evolve to exploit ligand expression to prevent a protective immune response. Desensitization of NKG2D as a result of shedding of NKG2D ligands is probably one example, but there are likely to be others.

A thorough understanding of the pathways that are involved in the activation of the immune response by tumours may enable the design of therapeutic drugs. In cases in which tumours acquire mutations that disable pathways which induce the expression of ligands for immune cells, a useful approach could be to design drugs that bypass the missing steps or re-induce ligands by another mechanism. It is possible, for example, that the efficacy of some chemotherapy drugs such as 5-fluorouracil and cisplatin may be related to increasing NKG2D ligand expression by tumour cells through the activation of the DNA damage response[8,129]. In addition, proteasome inhibitors and/or histone deacetylase inhibitors are promising candidates for enhancing the expression of NKG2D ligands[130,131]. Given the probability that ligand expression is induced by the synergistic action of several signals that are associated with the cancerous state, such interventions may be selective in targeting tumour cells and not normal cells. In cases in which inhibitory cytokines have a role in suppressing ligand expression on tumour cells, blockade of cytokine action may serve as a useful therapeutic intervention. As an alternative approach, although NKG2D ligands are absent from some tumour cells *in vivo*, vaccines comprising cells that express tumour antigens and NKG2D ligands may nevertheless be effective at inducing effective adaptive immune responses against tumours in some cases[70,132].

However, many, if not most, advanced cancers continue to express ligands for NKG2D and other activating receptors on NK cells with impunity (FIG. 2). Tumour escape in these instances may be due to impaired functioning of NK cell receptors owing to inhibitory cytokines in the microenvironment, in which case cytokine blockade may be beneficial. Shedding of ligands, such as NKG2D ligands, are thought to repress protective immune responses, suggesting that antibodies that systemically block or remove the shed proteins may be of therapeutic benefit[111]. In other cases, persistent stimulation of the NK cells through NKG2D and possibly other activating receptors may result in NK cell anergy. A deeper understanding of how such persistent stimulation inactivates NK cell receptors and NK cell activity may provide approaches to reverse the unresponsive state of NK cells. Finally, NKG2D ligands are an inviting target for therapeutic antibodies that are designed to eliminate ligand-expressing tumour cells in cases in which they continue to be expressed by tumour cells.

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