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Beyond Stressed Self: Evidence for NKG2D Ligand Expression on Healthy Cells

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Abstract

The activity of cytotoxic lymphocytes is regulated by the opposing function of stimulatory and inhibitory cell surface receptors. According to the now classical model of Natural Killer (NK) cell activity, the ligands for inhibitory receptors are constitutively expressed on healthy cells but can be lost on infection and on malignant cells. Loss of inhibitory checks will then allow activating signals to predominate, forming the basis of 'missing self recognition'. Natural Killer Group 2D (NKG2D) is an important member of the cohort of activating receptors expressed on Natural Killer (NK) cells and subsets of T cells. Ligands for the NKG2D receptor comprise a diverse array of self-proteins structurally related to MHC class I molecules. Expression of NKG2D ligands can be induced in cells during infection with pathogens, tumourigenesis, and by stimuli such as DNA damage, oxidative stress, and heat shock. Consequently NKG2D has been widely described as participating in 'stressed self' or 'damaged self' recognition. However, a body of evidence has recently emerged to suggest that this intuitive model of NKG2D function may be an oversimplification. NKG2D ligand expression has now widely been reported on cells that could not be described as stressed or damaged. For example activated T cells can express NKG2D ligands, and constitutive expression of NKG2D ligands has been reported on normal myelomonocytic cells, dendritic cells, and epithelial cells of the gut mucosa. In this article we will review the literature suggesting that NKG2D may function to recognise non-stressed cells and discuss the role NKG2D ligands could be playing in apparently healthy cells.

INTRODUCTION

The balancing act between protecting civil liberties and innovations by the state to protect its population - through CCTV surveillance, phone tapping, databases of personal data and so on - is one of key debates that animates politics today. In the immune system Natural Killer (NK) cells engage in immunosurveillance unconstrained by concerns over impinged liberties. Whilst NK cells have a free reign to conduct surveillance, they are subject to strict checks and balances that control their cytotoxic function. On encountering host cells, NK cell activity is determined by the balance of inhibitory and stimulatory signals it receives from the array of receptors it expresses at its cell surface [1]. Generally the cytotoxic activity of NK cells is held in check by inhibitory receptor binding to ligands that are constitutively expressed on a host cell. In humans, it is receptors of the killer-cell immunoglobulin-like receptor (KIR) family binding to MHC class I molecules that provides the predominant inhibitory restraint on NK cells. Loss of cell surface MHC class I, for example due to the action of viral immunoevasins, or genetic mutations that occur during tumour development,

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will remove inhibitory checks. Activating signals can then predominate resulting in NK cell killing of the infected or malignant cell, and inflammatory cytokine secretion by the NK cell. This basic model of NK cell function is known as “missing self” recognition [1, 2].

Natural Killer Group 2D (NKG2D) is a key member of an array of receptors that can activate or co-stimulate Natural Killer (NK) cells, and subsets of T cells [3, 4]. NKG2D is a homodimeric molecule that signals *via* association with adaptor molecules (Fig. 1). In humans NKG2D exclusively signals *via* an association with the DAP10 adaptor molecule [5]. In mice NKG2D can be alternatively spliced adding additional complexity to NKG2D mediated signalling pathways [6, 7]. The murine NKG2D variant with a long cytoplasmic tail associates with DAP10 only, however a shorter variant can associate with DAP10 or DAP12 [6, 7].

The ligands for NKG2D comprise a diverse array of proteins that are structurally related to MHC class I (Fig. 1). In humans the complement of ligands stands at seven proteins, including two members of the MHC-Class-I-polypeptide-related sequence family (MICA and MICB), and six members of the UL16-binding protein (ULBP) family that are also known as retinoic acid early transcript (RAET) proteins [8-11]. In mice there is an even greater diversity of NKG2D ligands, with five retinoic acid early transcripts (RAE-1 α - ϵ), three variants of the H60 minor histocompatibility antigen, and murine UL16-binding-protein-like transcript-1 (MULT1) [12-15]. Both human and mouse NKG2D ligands vary in their amino acid sequence (with only 20-25% identity between some), domain structure, expression pattern, binding affinity to NKG2D, cellular localisation, and trafficking. This baffling diversity of ligands is a unique feature of NKG2D and we have discussed the evolutionary and function implications elsewhere [16].

The human NKG2D ligand MICA was initially described as a stress response molecule, as it could be induced on cells by heat shock. It was subsequently discovered that expression of both human and murine NKG2D ligands can be induced on infection of cells with a wide range of different viruses, including human cytomegalovirus, influenza A, hepatitis B, Epstein-Barr virus, and adenovirus [17-20]. NKG2D ligands are also widely expressed on solid tumours and some leukaemias [21], and are induced by cancer related pathways; for example by DNA damage response pathways and by oncogenes such as BCR/ABL [22, 23]. There is also evidence that NKG2D participates in anti-cancer immune responses *in vivo* [24-26].

The importance of NKG2D in mediating anti-viral and anti-tumour immune responses is emphasised by the fact that viruses and cancers employ a variety of different strategies to prevent NKG2D binding to its ligands on the infected or malignant cell surface. For example human cytomegalovirus (HCMV) encodes at least two immunoevasins that act to prevent NKG2D ligands reaching the cell surface, murine cytomegalovirus (MCMV) encodes at least four [16, 27]. Viruses may also encode microRNAs that can suppress NKG2D ligand expression [28]. Tumours may evade NKG2D mediated immune responses by cleaving NKG2D ligands from the cell surface *via* protease enzymes, by releasing cytokines such as TGF β that downregulate NKG2D expression, and by simply evolving ways to switch off NKG2D ligand expression as they grow and metastasise [29-32].

In a number of studies aberrant expression of NKG2D ligands has been implicated as a causative or contributory factor in autoimmune diseases. Examples include reports of increased MICA expression in the gut of patients with Crohn's disease and celiac disease [33-35]. Recognition of MICA by NKG2D⁺ intraepithelial lymphocytes (IELs) is associated with the autoimmune attack of intestinal epithelial cells, leading to villous atrophy.

These data strongly suggested a model of NKG2D function where it participates in the immune recognition of a various forms of 'cellular stress' (Table 1 presents some of the key stress stimuli known to induce NKG2D ligand expression). The upregulation of NKG2D ligands appeared to have evolved as an innate mechanism by which the host cell could signal to the immune system that something was wrong and allow the cell to be eliminated. As a result NKG2D has been widely described in participating in 'stressed self' or 'damaged self' recognition, an intuitive counterpoint to the classical model of 'missing self' recognition. However there is now a substantial body of evidence that NKG2D ligands can be both induced, and constitutively expressed, on cells that could not be described as damaged or stressed. In this article we will review some of the recent work reporting NKG2D ligand expression on healthy cells that calls for an adaptation of our models for the role of NKG2D in the immune system.

NKG2D LIGAND EXPRESSION ON HEMATOPOEITIC CELLS

T Cells

One of the most striking examples of NKG2D ligand expression on healthy cells has been the observation that activated peripheral blood T lymphocytes can express NKG2D ligands and become susceptible to NK cell lysis *in vitro*. Human CD4⁺ and CD8⁺ T cells were shown to express MICA in response to CD3 and CD28 antibody crosslinking, and on activation with allogeneic peripheral blood mononuclear cells (PBMC) [36]. NF- κ B was implicated as treatment with the NF- κ B inhibitor sulphasalazine resulted in a dose dependent reduction in MICA expression on T cells stimulated through CD3 and CD28 [37].

In mouse it was shown that OVA specific T cells stimulated by OVA antigen presented by antigen presenting cells (APC) became susceptible to killing by syngeneic NK cells. This killing was shown to be NKG2D dependent, and stimulated T cells were shown to upregulate expression of H60 [38].

In the most extensive study to date, Cerboni *et al.* show that MICA and ULBP1-3, but not ULBP4, are induced on T cells stimulated by alloantigen, superantigen, or by a specific antigenic peptide [39]. MICA was expressed earlier following stimulation than ULBP1-3. This study also present data calling for a role of the ataxia-telangiectasia mutated (ATM) protein in NKG2D ligand induction on T cells. They show that ATM becomes phosphorylated on T cell activation and that treatment with the ATM inhibitor caffeine prevents MICA induction. ATM has also been shown to activate NF- κ B.

In addition to peripheral blood T cells subsets of thymocytes from BALB/c mice express NKG2D ligands [13, 40]. One study reports that approximately 35% of thymocytes that are uncommitted, and have not undergone selection, are high in NKG2D ligand expression and express intermediate levels of MHC class I (NKG2D ligand^{High}/MHC class I^{Mid}) [40]. A second subset of thymocytes committed to cell death were NKG2D ligand^{Low}/MHC class I^{Low}. A third subset, which mostly consisted of thymocytes that had undergone positive selection, were NKG2D ligand^{Low}/MHC class I^{High}. Therefore NKG2D ligand expression appeared to be lost after the selection of thymocytes had occurred.

NKG2D ligand expression on T regulatory cells (Treg) has also been reported [41]. When monocytes and CD4⁺ T cells were cultured with *Mycobacterium tuberculosis* an expansion of Tregs is normally observed. However this expansion was reduced by addition of activated NK cells, which could kill the Tregs. NKG2D recognition of ULBP1, but not other NKG2D ligands, on the target cell was shown to be important in Treg lysis. Freshly isolated Tregs - rather than *in vitro* expanded - were not killed by NK cells, suggesting other signals were influencing NKG2D dependent killing of Tregs.

Bone Marrow

The bone marrow is a specialised microenvironment in which immune progenitors are exposed to a complex array of developmental signals that shape their differentiation. Bone marrow stromal cells (BMSC) have been reported to express NKG2D ligands [42, 43]. In a study of human BMSC–NK cell interactions resting NK cells were reported to not kill BMSC, but were induced to produce IFN- γ and TNF- α [42]. When activated with IL-2, autologous NK cells would kill BMSC, with NKG2D recognition of MICA and ULBP3 playing a role. In mice the expression of RAE-1 on cells of the bone marrow was strain specific, with expression absent on C57BL/6 but present on BALB/c bone marrow [43]. This study progressed to describe a role for NKG2D mediated recognition of RAE-1 in bone marrow graft rejection. As interactions between haematopoietic stem cells and NK precursors with BMDC are thought to be important during haematopoiesis [44] it is plausible that NKG2D: NKG2D ligand interaction could play a role.

Pluripotent mesenchymal stem cells (MSC) that reside in the bone marrow have also been shown to express ligands for activating NK cell receptors, including MIC and ULBPs [45]. Despite this it is known that MSC can inhibit T cell responses induced by mitogens and alloantigens, and can also inhibit IL-2 induced NK cell proliferation when co-cultured *in vitro*. MSCs not only inhibit the cytokine-induced proliferation of freshly isolated NK cells but also prevented induction of effector functions, such as cytotoxicity and cytokine production. When NK cells were activated with IL-2 in the absence of MSC they then acquired cytolytic activity against MSC in killing assays. The inhibitory effect of MSC was related to an acute down-regulation of the activating NK receptors Nkp30, Nkp44, and NKG2D which was mediated by soluble factors produced by MSC such as indoleamine 2, 3-dioxygenase and prostaglandin E2 [46].

Nowbakht *et al.* report that NKG2D ligand expression on BM haematopoietic CD34⁺ progenitors is low, but upon myelomonocytic differentiation – defined by loss of CD34 expression and acquisition of CD33 and CD14 – ULBP1 expression could be detected [47]. This observation held for both directly isolated bone marrow cells, and *in vitro* differentiated myeloid cells.

Monocytes, Macrophages and Dendritic Cells

Peripheral blood and cultured monocytes have been reported to show variable expression of ULBPs [47, 48]. This variation is likely to be due to differences between donors. ULBP^{ve} CD14⁺ monocytes could be induced to express ULBPs in response to stimulation with the myeloid growth factors FLT-3 ligand, SCF, and GM-CSF [47].

Mature dendritic cells (DC) have been reported to express ULBP1 both *in situ* in the lymph node, and after *in vitro* maturation [49]. Jinushi *et al.* report that both IFN- α and IL-15 can upregulate MICA expression on mature DC and that MICA is involved in the activation of NK cells by DCs *in vitro* [50, 51]. In macrophages LPS recognition through toll-like receptor 4 (TLR4) has been shown to induce NKG2D ligand expression in both mice and humans [52, 53]. On expression of NKG2D ligands Macrophages become sensitive to killing by NK cells.

Other Peripheral Blood and Splenic Subsets

There is one report of peripheral blood B cells, platelets, and granulocytes expressing ULBP molecules [47]. NKG2D ligand expression on these subsets was shown to be variable between donors but the functional consequences of ULBP expression were not explored. In another more extensive study expression of the mediator of somatic hypermutation, Activation-Induced Cytidine Deaminase (AID), was shown to be involved in triggering

RAE-1 protein expression in immunoglobulin (Ig) class switching B cells located in the spleen [54]. chk1 kinase was shown to be phosphorylated in wildtype mice but not AID knockout mice. Thus AID links the Ig class switching pathway to that involved in NKG2D ligand induction in response to DNA damage in solid tumour growth, where the ATM protein is responsible for chk1 phosphorylation [22].

NKG2D LIGAND EXPRESSION IN NON-HEMATOPOEITIC TISSUES

The Gut Epithelium

Constitutive expression of MICA in intestinal epithelial cells was first reported over ten years ago [55]. In a normal state this does not result in immune responses being directed against the gut epithelium. However in the autoimmune conditions Crohn's disease, and celiac disease, intestinal epithelial cells are attacked and killed by NKG2D⁺ IELs [33, 35]. Infection of epithelial cells with pathogenic *Escherichia coli in vitro* upregulates MICA expression [56], and signalling through Toll-like receptor (TLR)-3 on mouse intestinal epithelial cells induces expression of RAE-1 molecules *in vivo* [57]. Therefore it is likely that NKG2D ligand expression level on gut epithelial cells is responsive to changes in the gut flora. Crohn's disease is characterised by uncontrolled chronic immune responses stimulated by gut flora, and elevated MICA expression on the gut epithelium. In celiac disease chronic inflammation and elevated MICA levels in the gut is induced by immune responses against wheat gliadin, a component of gluten. Therefore elevated MICA expression on intestinal epithelial cells is implicated in the breakdown of immune tolerance, but low level constitutive expression is not.

Airway Epithelial Cells

The airway epithelium is another interface between the body and its environment and has a high risk of exposure to pathogens. Two studies have shown that both cell lines derived from bronchial epithelial cells (BEC), and primary BEC, express MICA, and ULBP molecules. NKG2D contributed to the killing of primary BEC by alloreactive CD8⁺ T cells [58]. Borchers *et al.* also show that oxidative stress can increase cell surface levels of NKG2D ligands on BEC; a phenomenon they attribute to the mobilisation of NKG2D ligand from intracellular stores to the cell surface, rather than an overall increase in protein expression [59].

Other Tissues; Embryonic Tissues, Muscle, Neuron, Liver, Skin

A number of other tissues have been reported to express NKG2D ligands, although in most cases there is less functional data available. Primary muscle cells (myoblasts) have been shown to upregulate NKG2D ligand expression in response to TLR signalling [60]. In mouse, liver hepatocytes have a degree of constitutive expression of RAE-1 that is significantly increased on hepatitis B infection [18]. ULBP4 expression has been reported in the skin, although only at the transcript level [11]. Cultured neurons from dorsal root ganglia, but not hippocampus, could be killed *via* NKG2D mediated recognition of RAE-1 [61].

It has also been noted that retinoic acid - a regulatory compound involved in development - can upregulate expression of both human and murine NKG2D ligands [62, 63]. Indeed the mouse *RAE-1* genes were first identified as encoding retinoic acid inducible transcripts in mouse embryonic cells [63]. Recently it has been shown that subcutaneous injection of mouse embryonic stem (ES) cells into syngeneic or allogeneic immunodeficient mice resulted in teratomas in about 95% of recipients [64]. Tumours did not arise in immunocompetent allogeneic mice or xenogeneic rats. ES cells were highly susceptible to

killing by NK cells and expressed high levels of NKG2D ligands, leading the authors to propose a role for NKG2D in preventing teratomas.

A degree of caution is required when interpreting reports of tissue specific expression of NKG2D ligands where mRNA only has been detected. It is known that expression of NKG2D ligands at the protein level does not always correlate with mRNA expression patterns. For example MICA transcript can be readily detected in many different cell types where there is no evidence of protein expression [65, 66].

HOW CAN WE ACCOUNT FOR NKG2D LIGAND EXPRESSION ON HEALTHY CELLS?

As described above there is substantial evidence that NKG2D ligands can be expressed on healthy cells. It is striking how the ATM/ATR pathway – which plays a crucial role in the induction NKG2D ligands in response to activation of DNA damage response pathways during tumour development – is shown to be involved in NKG2D ligands expression in activated T cells, in viral infection, and in class switching B cells (Fig. 2). Therefore it is clear that the pathways responsible for induction of NKG2D ligand expression in response to cellular stress can be the same as, or closely related to, the pathways leading to NKG2D ligand expression on healthy cells.

We still need to address the functional consequences of NKG2D ligand on healthy cells. One explanation that applies to hematopoietic cells is that NKG2D dependent killing of activated immune cells by NK cells is a regulatory mechanism leading to a dampening down of immune responses.

In the case of T cells it has been shown that NK cell function can restrain T cell responses during viral infections and eliminate developing T cells *in vivo* [67]. Perforin deficient mice exhibit much larger expansion of T cells on lymphocytic choriomeningitis virus (LCMV) infection than do wild type mice [68]. Perforin deficient patients also exhibit lymphoproliferative diseases [69]. Therefore NKG2D binding to its ligands on activated T cells may be an important factor in NK cell dependent repression of T cell mediated adaptive immunity. NKG2D ligand expression during thymocyte development is intriguing, suggesting that NKG2D could play a role in T cell repertoire selection. However more work will be needed to elucidate the precise mechanisms involved.

Macrophages are known to co-operate with NK cells in immune responses to pathogens such as *Plasmodium falciparum*, and NK cells proliferate and produce IFN- γ on contacting macrophages [70]. However, as discussed earlier, NK cells will kill macrophages stimulated with high doses of LPS *via* NKG2D [52, 53]. Therefore it has been proposed that NK cells may act to dampen down excessive inflammatory responses and reduce the risk of septic shock by targeting macrophages [53].

A rationale for NKG2D dependent killing of activated immune cells has been established. However this is not the complete story of NKG2D ligand expression on healthy cells. In the case of non-hematopoietic tissues it is difficult to conceive of a role for NK cell killing as a regulator of the function of healthy cells, and indeed NKG2D co-ordinated immune responses against epithelial cells have been linked to autoimmune disease. It may also be the case that NKG2D ligands could be recognised by receptors other than NKG2D. There is little evidence for this at present, however Krieger et al. have reported an NKG2D independent effect of MICA and H60 in repressing the proliferation of T cells [71]. There is also evidence that expression of NKG2D ligand alone is not always a signal to trigger immune cell attack. Other factors need to be taken into account, such as the intracellular

location of the NKG2D ligand and the synergy or antagonism of NKG2D mediated signalling in conjunction with other signals delivered to the effector cell within its particular microenvironment.

NKG2D LIGAND EXPRESSION AT THE CELL SURFACE IS HIGHLY REGULATED

Post-Translational Regulation of Cell Surface NKG2D Ligand Expression

Primary epithelial cells of the gut and airways have been shown to express NKG2D ligands. In the case of the gut MICA expression in normal intestinal epithelium was demonstrated by immunohistochemistry with specific monoclonal antibodies, although little cell surface expression was observed by flow cytometry [33]. We also have unpublished data showing that RAET1G is found in the gut, but is predominantly expressed inside the cell. Conversely, in the gut epithelial of celiac disease patients and Crohns disease patients MICA expression is increased and can be found at the cell surface [33-35]. It has also been shown that infection of epithelial cell with pathogenic *Escherichia coli* upregulates MICA expression [56]. By intracellular flow cytometry and western blot Borchers *et al.* reported that primary bronchial epithelial cells express MICA and ULBP1-4 mainly inside the cell, until stimulated by oxidative stress when NKG2D ligands can be found at the cell surface [59].

These data suggest that in some cell types post-translational mechanisms exist to regulate levels of NKG2D ligands at the cell surface by retaining them in intracellular compartments. Additional evidence for the existence of these mechanisms has emerged from a study of melanoma cell lines where some lines were shown to express MICA predominantly in the endoplasmic reticulum whereas in other lines MICA was expressed at the cell surface [72].

The precise nature of these mechanisms has yet to be defined but it is clear that NKG2D ligand protein expression does not always correlate with cell surface expression. Hence an additional regulatory step may exist to control NKG2D mediated immune responses. In the case of both MHC class II molecules and TLR9 in monocyte derived dendritic cells it has been shown that they are both expressed in immature cells but held in cellular compartments where they do not function [73, 74]. On DC activation MHC class II molecules are stabilised at the cell surface where they can be recognised by CD4⁺ T cells and TLR9 redistributed to lysosomes where it can signal on binding to CpG DNA. The rationale for this mode of regulation is that by expressing MHC class II and TLR9 protein in a non-active form they can be much more rapidly mobilised on receipt of danger or activation signals than would be possible if they were regulated only at the level of gene transcription. This form of regulation may well apply to NKG2D ligands, however more work needs to be done firmly establish whether this is the case and the stimuli and mechanisms that regulate the cellular localisation of NKG2D ligands.

Another additional element of complexity that needs to be considered is the strict polarity maintained by epithelial cell layers. The functional roles required from the apical side of the epithelial cell layer that borders the gut lumen or bronchial airways will be quite different from the function of the basolateral side, where IELs are found. Proteins selectively partition to either the apical, or the basolateral side, based on the presence or absence of specific targeting signals [75]. MICA has clearly adapted to this system as it has been shown to encode a basolateral targeting motif in its cytoplasmic tail [76]. Therefore if cell surface MICA expression was upregulated in an epithelial cell layer on receipt of stress signals it would be found at the correctly location to be recognised by NKG2D⁺ IELs. Conversely human ULBP1-3 and mouse RAE-1 molecules are GPI anchored to the cell membrane and by convention GPI anchored molecules traffic to the apical membrane by default [75]. In this case GPI anchored NKG2D ligands would only become available to stimulate IELs if

the epithelial cell layer polarity was disrupted, as is known to happen during infection and in autoimmune diseases of the gut. We have discussed models of NKG2D ligand function in gut epithelium in more detail elsewhere [16].

In the case of NKG2D ligand expression on neurons, in a normal state NKG2D⁺ lymphocytes would be excluded from this tissue *in vivo* and be unable to interact with NKG2D ligand expressing cells [61]. However in a rat model, guanethidine treatment causes NK cell infiltration and attack of cervical ganglia, showing that NKG2D recognition of ligand could be a factor in autoimmune neurodegeneration [77]. In the case of embryonic tissue it is not clear what the purpose of NKG2D ligand expression could be, however as NKG2D ligands are responsive to retinoic acid it seems likely that they are playing an as yet undefined role in development.

The conclusion arising from these observations is that expression of NKG2D ligands in specialised cell types and tissues may not necessarily mean they are always accessible to NKG2D expressing lymphocytes. Control of the cellular location and trafficking of NKG2D ligands may provide important mechanisms to regulate NKG2D mediated immune responses (Fig. 3).

Post-Transcriptional Regulation of NKG2D Ligand Expression

In addition to post-translational regulation of NKG2D ligand cellular localisation and trafficking it has recently emerged that there can be post-transcriptional regulation of NKG2D ligand expression by microRNAs. A recent study has revealed the existence of several endogenously expressed human microRNAs that can bind to conserved sequences in the 3'UTR of *MICA* and *MICB*, effectively reducing *MICA* and *MICB* expression levels [78]. The microRNAs themselves had a variable expression profiles in normal human tissues, and some had elevated expression levels in cancer cells. Further research needs to be done to elucidate specific scenarios where endogenous microRNAs could be important in regulating NKG2D ligand expression. Nonetheless it is clear that this mechanism has the potential to allow fine tuning and modulation of NKG2D mediated immune responses in non-cancerous tissues, and also represents another route that cancer cells could evade NKG2D mediated immune recognition.

DIVERSE RESPONSES TO NKG2D SIGNALLING

Cellular Activation Must Be Considered in Terms of the Synergy/Antagonism of NKG2D Associated Signalling with Other Pathways

It is widely reported that the NKG2D/DAP10 receptor signalling complex can activate cytotoxicity in NK cells and costimulate cytotoxicity in antigen-specific effector T cells. This now appears to be an over-simplification of the complexity of NKG2D signalling pathways, which appear to be dependent upon various combinations of cell-, receptor- and species-specific signalling factors as well as soluble microenvironmental signals, such as cytokines. Additional complexity is present in mouse, where NKG2D has the potential to associate with both DAP10 and DAP12.

NK cell and T cell stimulation by microenvironmental signals markedly affects their cytotoxic activity. For example, There is a dramatic difference in the cytotoxicity of 'resting' versus IL-2 activated human NK cell isolated from peripheral blood and NK cells isolated from various mouse tissue e.g. spleen, which are readily cytotoxic. Cytokines, such as IL-2, IL-15, IL-18 are all known to potentiate the cytotoxicity of NK cells, with various reported effects on the stabilisation and longevity of NKG2D and DAP10 at the transcript and protein level [79]. IL-21 has been described as enhancing NK cell function and NKG2D mediated tumour rejection *in vivo* [80, 81], however another study has reported that it

causes NKG2D downregulation on IL-21 treatment *in vitro* [82]. Cytokines such as TGF- β , and macrophage inhibitory factor (MIF) are implicated in down-regulating NKG2D expression [30, 83]. Therefore it is vitally important to recognize the microenvironmental factors that may regulate the response of an NKG2D expressing cell.

An example of the intimate relationship between NKG2D signalling pathways and those induced by microenvironmental factors comes from work recently published on the axis between IL-15 and NKG2D/DAP10. A transgenic approach utilising ubiquitin-tagged DAP10 (DAP10-Ub) under the control of the lymphocyte-specific CD2 promoter, revealed a novel role for DAP10 in IL-15 regulation that was not apparent from DAP10-deficient mice [84]. The DAP10-Ub transgene worked as a dominant negative suppressor of NKG2D expression and DAP10-Ub killer cells failed to produce IFN- γ or eliminate tumours expressing NKG2D ligands. However, the DAP10-Ub mouse phenotype was more generalized and DAP10-Ub mice had deficiencies in NK- and NKT-cell numbers, which resembled that of IL-15 deficient mice. Unlike wild-type, DAP10-Ub transgenic NK cells could not be maintained in *ex vivo* cultures containing IL-15, despite normal levels of IL-15 receptor. DAP10 co-immunoprecipitated with the IL-15R β and γ chains and the IL-15 JAK3-STAT5 signalling pathway was also abolished in DAP10-Ub transgenic NK cells, showing that IL-15 receptor signalling required DAP10 [84].

In a recent study NKG2D signalling alone appears to be a weak inducer of cytotoxicity and cytokine production in resting NK cells [85]. This also appears to be the case for a number of other NK cell receptors. The authors then went on to examine the cooperation of NKG2D with different ITAM and non-ITAM receptors in both resting and IL-2 activated human NK cells. NKG2D was shown to synergise with either NKp46 or 2B4, to induce cytotoxicity and cytokine production in resting NK cells. In contrast, neither CD2 nor DNAM-1 synergized with NKG2D in resting NK cells. These results suggest that NK cell receptors generally have to cooperate with additional receptors to orchestrate release of cytotoxic granules by NK cells. This study also highlights that there are checks and balances on NK cell activation where some receptors can specifically synergise with NKG2D to induce cytotoxicity and cytokine release, but importantly some receptors do not induce cytotoxicity.

How then is the complexity of these receptor interactions reconciled with expression of 'stress-inducible' ULBP/RAET family of molecules on healthy cells? Is the NK surveillance response simply binary; killing or no killing? Or is there a degree of sophistication to NK cells that may mediate regulatory or homeostatic responses *via* NKG2D that is consistent with expression of NKG2D ligands on otherwise healthy cells? It is also important to consider that NKG2D+ lymphocyte populations will vary between different tissues and this will lead to different outcomes of NKG2D ligand recognition. For example NK cells in the lymph node are known to be poorly cytolytic [86]. Therefore NKG2D ligands expressed on dendritic cells and T cells in the lymph node are unlikely to contribute towards the triggering of a cytotoxic NK cell response, but could trigger NK cells to release cytokines.

NKG2D is not just restricted to cytotoxic NK cells and CD8+ T cells. It is also expressed on CD4+ T cells, $\gamma\delta$ T cells, NKT cells, and Tregs [3, 87]. This provides a premise for a role for NKG2D in tissue homeostasis and regulatory responses designed to suppress the immune system to avoid autoimmunity.

NKG2D Ligand Recognition Can Lead to Repressive/Regulatory Outcomes

A direct role for NKG2D in suppressive, rather than cytolytic, responses has, unexpectedly, arisen from extended studies of its role in surveillance of tumours. In contrast to the activating properties mediated by NKG2D, one study found that soluble MIC, derived from proteolytic shedding from tumours, induced endocytosis and degradation of the NKG2D-

DAP10 complex, resulting in reduced NKG2D expression on tumour-infiltrating and peripheral blood T cells from cancer patients [29]. This mechanism of NKG2D-DAP10 downregulation and subsequent effector T cell suppression was proposed to be a novel tumour evasion tactic that could also leave the host susceptible to possible secondary infections. Subsequently it has been shown that sustained localised expression of NKG2D ligands can result in NKG2D downregulation and impaired cellular cytotoxicity [88-90]. Indeed, Coudert *et al.*, have recently shown that sustained NKG2D-DAP10 signaling can cross-tolerise unrelated NK cell activation pathways [91].

Expression of NKG2D ligands can have profound effects on NKG2D+CD4+ T cell responses against tumour growth. For example, in the late-stage tumour environment, NKG2D recognition of MIC ligands drove proliferation of a subpopulation of NKG2D+CD4+ T cells [87]. These costimulated NKG2D+CD4+ T cells expressed Fas ligand (Fas-L), which suppressed the proliferation of bystander NKG2D-CD4+ T cells. Despite expression of Fas-L, these unconventional NKG2D+CD4+ T regulatory cells (Tregs) were themselves refractory to the effects of Fas-mediated growth arrest. These results suggest that at early stages of tumour development, involving limited NKG2D ligand expression, the production of Fas-L may be beneficial, by promoting tumour growth arrest and cell death. However, these conditions may be detrimental, in late-stage tumours, where gross expression and shedding of MIC may costimulate NKG2D and so drive proliferation of a population of unconventional immunosuppressive CD4+ Tregs that could promote tumour survival [87].

Studies using mice engineered to express RAE-1 under a constitutively expressed promoter, or an inducible promoter, highlight the potential difference in responses to acute or sustained expression of NKG2D ligands. Sustained expression of transgenic RAE-1 from a globally active promoter resulted in systemic reduction in NK cell cytotoxic function, and milder reduction in CD8+ T cell function [90]. A similar result was shown for MICA transgenic mice [89]. Conversely the acute expression of RAE-1, under control of an inducible epidermis-specific promoter, resulted in the rapid orchestration of a localised immune response [92]. The sustained expression of membrane bound or soluble forms of NKG2D ligands could conceivably induce NK cell hyporesponsiveness in other physiological scenarios, not just the tumour environment. For example a role for soluble MICA expression has been proposed in maternal-fetal tolerance [93], although this model has been recently been challenged [94].

The subtlety of NKG2D signalling may not only be restricted to the duration of binding to its cognate ligands. NKG2D signalling may be cell-type specific. For example, DAP10-deficient mice exhibit perturbations in the Treg compartment and display enhanced immunity towards melanoma malignancies due to the hyperactive functioning of NKT cells [95]. NKT cells exhibited increased cytokine production and enhanced cytotoxicity towards melanomas. This NKT anti-tumour response correlated with impaired activation of CD4+CD25+ Tregs, which maintained high IL-2 levels but expressed low levels of IL-10 and IFN- γ compared with wild-type Tregs [95]. Therefore, DAP10 signalling is not only involved in regulating anti-tumor responses but also in adjusting the activation thresholds and generation of NKT cells and Tregs to avoid autoreactivity and maintain tolerance [95, 96]. Whilst this study is complicated by the possible NKG2D association with DAP12 in DAP10-deficient mice, or the association of DAP10 with the IL-15R, a possible role for NKG2D signalling in Treg and NKT cell responses in the control of autoimmunity and tolerance to self have been implicated.

Glazka *et al.* have hypothesized that inflammation may induce tissue stress conditions that might contribute to the generation of mechanisms limiting ongoing immune responses,

rather than promoting them [97]. This putative mechanism may contribute to immune tolerance and immune privilege at physiologically sensitive sites like the central nervous system. The Selmaj group have shown that brain-derived heat shock protein 70-peptide complexes (Hsp70-pc) can induce NK cell-dependent tolerance to experimental autoimmune encephalomyelitis (EAE) [97, 98]. Further investigation into this phenomenon revealed that Hsp70-pc-induced EAE inhibition was determined by the NKG2D ligand, H60. Hsp70-pc led to significant and selective upregulation of H60 in SJL/J mice, and antibody blocking of H60 led to loss of EAE tolerance. Similarly, antibody blocking of NKG2D also reversed the Hsp70-pc-induced EAE inhibition. This phenomenon was strain-specific, since in C57BL/6 mice H60 was not expressed and Hsp70-pc-induced tolerance was not detected. The NK cell mediated Hsp70-pc-induced tolerance to EAE in SJL/J mice appeared to be dependent on the modulation of dendritic cell function leading to diminished T cell reactivity.

CONCLUSION

The number and variety of different tissues now reported to express NKG2D ligands has grown enormously in recent years. As a result it has become untenable simply to describe NKG2D as a receptor that recognises “stressed self”. We must now consider NKG2D-NKG2D ligand interactions as playing complex roles in both the activation and regulation of immune responses. In order to understand these roles it is necessary to have local knowledge of the prevailing microenvironmental and cellular signals in a particular tissue that will synergise with - or antagonise NKG2D.

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Fig. (1). NKG2D and its ligands

NKG2D is a C-type lectin-like molecule expressed on NK cells and subsets of T cells. In humans NKG2D associates exclusively with the transmembrane adaptor molecule DAP10 [5]. Both NKG2D and DAP10 form disulphide-linked dimers *via* extracellular cysteines, forming a hexameric complex of one NKG2D homodimer and four DAP10 proteins [99]. The cytoplasmic tail of DAP10 encodes a YXNM motif that, upon tyrosine-phosphorylation, recruits the SH2 domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). DAP10 can also recruit Grb2 and Vav1 to activate Rho family guanine triphosphatases and PLC γ [8, 100, 101]. Mice, but not humans, encode two NKG2D splice variants, NKG2D-short (NKG2D-S) and NKG2D-long (NKG2D-L). NKG2D-S can associate with either DAP10 or another transmembrane adaptor DAP12, whilst NKG2D-L only associates with DAP10 [6, 7]. In contrast to DAP10, DAP12 encodes an Immunoreceptor Tyrosine-based Activation Motif (ITAM) in its cytoplasmic tail. Intracellular signalling by DAP12 is initiated when the ITAM is tyrosine-phosphorylated by Src-family kinases [102]. Dual phosphorylated ITAM can recruit the Syk family tyrosine kinase that can activate PI3K and the signalling scaffold proteins LAT and NTAL, which provide a platform to recruit other signalling effectors, such as PLC γ , SLP76/VAV, and the Grb2/SOS complex [102]. The ligands for NKG2D all have MHC-class-I-related $\alpha 1$ and $\alpha 2$ domains, in addition MICA/MICB has an additional $\alpha 3$ domain [103]. ULBP1-3 and RAETIL in humans and RAE1 α -RAE1 ϵ and H60c in mouse are glycosylphosphatidylinositol (GPI) anchored to the cell membrane [9, 15, 63, 104]. MICA, MICB, ULBP4 (also known as RAETIE), RAETIG, H60, H60b and MULT1 (murine UL16-binding protein like transcript 1) possess transmembrane domains and cytoplasmic tails [10, 11, 14, 15, 103].

Fig. (2). Commonalities between the pathways that induce NKG2D ligand expression in cancer, viral infection, and healthy cells

There is evidence that the pathways leading to expression of NKG2D ligands in “stressed cells” could be closely related to those involved in NKG2D ligand expression on healthy cells. A role for ATM/ATR mediated phosphorylation of checkpoint kinase-1(chk1) in NKG2D ligand induction was first reported to occur in response to DNA damage [22]. Subsequently it has been shown that RAE-1 expression in class switching B cells, and on infection with a transforming retrovirus (Abelson murine leukaemia virus), is dependant on activation-induced cytidine deaminase (AID) mediated chk1 phosphorylation; a pathway that implies a role for ATM [54]. ATM/ATR and chk1 have also been implicated in the expression of NKG2D ligands on activated T cells [39]. The precise mechanism by which chk1 induces NKG2D ligand gene expression is unknown. ATM/ATR and chk1 are known to be regulators of cell division. Therefore increased cellular proliferation could be the crucial commonality between these four scenarios that result in NKG2D ligand expression.

Fig. (3). Steps in the regulation of NKG2D ligand cell surface expression

(1) The most fundamental mechanism controlling NKG2D ligand expression is at the level of gene transcription. A wide array of different stimuli have now been described that can switch on NKG2D ligand transcription in cells that do not normally express them [105, 106]. (2) It has recently been shown that human and viral microRNAs exist that can bind to the 3'UTR of NKG2D ligand mRNA, effectively preventing them being transcribed [28, 78]. (3) It has been noted that gut and bronchial epithelial cells can express NKG2D ligands constitutively, but they are not always expressed at the cell surface until a stress or inflammatory signal is received [33, 59]. Expression of MICA inside the cell has also been shown to not necessarily correlate with expression at the cell surface of melanoma cell lines [72]. The implication is that a mechanism exists can prevent the export of NKG2D ligand protein to the cell surface, or to internalise NKG2D ligand rapidly from the cell surface. (4) Regulation of the targeting of NKG2D ligands to specific plasma membrane domains. In polarised epithelium MICA is known to traffic to the basolateral membrane due to a targeting motif in its cytoplasmic tail [76]. GPI-anchored NKG2D ligands and an allele of MICA with a truncated cytoplasmic tail would not be targeted to the basolateral surface, but traffic to the apical membrane by default [75, 76]. NKG2D ligands have also been shown to have variable affinity to cholesterol rich microdomains, or lipid rafts, which may lead to further partitioning of NKG2D ligands at the cell surface [107].

Fig. (4). NKG2D signalling pathways can induce a sophisticated array of immune responses
 An overview of the different NKG2D signalling pathways (arrows) in mice and humans and the consequences of their interaction with different receptor pathways, e.g. ITAM, and microenvironmental signals, e.g. cytokines, is presented. In mice, NKG2D can associate with either DAP10 (NKG2D-S or -L) or DAP12 (NKG2D-S) [6, 7]. In humans, NKG2D only associates with DAP10 [5]. In resting human NK cells, some receptors can induce cellular activation directly, e.g. CD16 [85]. Other NK receptors, such as NKG2D, NKP46, 2B4 or DNAM require priming with cytokines, such as IL-2, IL-15 and IL-18 to induce activation in resting NK cells [79]. NKG2D can also induce cellular activation by synergising with other receptor pathways (Synergy), in this case ITAM receptors, e.g. NKP46 or NKG2D-S/DAP12 complexes [85]. The consequences of classical activation in NK cells is induction of cellular cytotoxicity or cytokine production e.g. interferon- γ . It is not known whether these pathways can induce regulatory activity directly in different NKG2D⁺ immune cell-types e.g. Treg. In some scenarios (*) this cytotoxicity may not just being directed towards tumours or virus-infected cells and may be targeted towards activated T cells or activated macrophages to downregulate potentially harmful immune responses [39, 52, 53]. Sustained/chronic NKG2D signalling can induce NKG2D receptor endocytosis and downregulation or cross-tolerisation of unrelated NK receptor pathways [89-91]. Evidence exists for an NKG2D-mediated pathway that could be involved in regulatory responses or tolerance induction in different cell-types, such as regulatory T cells, NKT and $\gamma\delta$ T cells. It is not known whether an NKG2D signalling pathway exists that can induce these non-classical responses directly in different immune cell-types (dotted arrow).

Table 1
Examples of “Cellular Stress” Signals Implicated in the Expression of NKG2D Ligands

Cellular Stress	Example	Notes	Ref.
Classical stress pathways	Heat Shock	Upregulation of MICA on epithelial cells	[55]
	Oxidative Stress	Upregulation of multiple NKG2D ligands in primary epithelial cells.	[59, 108]
Cancer related pathways	DNA Damage response	Multiple NKG2D ligands upregulated on receipt of DNA damaging chemicals and UV irradiation	[22]
	Oncogene Expression	For example MICA expression on chronic myeloid leukaemia is regulated by the BCR/ABL fusion oncoprotein	[23]
	Human and mouse cytomegalovirus (HCMV and MCMV)	Upregulation of multiple NKG2D ligands, which are targeted by immune evasion molecules	[27]
Viral Infection	Influenza A	Upregulation of ULBP1-3 on the cell surface of infected DCs	[109]
	Epstein Barr Virus (EBV)	Lytic infection in B cells leads to ULBP1 expression, but not other NKG2D ligands	[19]
	Adenovirus	E1A increases the expression of NKG2D ligands on the surface of mouse and human cancer cells	[20, 110]
	HIV	CD4+ T lymphocytes infected with HIV express ULBP1-3 at the cell surface	[111]
Bacterial Infection	<i>Mycobacterium Tuberculosis</i>	Infection causes upregulation of ULBP1 on monocytes	[112]
	<i>Escherichia coli</i>	Upregulation of MICA expression on the surface of epithelial cells on infection with pathogenic strain	[56]
	Proteasome inhibitors	Upregulation of ULBP2 in multiple cell lines. Bortezomib, activates DNA damage response and augments MICA expression in some multiple myelomas.	[113, 114]
Pharmacological	Trichostatin A (TSA) (HDAC inhibitor)	Increased upregulation of MICA/B and ULBP2 in cell lines	[113]
	Valproic Acid/ Sodium Valproate (VPA) (HDAC inhibitors)	Upregulates NKG2D ligand expression in leukemic blasts. Increased expression of MICA/B and lysis in hepatocellular carcinoma	[115, 116]
	Cisplatin	Activates DNA damage response pathways	[22]