New twist on the regulation of NKG2D ligand expression

Adelheid Cerwenka

The NK cell–activating receptor NKG2D plays a prominent role in antitumor immune responses. Expression of the multiple NKG2D ligands must be tightly controlled to guarantee that NK cells attack tumors but not healthy cells. New data reveal a novel mechanism of posttranslational regulation of the mouse NKG2D ligand MULT1, in which MULT1 is ubiquitinated and degraded in healthy cells. In response to UV stress or heat shock, ubiquitination of MULT1 decreases and cell surface expression increases. Thus, targeting the ubiquitination machinery in cancer cells might increase the susceptibility of tumors to NK cell–mediated killing.

NK cell activation is fine tuned by integrated signals delivered via activating and inhibitory receptors. The activating receptor NKG2D, which is expressed on NK cells, NKT cells, $\gamma\delta^+$ T cells, and some $CD8^+$ T cells, is critical for NK cell activation (for review see reference [1]). Crossing NKG2D-deficient mice with transgenic mouse models of cancer, for example, accelerated tumor growth, demonstrating the importance of NKG2D in tumor immunosurveillance (2).

NKG2D binds to a variety of ligands that resemble MHC class I proteins (for review see reference [3]). Mouse NKG2D ligands include the family of retinoic acid inducible genes-1 (RAE-1$\alpha-\varepsilon$), the minor histocompatibility antigen H60, two H60 variants (H60b and H60c), and mouse UL16-binding protein–like transcript 1 (MULT1) (4, 5). Human ligands include retinoic acid early transcript-1 proteins (RAET-1, originally called UL16-binding proteins [ULBPs]) and the highly polymorphic MHC class I chain–related proteins A and B (MICA and MICB). To date, at least nine ligands for mouse NKG2D and seven ligands for human NKG2D have been reported, and it is likely that this list is not yet complete.

NKG2D ligands are selectively detected on the cell surface of distressed, virus-infected, or malignant cells, but rarely on healthy cells, and their expression must be tightly controlled to avoid destruction of healthy cells. On page 287 of this issue, Nice et al. describe a new way in which MULT1 expression is controlled in healthy cells that involves ubiquitin-dependent lysosomal degradation of MULT1 protein (6).

Why so many ligands for one receptor?

When NKG2D ligands were first discovered, it was astonishing that there were multiple polymorphic ligands for one single nonpolymorphic receptor. Researchers initially suspected that more receptors for these ligands must exist, but so far none have been described. The concept thus emerged that the multiple NKG2D ligands help ensure that virus-infected and malignant cells are efficiently recognized by the NKG2D receptor. Evolutionary pressure to avoid escape mechanisms devised by certain viruses and cancers may have driven the diversity of NKG2D ligands. Moreover, different NKG2D ligands bind with distinct affinities to the NKG2D receptor, which may fine tune the extent of NK cell activation via NKG2D (7).

The expression of NKG2D ligands falls into two general categories. Transcripts of some ligands, such as RAE-1, are rarely expressed in healthy tissues but are detectable in tumors, virus-infected cells, and during embryogenesis. Transcripts of other ligands, including the MICs, ULBPs, and MULT1, are widely detectable in both healthy and diseased tissues. The extent of cell surface expression of these ligands, however, has not been conclusively addressed. Mechanisms that ensure cell surface expression of NKG2D ligands on distressed, but not on healthy cells, include cell- and tissue-specific stimuli that control both transcriptional and posttranscriptional processes.

Transcriptional regulation of NKG2D ligands

The molecular mechanisms that control NKG2D ligand expression at the transcriptional level are incompletely understood, and most promoters of NKG2D ligands remain poorly characterized (Fig. 1 A). RAE-1 molecules were initially identified by their induction in response to retinoic acid (RA) in a teratoma cell line, and a retinoic acid–inducible element was mapped in the promoter of *Rae-1γ*, suggesting that gene expression is directly induced by RA (8). Human MICA and MICB proteins were also up-regulated upon treatment with RA in hepatocellular carcinoma cells (9). The promoters of the *MICA* and *MICB* genes contain heat shock elements similar to those found in *HSP70* genes, which inducibly bind to heat shock factor-1 (10). Mouse embryonic fibroblasts deficient in JunB, a subunit of the transcription factor AP-1, also

A.C. is at Group of Innate Immunity, German Cancer Research Center, 69120 Heidelberg, Germany

CORRESPONDENCE
A.C.: a.cerwenka@dkfz.de

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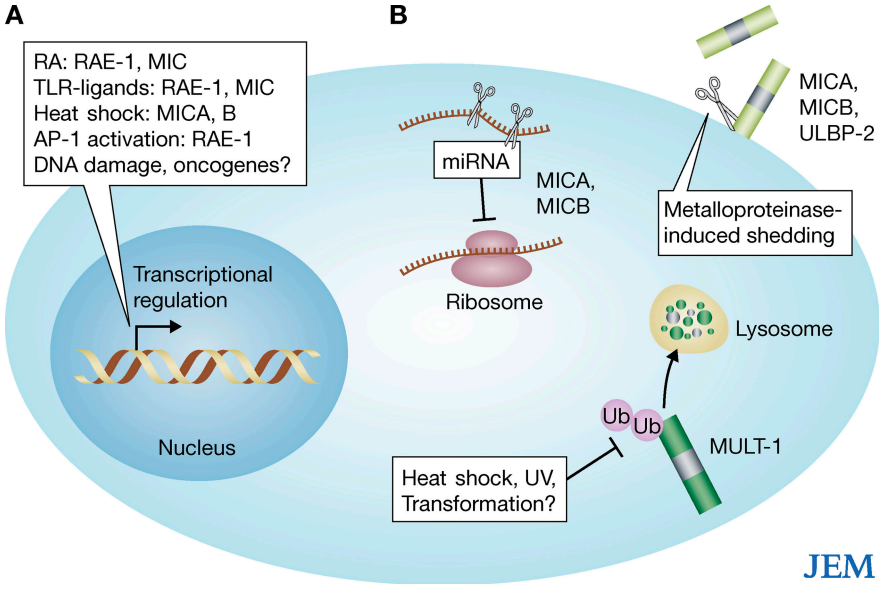

Figure 1. Transcriptional and posttranscriptional regulation of NKG2D ligands. (A) RA, TLR ligands, heat shock, and the transcription factor AP-1 induce the expression of certain NKG2D ligands, most likely via direct transcriptional regulation. The molecular mechanisms behind DNA damage– or oncogene-induced regulation of NKG2D ligands are incompletely understood. (B) Mechanisms of posttranscriptional regulation of NKG2D ligands include inhibition by microRNAs (miRNA) and metalloproteinase-induced shedding from the cell surface. Nice et al. report in this issue that MULT1 is ubiquitinated and degraded by the lysosome in healthy cells. Heat shock or UV irradiation decreases ubiquitination and induces MULT1 cell surface expression. It is currently unknown how tumor cells achieve cell surface expression of MULT1.

up-regulate *Rae-1ε* transcripts (11). Although several AP-1 binding sites were identified within the *Rae-1ε* promoter, the function of these sites was not investigated. Furthermore, transcripts of all RAE-1 family members, but not of MULT1 or H60, are induced in macrophages by Toll-like receptor ligands (12). DNA-damaging agents, including ionizing radiation, 5-FU, aphidicolin, cisplatin, and UV-C (13), induce the expression of NKG2D ligands via ataxia telangiectasia mutated (ATM) or ATM- and Rad3-related (ATR) protein kinases. Finally, oncogenes such as adenovirus E1A (14) up-regulate NKG2D ligand expression. To date, however, the exact molecular events linking the DNA damage pathway or the expression of oncogenes to up-regulation of NKG2D ligand transcripts and cell surface expression remain elusive and may involve both transcriptional and posttranscriptional events (Fig. 1).

Posttranscriptional and posttranslational regulation of NKG2D ligands

The recent discovery of posttranscriptional and posttranslational mechanisms of NKG2D ligand regulation has added more complexity to the picture (Fig. 1 B). IFN-γ was reported to decrease the expression of human MICA and ULBP-2 on melanoma cells (15) and of the mouse NKG2D ligand H60 on sarcomas (16). An IFN-γ–inducible microRNA was shown to mediate the down-regulation of MICA (17). MICA and MICB can also be shed from tumor cells by metalloproteinases (18, 19) in a process that requires the cell surface endoplasmic reticulum 5 protein (ERp5) (20). Soluble MICA/B and ULBP-2 can be detected in the serum of cancer patients, but not in healthy individuals, suggesting that tumors may exploit down-regulation of NKG2D ligands to escape from NK cell recognition.

Intriguingly, transcripts of mouse MULT1 have been detected in healthy tissues, including the thymus, although cell surface expression on thymocytes was not detected. In this issue, Nice et al. show that mouse MULT1 is ubiquitinated in normal cells, leading to its lysosomal degradation and preventing its expression at the cell surface (6). In response to heat shock or UV irradiation, ubiquitination of MULT1 was reduced. Genotoxic shock, on the other hand, had no effect on MULT1 ubiquitination. Therefore, heat shock might operate by two distinct mechanisms to up-regulate different NKG2D ligands. It activates the transcription of ligands such as MICA and MICB that contain heat shock promoter elements, and it inhibits the posttranslational degradation of other ligands, such as MULT1.

UV irradiation might likewise control different NKG2D ligands by different mechanisms. UV irradiation induces the activity of AP-1, which has been shown to control RAE-1ε expression, most likely by direct transcriptional regulation. And, as shown by Nice et al., UV irradiation also inhibits the ubiquitination and degradation of MULT1 (6). Whether UV irradiation and heat shock operate via similar mechanisms to induce MULT1 cell surface expression is currently unknown. Some tumor cells, such as YAC-1 cells, constitutively express MULT1, although it is unclear why MULT1 is not degraded in these cells. Tumor cells might constitutively activate heat shock responses that potentially counteract the degradation of MULT1, or they might be missing thus far unidentified components of the ubiquitination and/or degradation pathways. Future studies will be required to determine whether proteasome inhibitors further up-regulate MULT1 expression on tumor cells that already constitutively express the protein.

ULPB transcripts were initially found in various tissues in healthy individuals, including heart, lung, liver, and testis (21). MICA and MICB transcripts were also found in multiple tissues, with the exception of the central nervous system (22). Notably, MICA protein expression was exclusively detected on

enterocytes by immunohistochemistry staining (23). It is possible that cell surface expression of the transmembrane proteins MICA, MICB, and RAET-1G, which possess lysine residues within their cytoplasmic tails, is also controlled by ubiquitination. An additional post-translational mechanism must exist to control the cell surface expression of ULBP family members that are GPI-anchored proteins.

The concept that NKG2D ligand expression can be controlled by ubiquitinylation is not completely new. The E3 ubiquitin ligase K5 from Kaposi sarcoma virus was shown to down-regulate cell surface expression of MICA by ubiquitination of lysine residues in the protein's cytoplasmic tail (24). However, this ubiquitination did not correlate with an increased rate of degradation, but rather in a redistribution of MICA from the plasma membrane to an intracellular compartment. Therefore, even subsequent to ubiquitination, distinct mechanisms exist to control expression of different NKG2D ligands.

Multiple checkpoints operating at different levels of NKG2D ligand protein synthesis not only facilitate the expression of NKG2D ligands during different types of stress but might also fine tune the kinetics of cell surface expression. It is feasible that posttranslational regulation allows more rapid expression of NKG2D ligands on the surface of virus-infected or tumor cells than could be achieved via transcriptional regulation, thus ensuring rapid elimination of unhealthy cells.

NKG2D ligands in cancer therapy

Harnessing the natural ability of NK cells to attack tumors is a relatively new approach to cancer therapy (for review see reference [25]). Although NK cells are effective killers of most tumor cell lines in vitro, it is obvious that they do not effectively eliminate tumors in cancer patients. Strategies aimed at increasing both NK cell activation and tumor cell visibility to NK cells are currently being developed. These strategies will facilitate direct NK cell–mediated killing of tumor cells, and there is evidence that dying tumor cells subsequently alert the adaptive immune system.

The selective up-regulation of NKG2D ligands on tumor cells is one attractive approach. Small molecules that have been tested as antitumor agents, including the DNA damage-inducing cisplatin and 5-FU, up-regulate NKG2D ligands (13). The histone deacetylase inhibitor sodium valproate, currently being evaluated in clinical trials, also up-regulates NKG2D ligands (26). Moreover, low-dose application of the proteasome inhibitor bortezomib enhances expression of the human NKG2D ligands MICA and MICB on hepatocellular carcinoma cells (27). In this study, ligand induction occurred at both the transcriptional and cell surface protein levels, suggesting that multiple mechanisms were involved.

In their study, Nice et al. report that treatment of thymocytes with proteasome inhibitors induced MULT1 expression (6). However, it is unlikely that thymocytes are attacked by NK cells because they also express high levels of self-MHC class I molecules, which trigger inhibitory NK cell receptors. Tumors, on the other hand, are often characterized by low MHC class I expression. The treatment with proteasome inhibitors that further up-regulate NKG2D ligand expression on their cell surface might render these tumors highly susceptible to NK cell–mediated killing.

Open questions

10 yr after the discovery of NKG2D ligands, we are still far from a comprehensive understanding of their complex biology. Nice et al. (6) present a novel posttranslational control mechanism involving ubiquitination of the NKG2D ligand MULT1. The identification of the ubiquitin ligase and additional molecular events involved in this pathway will help us to understand why this mechanism operates in healthy cells, but not in tumor cells. It will also be crucial to provide experimental proof of the role of ubiquitination in the regulation of human NKG2D ligands.

Several small molecular compounds that are currently used in clinical trials up-regulate NKG2D ligand expression on tumor cells in vitro. Whether this also occurs in vivo, and whether the resulting increase in NKG2D ligand expression could activate NK cells in cancer patients, remains to be seen. In this context, it has been shown that chronic exposure to NKG2D ligands and soluble MIC that exists in the serum of some cancer patients down-regulates NKG2D expression and leads to deactivation of NK cells. This deactivation could be circumvented by therapeutic blockade of MIC shedding and/or subsequent adoptive transfer of NK cells. Small molecular compounds that induce NKG2D ligand expression on tumor cells combined with a NK cell–activating regimen could thus improve current clinical protocols in the treatment of cancer.

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