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Review

Ubiquitin and ubiquitin-like modifiers modulate NK cell-mediated recognition and killing of damaged cells

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Abstract: Efficient elimination of transformed and virus-infected cells by natural killer (NK) cells mainly depends on the recognition of "induced self" ligands by activating receptors, including NKG2D and DNAM1. The surface expression of these ligands in stressed or diseased cells results from the integration of transcriptional, post-transcriptional and post-translational mechanisms. Among post-translational mechanisms, recent findings indicate that ubiquitin and ubiquitin-like modifications, namely ubiquitination and SUMOylation, contribute to a very rapid negative regulation of NKG2D and DNAM1 ligand surface expression promoting either ligand degradation or ligand intracellular retention. On the other hand, accumulating evidences demonstrate that NKG2D receptor expression is down-regulated by ubiquitin-dependent endocytosis upon ligand stimulation. In this scenario, the overall consequence of the post-translational modifications of activating NK cell receptors and of their ligands on target cells is to impair effector cell-mediated recognition of damaged cells. Our review summarizes recent findings on the role of post-translational modifications in the modulation of target cell susceptibility to NK cell-mediated killing.

Keywords: NK cells; activating receptors; activating ligands; ubiquitination; SUMOylation

1. Introduction

Natural killer (NK) cells are innate lymphocytes rapidly activated during early stages of viral infections and tumor transformation [1,2]. Their cytolytic function against infected and transformed cells, as well as their ability to secrete cytokines and chemokines, is finely controlled by the integration of signals derived from inhibitory receptors which recognize major histocompatibility complex (MHC) class I molecules on healthy cells, and activating receptors, some ligands of which have been only partially characterized [3].

Among activating receptors, natural-killer receptor group 2, member D (NKG2D) and DNAX accessory molecule 1 (DNAM1/CD226) play a crucial role in "induced self" recognition in that their ligands are self-molecules up-regulated upon cellular stress, microbial infection and malignant transformation [4,5,6].

2. NKG2D and DNAM1 Activating Receptors and Their Ligands

NKG2D is a potent activating C-type lectin receptor expressed on all NK cells but also on CD8+ αβ T cells, γδ T cells, and activated CD4+ αβ T cells [7,8,9].

NKG2D-deficient mice show impaired surveillance towards spontaneous malignancies [10], and enhanced susceptibility to cytomegalovirus (CMV) infection [11], thus supporting a prominent role for NKG2D in NK cell-mediated immune responses.

Human NKG2D forms a hexameric complex with the transmembrane adaptor DNAX activating protein 10 (DAP10), which is required for signal propagation [12]. Ligand engagement promotes the phosphorylation of a tyrosine-based motif (YINM) in DAP10 intracellular domain that allows the recruitment of growth factor receptor-bound protein 2 (Grb2)/Vav1 complex and the activation of phosphatidyl-inositol-3-kinase (PI3K) [12,13]. Together, these signals are responsible for the consequent activation of phospholipase C gamma (PLCγ) 2, leading to cytotoxic granule secretion and cytokine synthesis [14]. A shorter NKG2D isoform that associates with either DAP10 or DAP12 adapters is expressed on activated murine NK cells and can initiate a signalling cascade involving the Syk/LAT pathway thanks to the immune tyrosine-based activation motif (ITAM) present in DAP12 cytoplasmic tail [15,16].

Remarkably, NKG2D can recognize multiple ligands that are all homologous to MHC class I molecules. NKG2D binds to two families of polymorphic ligands in humans: MHC class I related proteins (MIC) A/B and six UL16 binding proteins (ULBP1-6). MICA and MICB contain α1, α2 and α3 domains similar to MHC molecules and are transmembrane proteins, even though allelic variants of MICA exist that are linked to plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. ULBP proteins possess only α1 and α2 domains and are expressed either as transmembrane (ULBP 4 and 6) or GPI-linked (ULPB1-3 and 5) surface molecules [4,9,17].

Three subfamilies of ligands are known in mice (Rae-1α-ε, MULT1, and H60a–c). All are orthologous to the human ULPB family, and can be expressed as transmembrane (MULT1) or GPI-linked (Rae-1 and some H60 ligands) proteins [4,9,17].

DNAM1 is an immunoglobulin receptor expressed on the majority of NK cells as well as on monocytes, T cells and subsets of B cells [8,18]. It is an adhesion molecule with a clear role in

monocyte transendothelial migration [19] but it also contributes to tumor surveillance [20,21] and control of viral infections [22], as demonstrated in DNAM1-deficient mice.

DNAM1-mediated signalling cascade remains poorly defined. Ligand binding promotes the phosphorylation of a cytoplasmic serine by the protein kinase C (PKC) [23]. This phosphorylation allows the association of DNAM1 with the integrin LFA1 that is required for DNAM1-mediated signalling. Indeed, LFA1 crosslinking triggers the phosphorylation of a tyrosine in the DNAM1 cytoplasmic tail that, in turn, propagates intracellular signals required for cytotoxicity and cytokine production [24].

Both in humans and in mice DNAM1 interacts with the Nectin and Nectin-like family members Nectin2/CD112 and Nectin-like5/CD155 also known as poliovirus receptor (PVR) [25,26,27], even though murine DNAM1 binding to Nectin2 is still debated [28]. Nectin2 and PVR share a structure characterized by the presence of three immunoglobulin domains, while they differ in the cytoplasmic domain [27]. Like other members of the Nectin/Nectin-like family, Nectin2 and PVR are involved in cell-cell adhesion through the interaction with other members of the same family. Moreover, Nectin2 is also able to establish homophilic interactions.

2.1. NKG2D and DNAM1 ligands: molecular target for NK cell-mediated recognition

NKG2D ligands (NKG2DL) are almost absent in healthy cells but are found on the membrane of several tumors of epithelial and non-epithelial origin, including melanoma, leukemia, lymphoma, glioma, and hepatocellular carcinoma where their expression contributes to render tumor cells susceptible to NK cell-mediated killing [29–32]. Moreover, epithelial tumors that express MICA and MICB, including carcinomas of the lung, breast, kidney, ovary, prostate, and colon, are efficiently recognized by infiltrating γδ T cells [33].

Differently from NKG2DLs, DNAM1 ligands (DNAM1L) are widely expressed on normal cells, including neuronal, epithelial, endothelial and fibroblastic cells [27]. However, up-regulation of PVR and/or Nectin2 on solid and haematological cancers activates NK cell killing of these tumors in a DNAM1-dependent manner [34–38]. Notably, DNAM1-dependent cytotoxicity is particularly relevant against tumor cells that do not express NKG2DLs [39].

NKG2D and DNAM1 ligands play also a crucial role in infected cell elimination. They are rapidly induced on infected cells to alert the immune system. However, virus-encoded proteins often inhibit their cell surface expression in order to evade NKG2D and DNAM1-mediated NK cell recognition [5,18,40]. Both NKG2D ligands of the MIC and ULBP families are up-regulated on HCMV-infected fibroblasts [41–44], thus increasing the co-stimulation of CD8+ T cells by NKG2D and inducing NK cell activation. Concomitantly, DNAM1 participates to the NK cell-mediated killing of HCMV-infected cells since PVR is up-regulated upon infection [45]. Similarly to HCMV, HIV infection induces the expression of MICA, ULBP1-2 and PVR on virus-infected T cells rendering these cells susceptible to NK cell cytotoxicity [46,47,48]. ULBP1 and Nectin2 are up-regulated on EBV-infected B cells and activate NK cell cytotoxicity against these cells [49], while the expression of MICB on human macrophages upon infection with Influenza A or Sendai virus induces IFN-γ production in NK cells [50].

3. Mechanisms Regulating NKG2D and DNAM1 Ligand Expression on Stressed Cells

Expression of both NKG2D and DNAM-1 ligands is induced by different stress pathways associated with hyper-proliferation, malignant transformation and virus infection [4,5,6]. Notably, ligand expression can be the result of transcriptional, post-transcriptional and post-translational levels of regulation depending on the type of stress and cell context (Figure 1). Therefore, unravelling these different layers of regulation may allow the development of new therapeutic approaches aimed at improving NK cell-mediated immune surveillance.

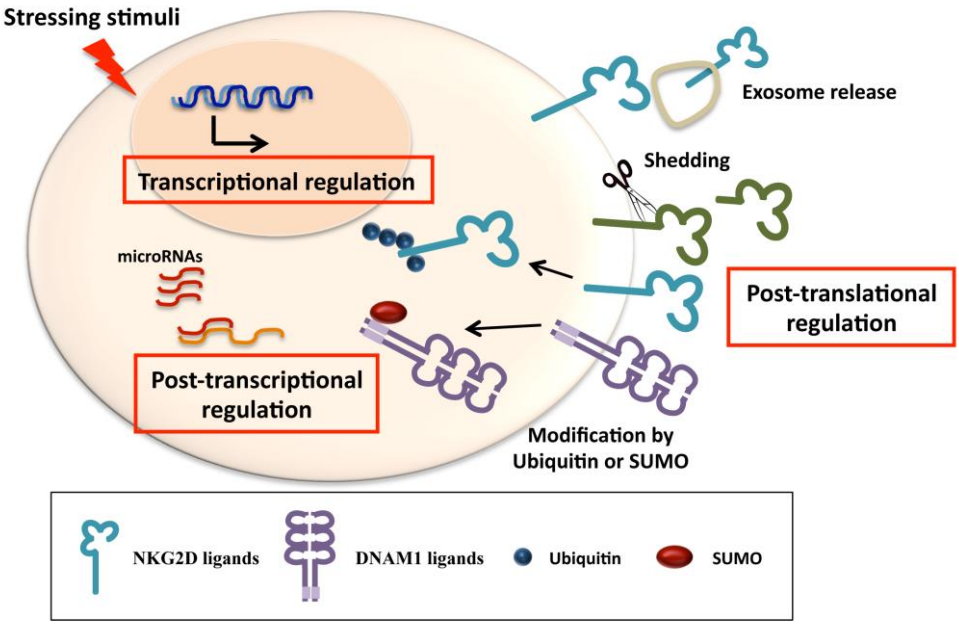

Figure 1. Regulation of NKG2D and DNAM1 ligand expression in response to stress stimuli. Mechanisms of post-transcriptional regulation include inhibition by microRNAs; mechanisms of post-translational regulations include metalloproteinase-induced shedding from the cell surface, release inside exosomes, and intracellular retention by ubiquitin or SUMO modification. (Figure modified from [6]).

Until now, much efforts have been mainly concentrated in the identification of molecular pathways involved in the transcriptional regulation of NK cell activating ligand expression in response to different types of stress. One of the best-characterized pathways is the DNA damage response (DDR), a signalling pathway involved in the maintenance of DNA integrity. In fact, DDR is activated in response to DNA lesions produced during replication or induced by stress-related stimuli including virus infection and genotoxic agents [5]. This pathway is implicated in the induction of MICA and PVR in healthy proliferating T cells [51,52] and in the up-regulation of MICA, ULBP2 and PVR on CD4+ T lymphocytes upon HIV infection [53,54,55]. Several lines of evidence demonstrated that up-regulation of both NKG2D and DNAM1 ligands is mainly driven by the DDR pathway also in cancer cells, further supporting a role for this pathway in alerting immune system against damaged cells [56–59].

In addition to DDR pathway, stressful stimuli including the heat shock pathway, the oxidative stress pathway and the endoplasmic reticulum stress response, as well as stimulation through Toll-like receptor (TLR) are responsible for NKG2D ligand and PVR transcriptional up-regulation [4,60,61]. In some cases, as during HCMV infection, viral proteins can directly be responsible for transcriptional regulation of these ligands [62]. Among transcription factors involved in NKG2D and DNAM1 ligand regulation, a role for NF-KB, AP-1, and E2F has been documented [51,61,63–66].

As formally demonstrated in the case of NKG2DLs, ligand transcript levels can also be regulated at mRNA level by different endogenous or virus-encoded microRNA [67–70].

Different post-translational mechanisms, including the release of soluble ligands and the Ubiquitin (Ub) and Ub-like modifications, are also implicated in the regulation of both NKG2D and DNAM1 ligand expression, and will be discussed further below.

3.1. Regulation of NK cell activating ligands by post-translational mechanisms

The best characterized post-translational mechanism is the production of NKG2DL soluble forms by tumor cells either by metalloproteinase-mediated shedding [31,71,72,73] or by their release on the membrane of exosomes [74,75], nanosized vesicles involved in intracellular communications (Figure 1). Regarding DNAM1Ls, soluble PVR forms, generated by alternative mRNA splicing, have been detected in sera of tumor patients [76]. However, the mechanism through which these soluble forms increase during transformation is largely unknown.

Post-translational mechanisms that repress surface expression of NKG2D and DNAM1 ligands have also been reported upon viral infection. Human CMV-encoded proteins UL16 and UL142 can cause NKG2DL intracellular retention [43,77,78], while UL141 promotes intracellular retention of an immature form of PVR [45] and the constitutive degradation of Nectin2 [79]. Similarly, HIV-Nef induces intracellular retention of PVR [48] and NKG2DLs of ULBP family [46]. Upon HSV-1 infection, MICA is down-modulated from the cell surface without any effect on total protein level [80], suggesting ligand intracellular retention.

These evidences support the conclusion that post-translational regulation of NKG2D and DNAM1 ligands may represent a general viral strategy to evade NK cell recognition.

Additional examples of post-translational regulation of NK cell activating ligands comprise the down-regulation of PVR surface expression and protein degradation in hepatocellular carcinoma upon activation of unfolded protein response (UPR) [81].

Regarding the molecular mechanisms implicated, Ub and small Ub-like modifier (SUMO) pathways appears to be deregulated in damaged cells, including virus infected and transformed cells [82,83,84]. In particular, the enzymes involved in ubiquitination and SUMOylation are often up-regulated in tumor cells, and several viruses exploit these pathways for their replication.

Interestingly, recent evidences reveal a previously unknown role for Ub and Ub-like modifiers as regulators of NKG2D and DNAM1 ligand expression, and provide novel insights in molecular mechanisms underlying expression of innate immune activating ligands on tumor cells [85–88].

3.2. NKG2D and DNAM1 ligand modification by ubiquitin and ubiquitin-like modifiers

Protein ubiquitination is a dynamic post-translational modification whereby the 76-amino acid protein Ub is covalently attached to a protein substrate. It is involved in nearly all aspects of eukaryotic biology due to the large number of proteins that may be subjected to this modification [89,90]. Ubiquitination, is catalyzed by the consecutive action of three classes of enzymes: the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2) and the Ub protein ligase (E3) that is responsible for substrate specificity. Once ubiquitinated, the substrate may undergo proteasome-dependent degradation as well as non-degradative fate, depending on the type of ubiquitin modification (Figure 2A). Indeed, Ub possesses seven lysine (K) residues that can be ubiquitinated, thus leading to the formation of different kind of Ub chains. In particular, whether the addition of K48-linked Ub chains signals for proteasomal degradation, modification by single Ub moieties (mono- and multi-ubiquitination) or by K63-linked Ub chains regulates endocytosis and trafficking of membrane proteins [89,91].

The first evidence of an NK cell activating ligand modified by ubiquitination came from studies on Kaposi's sarcoma-associated herpesvirus. The viral protein K5, endowed with E3 Ub ligase activity, has been shown to be responsible for MICA ubiquitination on cytoplasmic lysines [85], thus preventing ligand surface expression. However, MICA does not undergo Ub-dependent degradation but instead it is retained in intracellular compartments (Figure 3, left). Interestingly, MICB was shown to be subjected to the same mechanism of down-regulation, while a common MICA allele that lack the cytoplasmic tail (MICA*008) resulted resistant to K5-mediated down-regulation [85].

Murine NKG2D ligand MULT-1 has been shown to be constitutively ubiquitinated by the cellular orthologues of the Kaposi herpes virus-encoded Ub ligase on their cytoplasmic lysine residues [86,87]. Notably, MULT1 ubiquitination is responsible for ligand lysosomal degradation under normal conditions, and it is reversed in response to UV radiation and heat shock but not by other stressful stimuli [86], suggesting that only specific stressors impact on post-translational ligand modifications. No evidences are actually available in regard to DNAM1L ubiquitination.

Protein SUMOylation is a modification whereby the Ub-like modifier SUMO is covalently attached to the lysine residues of acceptor proteins. In analogy with Ub modification, SUMOylation occurs in three sequential steps catalyzed by different enzymes: a single E1, a single E2 and a small number of E3 enzymes [92,93]. Upon SUMOylation, target proteins do not necessarily undergo a degradative fate but instead they are subjected to conformational changes that modify their enzymatic activity or their ability to interact with other macromolecules (Figure 2B).

SUMO conjugation to protein substrates is frequently up-regulated in many tumors including breast and lung cancers, glioblastoma and multiple myeloma [92,93].

We have recently reported that this pathway impacts on activating ligand expression, thus impairing tumor cell recognition by NK cells [88]. Focusing on DNAM1Ls, we demonstrated that the SUMO pathway controls PVR, but not Nectin2 surface expression. PVR is directly subjected to SUMOylation, and this modification prevents its surface expression impairing DNAM1-mediated NK cell recognition [88] (Figure 3, left).

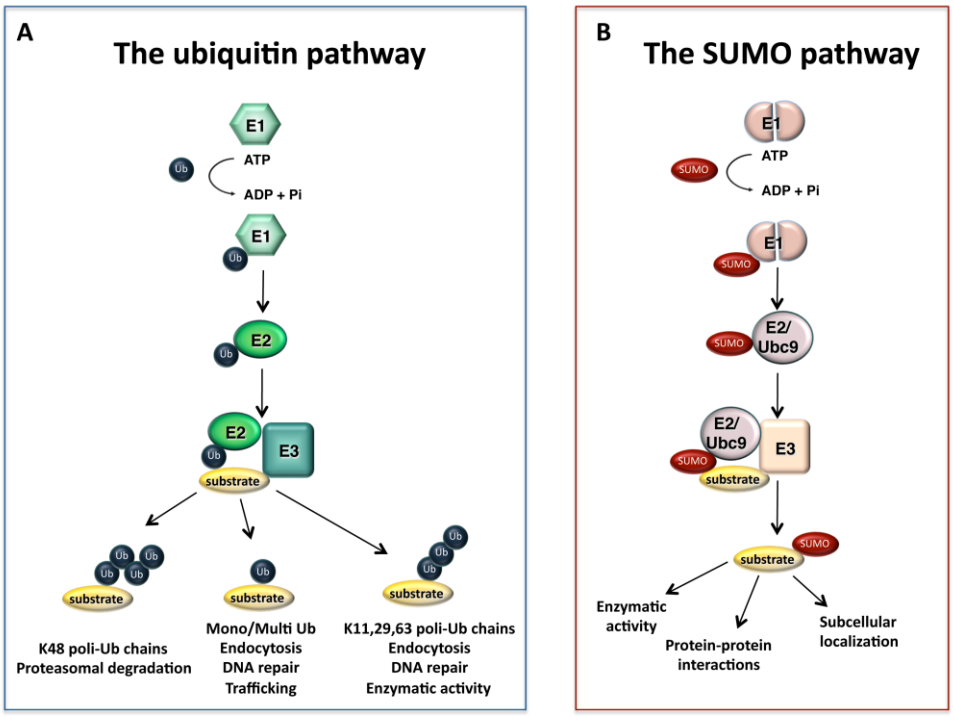

Figure 2. Comparison of ubiquitin and SUMO conjugation pathways. (A) The ubiquitin pathway. Attachment of ubiquitin (Ub) to a target protein requires three major enzymatic steps. In the first ATP-dependent step, Ub becomes covalently linked to the ubiquitin-activating enzyme E1 with a thiolester link. The activated Ub is then transferred to ubiquitin-conjugating enzyme (E2), which serves as a carrier protein. Ubiquitin ligase (E3) catalyzes the covalent attachment of Ub to the target substrate by the formation of isopeptide bonds. One or several Ub molecules can be conjugated to a substrate, determining its cellular fate. (B) The SUMO pathway. The conjugation pathway to SUMO is similar to the ubiquitin pathway, but it uses different E1 and E2 enzymes. The second step consists of a thiolester link that conjugates SUMO to Ubc9. This enzyme seems to have some intrinsic ligase activity for SUMO but the E3 protein might serve to increase the affinity between Ubc9 and the substrate allowing SUMOylation to occur at a maximal rate. SUMOylation may affect enzymatic activity, subcellular localization, and the ability to interact with other proteins. (Figure modified from Gasparrini et al., *Int Arch Allergy Immunol* 156: 16–26; 2011).

Intracellular retention of immature forms of MICA in endoplasmic reticulum followed by constitutive degradation has been observed in melanoma cells, and renders these cells able to evade NKG2D-mediated surveillance [94]. Similar results were obtained for MICB in a panel of tumor cell lines: this ligand is continuously internalized from plasma membrane and retained in intracellular compartments without being degraded [95]. Whether Ub or Ub-like modifications are directly implicated in MICA/B intracellular retention has not been investigated so far.

More recently, a similar behavior has been observed for ULBP1 that is continuously internalized and degraded in proteasome [96], suggesting that Ub or Ub-like modifications also affect GPI-linked molecules.

Altogether, these findings provide novel insights into the molecular mechanisms underlying NKG2D and DNAM1 ligand expression, and confer a role to post-translational modifications in preventing NK cell-mediated recognition and killing.

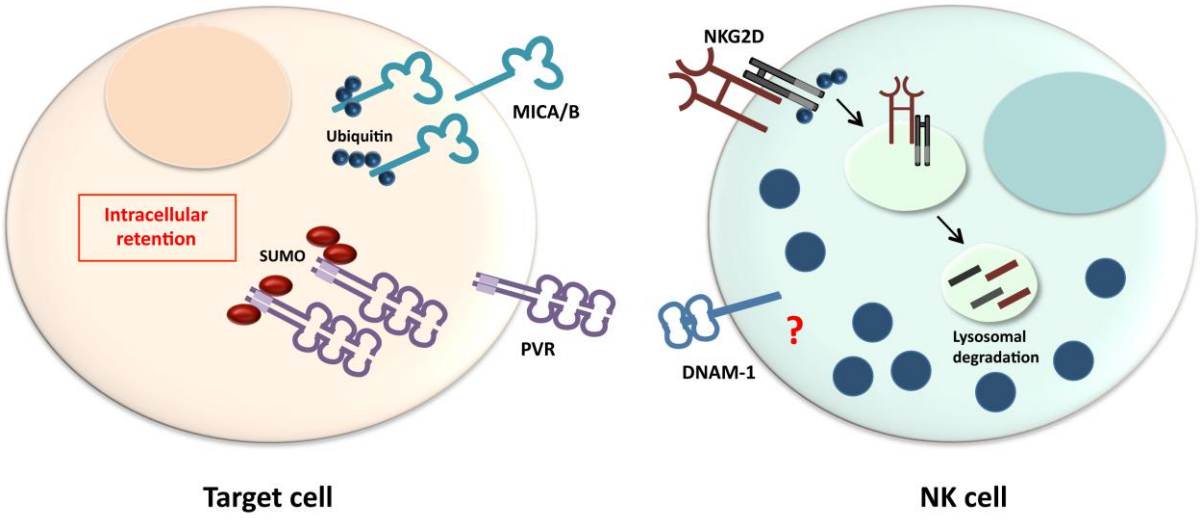

Figure 3. Model depicting how ubiquitin and/or SUMO modifiers prevent activating ligand surface expression on target cell (left), and provide a signal for internalization and trafficking of activating receptors on NK cell (right).

4. Regulation of NK Cell Activating Receptor Expression by Ubiquitination

The efficacy of damaged cell elimination also depends on the presence and relative abundances of activating receptors on the surface of NK cells.

In the case of NKG2D, its surface expression can be modulated under different conditions. For examples, several cytokines, i.e. interleukin-2 (IL-2), IL-7, IL-12 and IL-15, increase NKG2D/DAP10 transcripts and receptor surface expression, whereas others (i.e. TGF-β and IL-21) decrease NKG2D surface expression and promote a selective impairment of NKG2D-mediated cytotoxicity [97–104].

It is now well recognized that persistent exposure to NKG2D ligands also results in a decrease of NKG2D surface expression promoting receptor down-modulation and the consequent impairment of NK cell-mediated effector functions [72,105–111].

The main mechanism responsible for NKG2D down-modulation is ligand-mediated endocytosis [112]. Both human and murine NKG2D receptors are indeed removed from the plasma membrane by clathrin-dependent internalization [107,110], and then rapidly traffics through endosomal compartment till lysosomes where both NKG2D and its associated adaptor, DAP10, are degraded [105,111,113,114].

Recent findings indicate that ligand-dependent human NKG2D endocytosis requires DAP10 ubiquitination that provides a signal for both receptor internalization from plasma membrane and its sorting through the endocytic compartments to lysosomes for degradation [114] (Figure 3, right). Indeed, a mutated form of DAP10 that does not undergo ubiquitination strongly impairs both NKG2D/DAP10 internalization and lysosomal degradation [114]. Although not formally demonstrated in mouse, it is likely that the Ub pathway is also implicated in murine NKG2D endocytosis. Indeed, NKG2D/DAP10 receptor complex degradation was observed in a murine transgenic model overexpressing a DAP10-Ub fusion molecule [115].

Notably, some NKG2DLs possess intrinsic diverse ability to induce NKG2D endocytosis. In particular, MICA and ULBP2 differ in their capability to phosphorylate the Ub ligase c-Cbl and to activate the Ub pathway, being MICA the most efficient ligand [111]. Whether c-Cbl is the Ub ligase responsible for DAP10 ubiquitination is still unknown; however, these results suggest that the degree of NKG2D/DAP10 ubiquitination dictates the extent of receptor internalization and degradation.

Interestingly, Ub modification has been also previously demonstrated for CD16, the low affinity receptor for IgG responsible for NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) [116]. Ubiquitination of CD16 ζ subunit and the associated tyrosine kinases promotes endocytosis of engaged receptor complexes and render NK cells impaired in their ability to further perform ADCC [116–119].

Regarding DNAM1, less is known about the effect of cytokines on receptor surface expression. As in the case of NKG2D, IL-15 up-regulates DNAM1 mRNA level whereas TGF-β has the opposite effect [120]. Upon ligand binding, down-modulation of DNAM1 surface expression was observed on NK cells from patients affected by different tumors, including multiple myeloma, acute myeloid leukemia and ovarian carcinoma, as compared to healthy donors [35,36,121]. Notably, DNAM1 down-modulation leads to an impaired NK cell mediated natural cytotoxicity. The molecular mechanisms responsible for DNAM1 down-modulation have not been clarified yet. In particular, whether DNAM1 may undergo ubiquitination has not been investigated so far.

Altogether these results demonstrate that the Ub pathway may also negatively regulate the functional capability of NK cells by a direct covalent modification and down-regulation of their activating receptors.

5. Conclusion

Recent findings support a role for Ub and Ub-like pathways in the regulation of NKG2D and DNAM1 ligands. Those ligands undergo ubiquitination or SUMOylation, and are then degraded or subjected to intracellular retention with the consequent reduction of their surface expression. Of note, compared to transcriptional regulation this mechanism offer the opportunity for a much more rapid kind of regulation, since the ligands have been already transduced.

In healthy cells, these modifications may act to prevent ligand membrane expression and a potential dangerous NK cell recognition, while in viral infected or transformed cells may represent a mechanism to evade NK cell surveillance.

Upon contact with ligand-bearing targets, the Ub pathway may also contribute to down-regulate the surface expression of engaged activating NK cell receptors, as formally demonstrated for CD16 and NKG2D.

Altogether, these findings support the conclusion that post-translational regulation of NK cell activating receptors and their ligands may rapidly regulate the strength of NK cell-target interaction, and impair the ability of NK cells to eliminate dangerous cells.

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Conflict of Interest

All authors declare that they have no conflict of interest in this paper.

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