



# Post-translational Mechanisms Regulating NK Cell Activating Receptors and Their Ligands in Cancer: Potential Targets for Therapeutic Intervention

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Efficient clearance of transformed cells by Natural Killer (NK) cells is regulated by several activating receptors, including NKG2D, NCRs, and DNAM-1. Expression of these receptors as well as their specific “induced self” ligands is finely regulated during malignant transformation through the integration of different mechanisms acting on transcriptional, post-transcriptional, and post-translational levels. Among post-translational mechanisms, the release of activating ligands in the extracellular milieu through protease-mediated cleavage or by extracellular vesicle secretion represents some relevant cancer immune escape processes. Moreover, covalent modifications including ubiquitination and SUMOylation also contribute to negative regulation of NKG2D and DNAM-1 ligand surface expression resulting either in ligand intracellular retention and/or ligand degradation. All these mechanisms greatly impact on NK cell mediated recognition and killing of cancer cells and may be targeted to potentiate NK cell surveillance against tumors. Our mini review summarizes the main post-translational mechanisms regulating the expression of activating receptors and their ligands with particular emphasis on the contribution of ligand shedding and of ubiquitin and ubiquitin-like modifications in reducing target cell susceptibility to NK cell-mediated killing. Strategies aimed at inhibiting shedding of activating ligands and their modifications in order to preserve ligand expression on cancer cells will be also discussed.

**Keywords:** activating NK cell receptors, ligands for NK cell activating receptors, post-translational modifications, shedding, ubiquitin modification

## INTRODUCTION

Natural Killer (NK) cell activation is tuned by the integration of signals derived from inhibitory receptors for Major Histocompatibility Complex (MHC) class I molecules and from activating receptors that bind either non self-molecules associated to pathogens or self-molecules up-regulated in stress conditions including malignant transformation (1, 2).

Among activating receptors, Natural-Killer receptor group 2, member D (NKG2D), DNAX-associated molecule1 (DNAM-1), and the Natural Cytotoxicity Receptors (NCRs) play a pivotal role in NK cell-mediated tumor surveillance as revealed by an increased incidence of spontaneous malignancies or impaired tumor clearance

in mice deficient for these receptors (3–7). In human, NCR expression may represent a prognostic biomarker in acute myeloid leukemia (AML) and solid tumors (8–10). Moreover, the engagement of the low affinity receptor for IgG (CD16) by means of natural or therapeutic monoclonal antibodies can also contribute to tumor clearance through antibody-dependent cellular cytotoxicity (ADCC) (11, 12).

## NK Cell Activating Receptors and Their Ligands on Tumor Cells

NKG2D is a C-type lectin receptor not exclusively expressed on NK cells but also found on NKT, CD8<sup>+</sup>αβ T cells, γδ T cells, and activated CD4<sup>+</sup>αβ T cells (13–15). In humans, NKG2D binds to the adaptor DNAX activating protein 10 (DAP10), responsible for signal propagation. In murine activated NK cells, a shorter NKG2D isoform can either associate with DAP10 or DAP12, an alternative signal transducing adaptor (16, 17).

Human NKG2D ligands (NKG2DLs) belong to two families of polymorphic molecules structurally related to MHC class I: the MHC class I related proteins (MIC)A/B which possess α1, α2, and α3 domains similar to MHC molecules and six UL16 binding proteins (ULBP1–6) characterized by α1 and α2 domains (15, 18, 19). MICA and MICB are generally transmembrane proteins, while ULBP proteins can be transmembrane (ULBP4 and 6) or GPI-linked (ULPB1–3 and 5) molecules. Murine NKG2DLs include Rae-1α-ε, MULT1, and H60a-c and are expressed either as transmembrane or GPI-linked molecules (18).

DNAM-1 belongs to the immunoglobulin receptor family and is expressed not only on NK cells but also on monocytes, T cells, and subsets of B cells (14, 20, 21). It binds to Nectin2/CD112 and PVR/CD155 both members of the Nectin/Nectin-like family of adhesion molecules (22–24), and it associates with the integrin LFA1 to transduce intracellular signals.

Natural cytotoxicity receptors comprise NKp46, NKp44, and NKp30 immunoglobulin-like receptors that are not exclusively expressed on NK cells but also on innate lymphoid cells (ILCs) of group 1 (ILC1) and a subset of ILC3, γδ T cells, and a population of cytotoxic T lymphocytes (25, 26). Only ortholog of NKp46 is expressed in mice (26).

NKp30 and NKp46 associate with the signal transducing adaptors CD3ζ and FcεRIγ while NKp44 mainly signals through the DAP12 adapter. Splicing variants of NKp44 and NKp30 endowed with inhibitory signal capability have been described and are associated with worst prognosis in cancer patients (9, 27).

NCRs interact with several ligands that are either pathogen-encoded or self-molecules and include cell surface and intracellular proteins that reach the surface in infected or transformed cells (28). However, the ligands expressed on tumor cells have not been fully identified yet.

Each NCR has the ability to recognize a specific configuration of heparan sulfate proteoglycans expressed in the context of tumor microenvironment, and this binding can modulate receptor function (28).

Ligands for NKp30 include B7-H6 belonging to the B7 family and only expressed on tumor cells, the intracellular protein HLA-B associated transcript 3 (BAT3), also known as BAG6, and galectin-3 (29–31). The first two ligands bind to and activate NKp30 while the released form of galectin-3 inhibits anti-tumor NKp30 function.

NKp44 interacts with the Proliferating Cell Nuclear Antigen (PCNA), which is aberrantly expressed on the surface of tumor cells. This binding preferentially engages an inhibitory isoform of NKp44 and negatively regulates NK cell functions (32). Interaction between NKp44 and a subset of HLA-DP molecules has been recently reported (33) demonstrating that HLA class II molecules may impact on NK cell activity. Of note, NKp44 can be triggered by specific tumor-derived soluble growth factors (34) and by Nidogen-1, an extracellular matrix protein (35). NKp46 recognize viral ligands including hemagglutinins as well as tumor ligands of still unknown identity.

Most of the above mentioned NK cell activating ligands, including NKG2DLs and B7-H6, are absent in normal cells but their expression is induced upon neoplastic transformation, thus rendering tumor cells more susceptible to NK cell-mediated killing (29, 36–41).

On the other hand, PVR and Nectin2 are expressed on healthy cells (21) but their amount is up-regulated on epithelial and hematological tumor cells promoting NK cell cytotoxicity (7, 42–44).

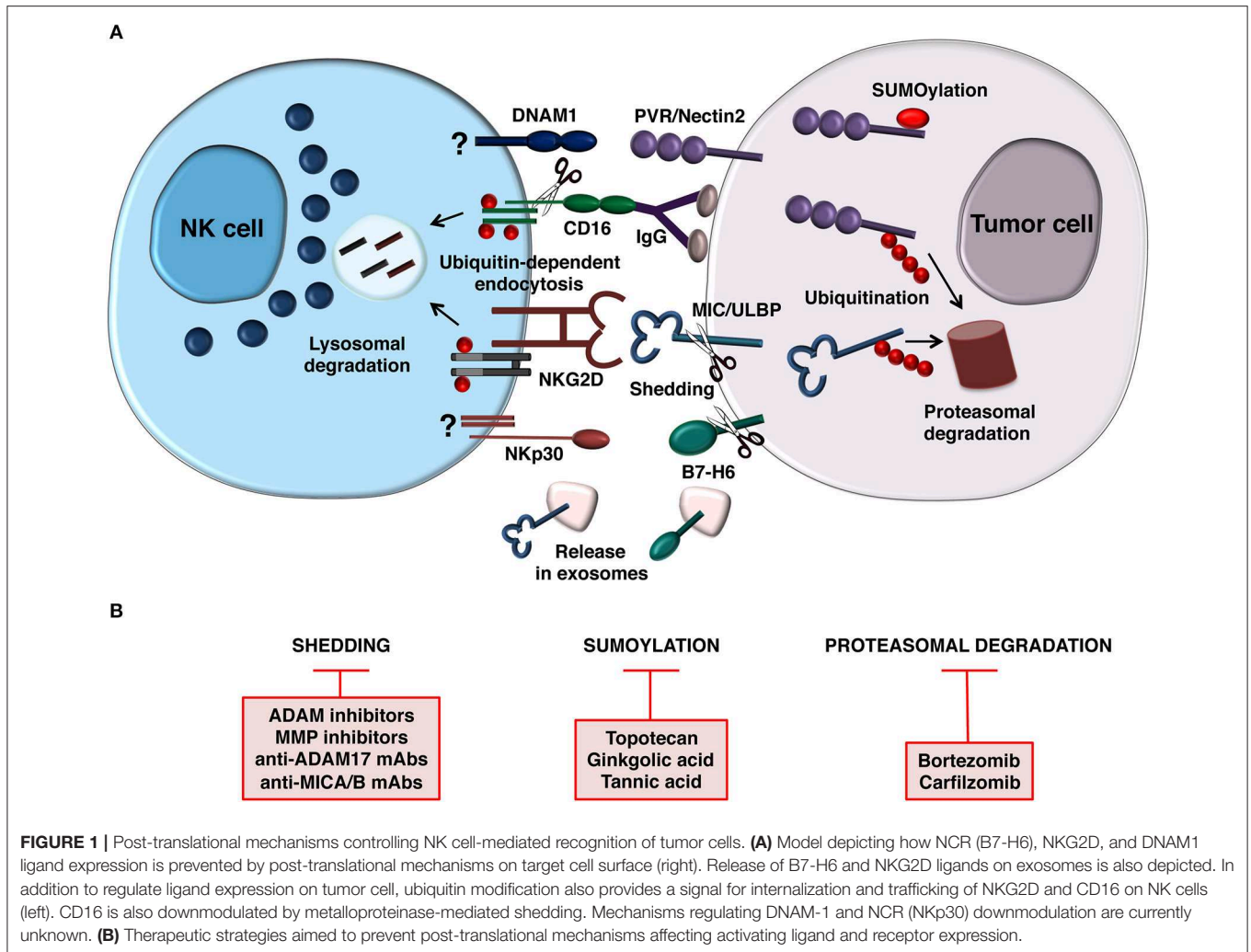
## POST-TRANSLATIONAL MECHANISMS MODULATING MEMBRANE EXPRESSION OF NK CELL ACTIVATING LIGANDS ON TUMOR CELLS

During malignant transformation different stressful stimuli are responsible for the induction of NK cell activating ligands at transcriptional and post-transcriptional levels and the molecular mechanisms implicated have been partially identified (18, 45). Moreover, increasing evidence demonstrate that post-translational mechanisms including the release of ligands for NK cell activating receptors as soluble forms as well as ligand modification by the Ubiquitin (Ub) or Ub-like pathways are used by tumor cells to dampen activating ligand surface expression in order to evade NK cell recognition (**Figure 1A**).

### Mechanisms Implicated in the Release of Ligands for NK Cell Activating Receptors

Most of the information regarding soluble ligands in cancer patients comes from studies performed on NKG2DLs. These molecules are present in the sera of patients affected by hematological or solid malignancies, and their level correlate with tumor stage and poor prognosis (46–53). More recently, B7-H6, BAG6, and PVR soluble forms have been found in the sera of patients affected by different type of tumors suggesting a relationship between soluble ligand expression and cancer progression (54–58).

Generation of soluble ligands relies on different mechanisms including alternative splicing, exosome secretion and proteolytic



cleavage. Soluble PVR isoforms are generated by alternative splicing (59) and have an inhibitory effect on DNAM-1 mediated tumor immunity (54). In addition, alternative splicing gives rise to ULBP-4/5 secreted ligands that can impair NK cell target recognition *in vitro* (60, 61).

Exosomes represents nanovesicles derived from the endosomal compartment (62) and have been involved in the secretion of NKG2D and NKp30 ligands but not of DNAM-1 ligands (63). Differently from the proteolytic-mediated release, expression of activating ligands on the exosome surface should retain their biological activity by keeping the integral-molecule. A number of studies have shown that NKG2DLs from both MIC and ULBP families are expressed on the surface of exosome-like vesicles released from ovarian cancer (63), melanoma (64), and prostate cancer cells (65). Remarkably, NKG2DLs such as ULBP3 and ULBP1 (66) or the allelic variant MICA\*008 (67, 68) that are glycosylphosphatidylinositol (GPI)-anchored proteins, are preferentially released via exosomes.

In regard to NKp30Ls, the nuclear protein BAG6 is secreted on exosomes and stimulates NK cell activity (69), whereas the

cell surface ligand B7-H6 can be released in its soluble form associated to exosomes or through protease-mediated cleavage (57, 70, 71). Although several stress conditions can increase exosome secretion from cancer cells (72–75), it is still uncertain whether the release of NKG2DLs or B7-H6 through exosome-like vesicles could result in the diminution of their expression on the cell surface.

Concerning the shedding process, MICA, MICB, and ULBP2 are cut by metalloproteinases belonging to two distinct families, the matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) (76–81), whereas the B7-H6 proteolytic cleavage occurs through a mechanism mainly dependent on ADAM enzymes (57). A recent study has shown that some ULBP4 isoforms are sensitive to the protease cleavage (82). Both MMPs and ADAMs proteases undergo modulation of their activity and expression in the course of neoplastic transformation (83, 84) and in response to cancer therapy (85–88). Disparate sensitivity to the proteases has been described for distinct NKG2DLs and/or allelic variants and isoforms. For instance, the generation of soluble MICA can be affected by

polymorphisms as shown for the MICA\*008 allele that is resistant to the protease-mediated cleavage. Moreover, the MICA-129 dimorphism, producing a valine to methionine swap at position 129, influenced the MICA cleavage process but the mechanism behind has to be defined (89, 90). In addition, proteolytic cleavage can be affected by fatty acylation and palmytolation that mediate MICA/B recruitment to membrane microdomains (78, 91).

Differently from the exosome-mediated release, the proteolytic cleavage of NKG2DLs and B7H6 has been associated to a reduction of cell surface ligands, thus its inhibition could be accomplished as a promising approach to keep the ligands on cancer cell surface and to promote anti-cancer immune response.

## Activating Ligand Modification by Ub and Ub-Like Pathways

Recent evidences reveal a role for ubiquitination and SUMOylation in the regulation of NK cell ligand expression on tumor cells.

Ubiquitination and SUMOylation are reversible modifications whereby Ub and small Ub-like modifier (SUMO), respectively, are covalently bound to a target protein through the action of enzymes frequently up-regulated during malignant transformation (92–95).

Once modified, proteins undergo different fate depending on the type of modification.

Proteins modified by poli-Ub chains are generally targeted to proteasomal degradation (95) whereas the addition of single Ub molecules to one or more lysine residues promote non-degradative fates including regulation of membrane protein endocytosis (96). SUMOylated substrates undergo conformational changes that in turn modify their interaction with other proteins or their enzymatic activity without inducing a degradative fate (94).

Little is currently known about the role of these modifications in the regulation of NK cell ligand expression during malignant transformation.

Ubiquitination of MICA/B has been demonstrated in Kaposi's sarcoma-associated herpesvirus infected cells: the viral E3 Ub ligase K5 induces modification of both NKG2DLs and their intracellular retention (97). Moreover, in healthy cells the murine ULBP-1 ortholog MULT-1 undergoes constitutive ubiquitination and lysosomal degradation (98, 99). Interestingly, stress conditions including UV radiation and heat shock prevent MULT-1 ubiquitination and increase its surface expression (98). Thus, these results support a negative role for the Ub pathway in the regulation of NKG2DL expression.

In tumor cells a direct implication of the Ub pathway has not been formally reported but several data demonstrate that surface expression of human NKG2DLs is regulated by a rapid protein turnover. In melanoma cells, an immature form of MICA accumulates in the endoplasmic reticulum and is targeted to degradation (100). MICB is internalized and retained intracellularly in several tumor cell lines (101), while in Multiple Myeloma (MM) cells the constitutive internalization of MICB is followed by its lysosomal degradation (102). Similarly, the GPI-linked ligand ULBP1 is continuously removed from

plasma membrane and targeted to proteasomal degradation (103). Regarding DNAM-1 ligands, in hepatocellular carcinoma the activation of Unfolded Protein Response (UPR) inhibits PVR surface expression and promotes protein degradation (104). In line with this result, ubiquitination and SUMOylation negatively regulates surface expression of Nectin2 and PVR on tumor cells (105, 106). Ubiquitinated Nectin2 is retained in intracellular compartments but also targeted to proteasomal degradation (106) whereas SUMOylation of PVR promotes its intracellular retention without inducing protein degradation (105). Inhibition of Ub and SUMO pathways increases Nectin2 and PVR surface expression and renders tumor cells more sensitive to NK cell-mediated killing.

Although these findings are currently limited to NKG2D and DNAM-1 ligands, they provide novel insights into the mechanisms underlying activating ligand expression in diseased cells and reveal novel potential targets for therapeutic intervention.

## LIGAND INDUCED DOWN-MODULATION OF NK CELL ACTIVATING RECEPTOR EXPRESSION

Tumor progression also implies the inability of NK cells to kill tumor cells as consequence of ligand mediated down-regulation of activating receptors (**Figure 1A**).

However, receptor down-regulation may be affected by the presence of soluble or membrane-bound ligands as well as by their affinity and/or avidity (19).

A decreased in NCR expression levels was observed in NK cells derived from patients affected by myeloid leukemia and other tumors upon the interaction with their respective ligands (8, 107).

Reduced Nkp30 surface expression has been also detected on NK cells derived from ovarian carcinoma and neuroblastoma patients as a result of chronic stimulation either with B7-H6-expressing tumor cells or soluble B7-H6 (55, 56, 108). Moreover, the presence of soluble BAG6 has been associated with a low transcriptional levels of different Nkp30 isoforms (58, 109).

DNAM-1 engagement, by membrane-bound ligands but not their soluble counterpart, is also followed by receptor down-modulation and impairment of NK cell functions in patients affected by different tumors including MM, ovarian carcinoma and AML (44, 110, 111). However, the mechanisms underlying these effects are still undefined.

For other activating receptors including NKG2D and CD16, mechanisms of ligand-induced down-modulation have been elucidated.

NKG2D stimulation by ligands expressed on tumor cells as well as by soluble ligands promotes receptor endocytosis and the decrease of NKG2D-dependent functions (46, 76, 112–115). In regard to released ligands, those associated to exosomes show a higher avidity and a more efficient ability to induce receptor down-regulation compared to shed ligands (66, 67).

In humans, internalization of ligand-engaged NKG2D receptors requires DAP10 ubiquitination and is followed by

lysosomal degradation (116). However, MICA is more efficient than ULBP2 in promoting receptor ubiquitination (114).

Ub modification has been also implicated in the down-modulation of CD16 in response to antibody-coated tumor cells (117–119). Indeed, CD16 clearance from NK cell surface is mainly induced by Ub-dependent endocytosis of aggregated receptors followed by degradation of CD16 $\zeta$  subunit and the associated kinases (114, 117, 120). However, CD16 down-regulation can also occur as a consequence of metalloproteinase-induced receptor shedding (121–124). Regardless, NK cell-mediated ADCC, natural cytotoxicity, and the efficacy of antibody-based therapies resulted impaired (118, 119, 125).

Altogether these results demonstrate that activating receptor expression is modulated in tumor microenvironment by the interaction with ligand-expressing cells, thus impairing NK cell ability to counteract tumor development.

## TARGETING POST-TRANSLATIONAL MECHANISMS REGULATING NK CELL-MEDIATED RECOGNITION AND KILLING OF CANCER CELLS

All these post-translational mechanisms represent potential targets for therapeutic intervention (**Figure 1B**). Ligand shedding blocking can be achieved by the usage of inhibitors of MMPs and ADAMs enzymes (57, 77, 126). Since ADAM10 and ADAM17 sheddases play a prominent role in B7-H6 (57) and NKG2DL cleavage (77, 78, 80, 127), the selective targeting of such enzymes might be promising for anticancer therapy. Recently, by performing an *in vitro* drug screen using an FDA-approved drug library, lomofungin was found to strongly decrease ADAM17 activity in hepatocellular carcinoma leading to the impairment of MICA shedding and has been proposed as new drug candidate for immunotherapy in liver cancer (128). Most of the compounds able to inhibit ADAM catalytic activity are hydroxamate-based and are either selective for ADAM17 or inhibitors of both ADAM10 and ADAM17 (129). Of interest, the synthesis of new selective ADAM10 inhibitors able to impair NKG2DL shedding in Hodgkin's lymphoma cell models has been reported (130). ADAM10 and ADAM17 are expressed at high levels on the surface of glioblastoma-initiating cells thus contributing to an immunosuppressive phenotype through the ULBP2 cleavage. Specific inhibition of these enzymes preserved cell surface ULBP2 leading to increased glioblastoma cell recognition and killing by NK cells (131). Remarkably, *in vivo* experiments using athymic nu-/nu- mice implanted with subcutaneous HeLa tumors demonstrated that systemic MMPi treatment resulted in the reduction of MICA serum levels and a concomitant augmentation of MICA expression on cancer cells reinforcing the immune cell therapy mediated by cytokine-induced killer cells (132). Of interest, adoptively transferred NK cells displaying high levels of surface NKG2D determined the clearance of soluble MICA in neuroblastoma patients by preserving NK cell cytotoxicity via non-occupied NKG2D (133).

Another appealing strategy to specifically inhibit MICA/B proteolytic cleavage concerns the generation of antibodies targeting the MIC protein domain involved in the proteolytic cleavage (134). Interestingly the usage of these antibodies limited MICA/B shedding in human cancer cells and repressed cancer cell growth in *in vivo* models (134). More recently, the glycosylation-engineered epitope mapping (GEM) method allowed to the identification of a number of epitopes relevant for MICA/B shedding inhibition (135).

In general, such antibodies as well as metalloproteinase inhibitors could be used in combination with other therapies aimed at the enhancement of ligand expression on the surface of cancer cells including DNA damaging agents (127), radiations (87), and chemotherapeutic drugs (132). Our group has shown that the combined use of metalloproteinase inhibitors and genotoxic drugs enhanced NK cell-mediated killing of multiple myeloma cells by preserving MIC molecules on the cell surface (127). To date, ADAMs inhibitors have been largely unsuccessful in clinical trials, but they remain a viable and desirable therapeutic target based on preclinical studies.

Strategies aimed at inhibiting ADAM17-mediated CD16 cleavage from the surface of NK cells could be also promising. Beyond the usage of inhibitors, recent advances in generating function-blocking antibodies of ADAM17 are emerging. The monoclonal antibody MEDI3622 has been shown to block CD16A cleavage from activated human NK cells allowing to an increased IFN $\gamma$  production in the course of ADCC (136).

Proteasome inhibitors can change the fate of ubiquitinated ligands. Bortezomib (Velcade) and Carfilzomib (Kyprolis) have been already used as chemotherapeutic drugs for relapsed MM patients (137–139) and for the treatment of mantle cell lymphoma (140).

In line with our findings (106), previous reports demonstrated that low doses of bortezomib increase NK cell activating ligand, including Nectin2 (141–143). Whether those drugs can directly affect ligand expression stabilizing ubiquitinated Nectin2 and/or SUMOylated PVR is currently unknown.

Regarding the SUMO pathway, the FDA-approved drug Topotecan has been shown to affect SUMOylation in glioblastoma multiforme (144). Moreover, natural compounds including ginkgolic acid and tannic acid (145, 146) have been found to possess anti-cancer activities by targeting the SUMO pathway (147).

All of these compounds hold great promise to be developed into novel and efficient anti-cancer drugs.

## CONCLUSION AND THERAPEUTIC PERSPECTIVES

On tumor cells, several activating ligands are subjected to protease-mediated cleavage with a consequent dramatic reduction of their surface expression. A similar effect is also achieved upon ubiquitination or SUMOylation of NKG2D and DNAM-1 ligands, which are retained intracellularly and/or degraded.

On NK cells, the Ub pathway may also contribute to down-regulate the surface expression of activating receptors.

In conclusion, all these post-translational mechanisms act to reduce NK cell-mediated surveillance against tumors and represent potential targets for therapeutic intervention.

Several inhibitors have been developed and their use in combination with conventional therapies represent a useful tool to potentiate NK-cell mediated recognition and killing of tumor cells.

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## AUTHOR CONTRIBUTIONS

RM, AZ, AS, and RP participated in the conception, writing, and elaboration of the final version of the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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