

# Key Activating and Inhibitory Ligands Involved in the Mobilization of Natural Killer Cells for Cancer Immunotherapies

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**Abstract:** Natural killer (NK) cells are the most potent arm of the innate immune system and play an important role in immunity, alloimmunity, autoimmunity, and cancer. NK cells recognize “altered-self” cells due to oncogenic transformation or stress due to viral infection and target to kill them. The effector functions of NK cells depend on the interaction of the activating and inhibitory receptors on their surface with their cognate ligand expressed on the target cells. These activating and inhibitory receptors interact with major histocompatibility complex I (MHC I) expressed on the target cells and make decisions to mount an immune response. NK cell immune response includes cytolytic activity and secretion of cytokines to help with the ongoing immune response. The advancement of our knowledge on the expression of inhibitory and activating molecules led us to exploit these molecules in the treatment of cancer. This review discusses the importance of activating and inhibitory receptors on NK cells and their clinical importance in cancer immunotherapy.

**Keywords:** natural killer cells, innate immune system, oncogenic transformation, cancer, activating and inhibitory receptors

## Introduction

Despite the rapid advancement of different therapeutic strategies, cancer is one of the most deadly diseases that affect millions of people worldwide.<sup>1</sup> Cancer develops in a host body by damaging the cells’ genetic component (mutation) beyond repair. These changes may occur spontaneously or by some agents like carcinogens or viruses.<sup>2</sup> Such changes lead to cellular signaling abnormalities that culminate in the growth and rapid proliferation of these “altered-self” cells to form tumors.<sup>3</sup> The host’s immune system recognizes these cancer cells as a foreign entity due to the expression of neoantigens on the cell surface.<sup>4</sup> These neoantigens are expressed on the surface of cancer cells through MHC class I molecules, taken up and processed by professional antigen-presenting cells (APCs). APCs present neoantigen to CD8<sup>+</sup> T cells and activate them. These activated CD8<sup>+</sup> T cells recognize and kill the cancer cells that express MHC class I molecules loaded with neoantigen-derived peptides.<sup>5</sup> Cancer cells can escape this immune surveillance despite these formidable defense mechanisms, develop tumors, and metastasize to distant organs.<sup>6</sup> Various mechanisms govern these immune-escape phenomena of cancer cells, including over-expression of immune checkpoint molecules, secretion of regulatory cytokines including TGF- $\beta$ , and downregulation of expression of MHC class

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I molecules on the cancer cell surface.<sup>7</sup> When the cancer cells reduced the expression of MHC class I bound neoantigens on their surface, they were no longer recognizable by the antigen-specific CD8<sup>+</sup> T cells.<sup>8</sup> Natural killer cells are innate immune cells that can eliminate foreign particles, including viruses and cancer cells. NK cells recognize the absence of MHC class I molecules on cells and eliminate them.<sup>9,10</sup> NK cells express different sets of activating and inhibiting receptors on their surface. Based on the activities of these receptors, NK cells are licensed to perform their cytotoxic action.<sup>11</sup>

Currently, researchers are trying to modulate the NK cells to promote antitumor immunity by activating the activating receptor or blocking the inhibitory receptors.<sup>12</sup> In this review, we have described the role of different activating and inhibitory receptors on antitumor functions of NK cells and how we can exploit NK cell activity to develop various therapeutic strategies to promote the antitumor function of NK cells.

## NK Cell Biology – A Brief Overview Development, Classification, and Memory of NK Cells

NK cells are innate lymphoid cells that show potent cytotoxic potential against virus-infected cells or altered-self cells like tumor cells.<sup>13</sup> In peripheral blood, mature NK cells lack expression of T cell receptors (TCR) and CD3 molecules.<sup>14</sup> In humans, the presence of the neural cell-adhesion molecule (NCAM or CD56) identifies mature NK cells, which is absent in mice.<sup>15</sup> NK cells in C57BL/6 and NZB mice express NK1.1 molecule (also known as CD161; Killer cell lectin-like receptor subfamily B, member 1, KLRB1), but other mice strains (such as BALB/c, SJL, AKR, CBA, and C3H) lack the expression of this molecules. The majority of NK cells in all the strains of mice can be identified using monoclonal antibody DX5. NK cells develop from CD34<sup>+</sup> hematopoietic stem cells known as common lymphoid progenitor cells (CLPs) in the bone marrow. CLPs originate in the bone marrow.<sup>16</sup> They mature in the primary and secondary lymphoid organs and can persist in the periphery for longer time.<sup>16</sup> These NK cells have cytotoxic or regulatory phenotypes depending on the expression of various activating and inhibiting receptors on their surface and interaction with their cognate ligands.<sup>17</sup> The expression of various surface molecules and their interaction with the surroundings classifies human NK cells into different

functional subsets. CD11b<sup>+</sup>CD27<sup>-</sup>CD56<sup>dim</sup> NK cells show a potent cytotoxic function. These cells also express several activating receptors on their surface, thus exhibiting potent activating signals. CD11b<sup>-</sup>CD27<sup>-</sup>CD56<sup>bright</sup> NK cells show a tolerogenic function and show inhibitory receptor expression. CD11b<sup>+</sup>CD27<sup>+</sup>CD56<sup>bright</sup> NK cells perform various regulatory functions and are widely distributed in various organs, including the liver and uterus.<sup>18–20</sup> Unlike CD4<sup>+</sup> or CD8<sup>+</sup> T cells, NK cells are also reported to show immunological memory. It has been shown that following mouse-cytomegalovirus (MCMV) virus infection, NK cells can show long-lived self-renewing antigen-specific memory phenotype and respond to recall response.<sup>21,22</sup> NK cell memory response is discussed elsewhere<sup>23–25</sup> and beyond the scope of this review.

## Licensing, Activation, and Function of NK Cells

NK cell activation and function depend on their engagement with different sets of activating and inhibitory receptors with their ligands.<sup>26</sup> Based on the type of receptor engagement, NK cells decide whether to kill or not to kill the target cells.<sup>27</sup> The primary signal for NK cell-mediated killing is triggered by the absence of MHC class I molecules on the surface of the target cells.<sup>28</sup> The killer cell immunoglobulin-like receptors (KIRs) recognize and bind to MHC class I molecules. The downstream signaling from KIRs suppresses the activation and subsequent cytotoxic killing of target cells. During the development of NK cells, the exposure of KIRs to self MHC molecules governs NK cell maturation and provides tolerance to the destruction of self-cells by NK cells. This complex process is called the “licensing” of NK cells.<sup>29</sup> The number of MHC class I molecules on the target cells dictates the strength of cytotoxicity by NK cells. In mature NK cells, the KIR-mediated inhibition is not an absolute phenomenon. One or more strong activating stimuli can reverse this inhibition.<sup>30</sup>

In addition, another mechanism of NK cell-mediated killing is antibody-dependent cellular cytotoxicity (ADCC).<sup>31</sup> In this process, the CD16 receptor (FcγRIIIA) on the NK cell surface recognizes the Fc regions of immunoglobulin<sup>32</sup> and targets to kill immunoglobulin-coated opsonized target cells.<sup>32</sup> Various cytokines are also known to govern the NK cell cytotoxicity.<sup>30</sup> IL-2 and IL-15 activate NK cells and promote their survival

and proliferation.<sup>33</sup> IL-12 and IL-18 also activate NK cells and are potent inducers of IFN- $\gamma$  production by NK cells.<sup>34</sup>

Once NK cells are activated and licensed to kill the target cells, they kill them in two ways. Either they are directly lysing the cells by releasing perforin or granzyme in the vicinity of the target cells or by receptor-induced target cell apoptosis. During NK cell degranulation, lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) and -2 (LAMP-2 or CD107b) transiently appear on the NK cell surface. The expression of LAMP-1 on the NK cells is a marker for indirect measurement of NK cell cytotoxicity.<sup>35</sup> Perforin produces pore in the target cell, and then granzyme enters the cell and destroys the target cells.<sup>27,36,37</sup> NK cells also express TNF receptor ligands which include FasL, TRAIL-L, and TNFR.<sup>38</sup> Interaction of these ligands present on NK cells with their cognate receptor on target cells induces apoptosis of the target cells.<sup>39,40</sup> The development, licensing, activation, and cytotoxic function of NK cells ultimately depends on the interaction between activating and inhibitory receptors on NK cells and their ligands on target cells. In the next section, we have described various activating and inhibitory receptors on NK cells and their role in antitumor immunity.

## Activating Receptors on NK Cells and Their Role in Antitumor Immunity

NK cells contain an extensive repertoire of activating and inhibitory receptors (Table 1). These germline-encoded receptors do not require “V(D)J” recombination for their diversity and functions. When these receptors interact with their ligands, they transmit either stimulatory or inhibitory signals to NK cells. In addition to this signal, the interaction of activating receptors on NK cells and their cognate ligand on the target surface is essential for optimum activation and cytolytic capacity of NK cells. The intracellular domain of the activating receptor contains immunoreceptor tyrosine-based activation motifs (ITAMs). Interaction of activating receptor-ligand complexes leads to phosphorylation of ITAM by the Src family of tyrosine kinases such as Lck, Fyn, Src, Yes, Fgr, and Lyn (Figure 1). These signaling pathways directly activate the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathway.<sup>41</sup> This signaling culminates in elevated intracellular Ca<sup>2+</sup> levels and actin cytoskeleton rearrangement, promoting the release of cytotoxic granules and the killing of target cells.

NK group 2 calcium-dependent lectin-like family of receptors performs both activating and inhibitory functions on NK cells. Among them, NKG2D has a stimulatory role on NK cell activity. Apart from NK cells, NK T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and  $\gamma\delta$  T cells also express NKG2D. In mice, NKG2D interacts with retinoic acid early inducible-1 family of proteins (Rae-1 $\alpha$ - $\epsilon$ ), murine UL16-binding protein-like transcript 1 (MULT1), and H60 group of proteins (H60a, H60b, H60c).<sup>42</sup> In humans, NKG2D recognizes MHC class I chain-related protein A (MICA) and B (MICB) and UL16-binding proteins (ULBP1-6). MICA/B was found to be expressed in cancer with epithelial origin<sup>43-45</sup> but less expressed on hematological malignancies.<sup>46-49</sup> In contrast, expression of ULBPs is predominantly known on leukemia<sup>49,50</sup> but relatively rare in solid cancers. The expression of Rae-1 and MULT1 is variable in different forms of cancers.<sup>51-54</sup> The broad expression of NKG2D ligands makes them a suitable target for developing many forms of NK-based antitumor therapy, which we have discussed in the later sections.

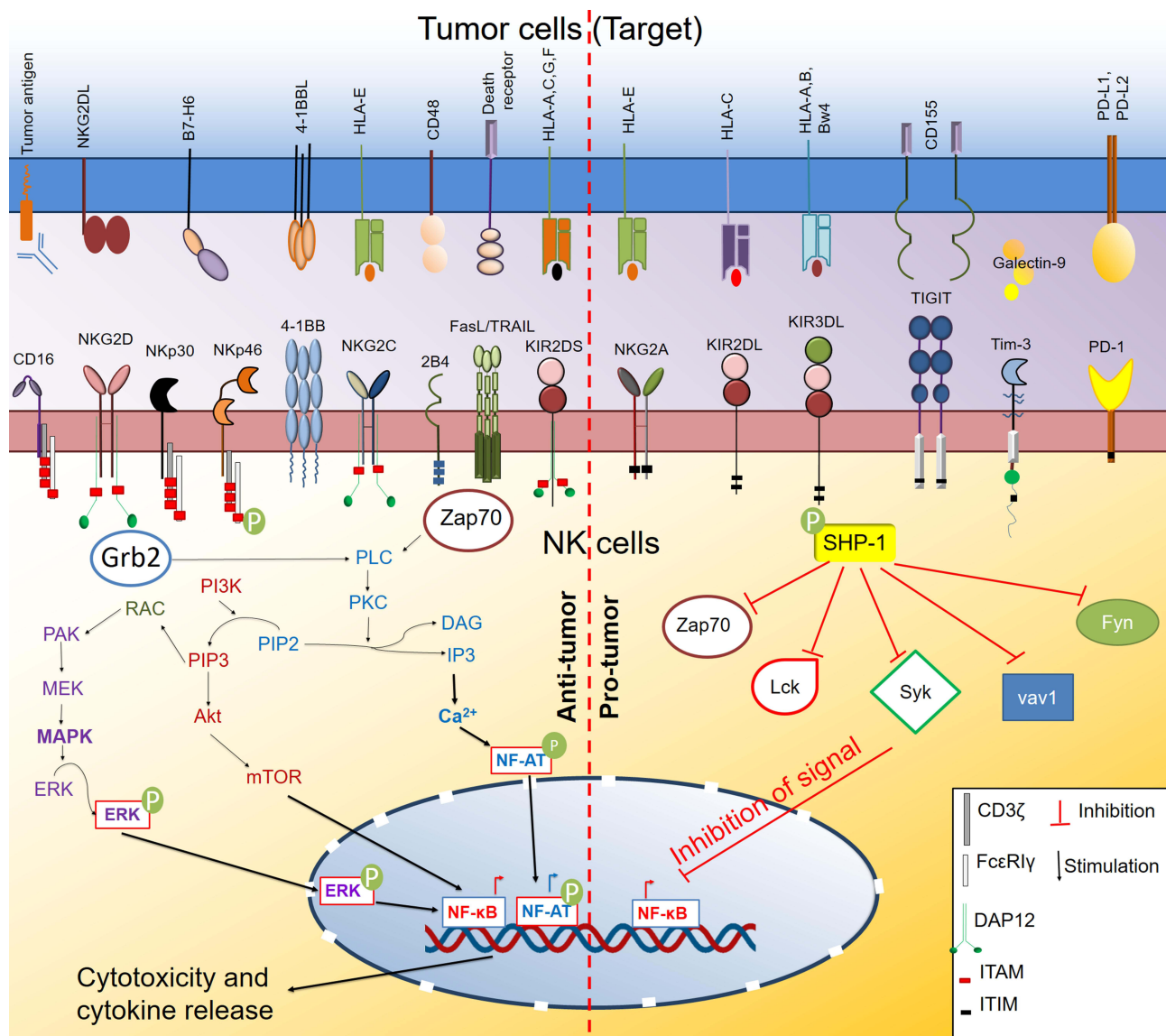
Ly49 group of genes is highly polymorphic and analog to the human KIRs. They follow two nomenclature systems Ly49 and Killer Cell Lectin-like Receptor subfamily A (KLRA).<sup>55</sup> Most of the Ly49 group of receptors (Ly49A, Ly49B, Ly49C, Ly49E, Ly49F, Ly49G, Ly49I, and Ly49O) are inhibitory, and some receptors (Ly49B, Ly49D, Ly48H Ly49P, Ly49R, and Ly49W) exhibit activation functions in mice.<sup>55</sup> They are associated with DAP12 adapter molecules and transduce signals through ITAMs. Ly49H binds to the glycoprotein m157 protein of murine cytomegalovirus (MCMV), responsible for the resistance to MCMV infection.<sup>56</sup> Other Ly49 activating receptors such as Ly49D, Ly49P, and Ly49W recognizes H-2D<sup>d</sup> in mice.<sup>57-59</sup> Ly49D engagement on T lymphocyte through MHC class I ligand expressed on target cells and known to promote CD4 and CD8 T cells functions and their cytotoxic potential.<sup>60</sup> NK cells also recognize MHC molecules on the tumor cells through Ly49D and involve in tumor rejection.<sup>61</sup> The few subtypes of the killer cell immunoglobulin-like receptors (KIRs) family, which are activating in nature, mostly HLA-specific activating receptors, are associated with DAP12 adapter protein for their signal transduction.<sup>62</sup>

The 2B4 receptor (CD244) is a group of molecules that act as both activating and inhibitory receptors. Though it is inhibitory in mice,<sup>63,64</sup> in humans, the expression of CD244 ligand (CD48) on the target cells enhances NK

Table 1 Activating Receptors on NK Cells and Their Targeting Approach for Anti-Tumor Immunity

Receptors	Ligands	Targeting Approach	Cancers	Targets	Clinical Trial	Clinical Trial Identifier
NKG2D	Mouse- RAE-1 $\alpha$ - $\epsilon$ , MULTI, H60 Human-MICA/B, ULBP1-4	BIKE	Multiple myeloma	CSI-NKG2D	Pre-clinical	-
		Monoclonal antibody to prevent MICA/B shedding CAR-NK	Melanoma and colon cancer (mice) Solid tumors	MICA/B NKG2D ligands	Pre-clinical Phase I	- NCT03415100
NKG2C	Mouse-Qa1b Human-HLA-E	NKG2C overexpressed NK cells	CD73 <sup>+</sup> tumors Human lung cancer xenograft	CD73 via NKG2D	Pre-clinical	-
			Solid tumors and leukaemia cell lines	NKG2D-DAP10-CD3 $\zeta$		
CD16	Fc region of IgG	BIKE	CD19 <sup>+</sup> HLA <sup>low</sup> target cells	NKG2C	Pre-clinical	-
		TriKE	Hodgkins lymphoma	CD16 and CD30	Phase II	NCT02321592
4-1BB	4-1BBL	Agonistic antibody (Utomilumab) monotherapy/in combination with rituximab	High risk haematological malignancies	CD16/IL-15/CD33	Phase II	NCT03214666
		Agonistic antibody (Urelumab)	Lymphoma	4-1BB	Phase I	NCT01307267
2B4 (CD244)	CD244 ligand	CAR-NK	Ovarian cancer xenograft in mice	NKG2D-2B4-CD3 $\zeta$	Pre-clinical	-
Ly49D	Mouse:H-2D <sup>d</sup>	-	-	-	-	-
Ly49H	Mouse:m157 of MCMV	-	-	-	-	-
NKp30	Human: B7-H6, BAT3, pp65 of HCMV, PIEMPI of plasmodium falciptarum, viral HA	CAR-T	B7-H6 expressing tumors	NKp30 and CD28	Pre-clinical	-
NKp46	Heparin, viral HA and HN	-	-	-	-	-
NKP44	Viral HA and HN, PCNA and proteoglycans	-	-	-	-	-

DNAMI	Mouse and human- CD112, CD155	Overexpression on NK-92 cells	Sarcoma	DNAMI	Pre-clinical	-
FasL	Fas	-	-	-	-	-
TRAIL-L	TRAIL	-	-	-	-	-
DAPI2 (coreceptor)	Interacts with many activating receptors	CAR-NK	Prostate cancer	Prostate stem cell antigen (PSCA)	Pre-clinical	-
KIR2DS1	Human: HLA-C2	-	-	-	-	-
KIR2DS2	Human: HLA-C1	-	-	-	-	-
KIR2DS3	Unknown	-	-	-	-	-
KIR2DS4	Human: HLA-A11	-	-	-	-	-
KIR2DS5	Unknown	-	-	-	-	-
KIR3DS1	Human: HLA-Bw4	-	-	-	-	-
KIR2DL4	Human: HLA-G	-	-	-	-	-



**Figure 1** Activating and inhibitory receptors on NK cells and their signaling pathway. NK cells express various activating and inhibitory receptors. Upon interaction with their cognate ligands on the target (tumor) cell surface, they initiate a downstream signaling pathway that ultimately dictates the effector and cytotoxicity function of NK cells. The interaction of the activating receptor with their ligand initiates primarily mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt)/Mammalian target of rapamycin (mTOR) pathway that activates nuclear factor- $\kappa$ B (NF $\kappa$ B) transcription factor. Activating receptors also activate the  $Ca^{2+}$  signaling pathway through phospholipase  $C\gamma$  (PLC $\gamma$ )-protein kinase C (PKC) axis that further activates the nuclear factor of activated T cells (NF-AT) transcription factor. Activated NF- $\kappa$ B and NF-AT drive the production of cytokines and regulate the cytotoxic functions of NK cells. In contrast, the interaction of inhibitory receptors with their ligand activates the Src homology region 2 domain-containing phosphatase-1 (SHP-1) that inhibits the functions of downstream signaling kinases and inhibits the activation of transcription factors like NF- $\kappa$ B and concurrently inhibits NK cell effector and cytotoxic function.

**Abbreviations:** 2B4, also known as Signaling lymphocyte activation molecule 4 (SLAMF4) or CD244; 4-1BB, also known as Tumor necrosis factor ligand superfamily member 9 (TNFSF9) or CD137; CD16, also known as type III Fc $\gamma$  receptor (Fc $\gamma$ RIII); CD48, also known as B lymphocyte activation marker (BLAST-1) or signaling lymphocytic activation molecule 2 (SLAMF2); CD155, also known as Poliovirus receptor (PVR); DAG, Diacylglycerol; DAP10/12, DNAX activating protein 10/12; DNAM-1, DNAX accessory molecule-1; ERK, Extracellular signal regulated kinase; FasL, also known as TNFSF6, CD95L or CD178; Fc $\epsilon$ R1 $\gamma$ , Fc epsilon receptor 1 gamma; Grb2, growth factor receptor bound protein 2; HLA, Human leukocyte antigen; IP3, Inositol triphosphate; NKG2D/A/C, natural killer group 2D/2A/2C member; ITAM, Immunoreceptor tyrosine-based activation motif; ITIM, Immunoreceptor tyrosine-based inhibitory motif; KIRs, killer cell immunoglobulin-like receptors; Lck, Lymphocyte-specific protein tyrosine kinase; MAPK, Mitogen-activated protein kinase; MEK, MAPK kinase; NKp30, also known as Natural cytotoxicity receptor 3 (NCR3) or CD337; NKp46, also known as Natural cytotoxicity receptor 1 (NCR1) or CD335; PAK, p21 (PAC1) activated kinase; PD-L1, programmed cell death ligand 1 (also known as CD274, B7H1); PD-1, programmed cell death protein-1; PIP3, phosphatidylinositol (3, 4, 5)-triphosphate; TIM-3, T cell immunoglobulin and mucin-domain containing 3; RAC, Ras-related C3 bolulinum toxin substrate; Syk, Spleen tyrosine kinase; TIGIT, T cell Immunoglobulin and ITIM-domain; TRAIL, Tumour necrosis factor (TNF)-related apoptosis-inducing ligand; Vav1, Vav guanine nucleotide exchange factor 1; ZAP70, Zeta-chain-associated protein kinase 70.

cell-mediated killing of the target cells.<sup>65,66</sup> The activating or inhibitory receptor's function also depends on the association of downstream adapter molecule EAT-2, ERT, SAP, and other signaling pathway.<sup>28</sup>

Natural cytotoxicity receptors (NCRs) are immunoglobulin superfamily receptors that recognize many ligands, including bacterial, viral, parasite proteins, and several molecules from tumor cells. Mouse NK cells only express NKp46 while human NK cells express NKp46 (NCR1 or CD335), NKp44 (NCR2 or CD336), and NKp30 (NCR3 or CD337), and their expression is quite maturity and activation-dependent. Scientists have observed that NKp46 deficiency led to the impaired clearing of T cell lymphoma, melanoma, and lung metastasis.<sup>67</sup> B7-H6, a ligand for NKp30, is essential for human NK cell-mediated killing of K-562 cell.<sup>68,69</sup> People have tried to target the NKp30 pathway via different approaches to improve antitumor functions like CAR-NK therapy.<sup>70,71</sup>

CD38 is another activation molecule expressed on the NK cell surface that enhances the cytotoxicity of NK cells. CD38 is an enzyme that catalyzes the conversion of beta-nicotinamide adenine dinucleotide (beta-NAD<sup>+</sup>) and beta-nicotinamide adenine dinucleotide 2'-phosphate (beta-NADP<sup>+</sup>) into cyclic adenosine diphosphate-ribose (ADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP).<sup>72</sup> Mallone et al showed that targeting CD38 with agonistic antibody enhanced the cytotoxic potential of NK cells by enhancing IFN- $\gamma$  and GM-CSF release.<sup>73</sup>

Costimulatory adhesion receptor DNAM1 is also responsible for tumor recognition by NK cells. It binds PVR and nectin-2, and its signaling leads to tumor recognition by NK cells and the migration of NK cell.<sup>74,75</sup> The importance of DNAM1 in colorectal cancer, breast cancer, AML, and melanoma has been described.<sup>76–79</sup> Not only cancer cells but cancer-associated fibroblasts can also inhibit NK cell function through DNAM1 signaling.<sup>80</sup>

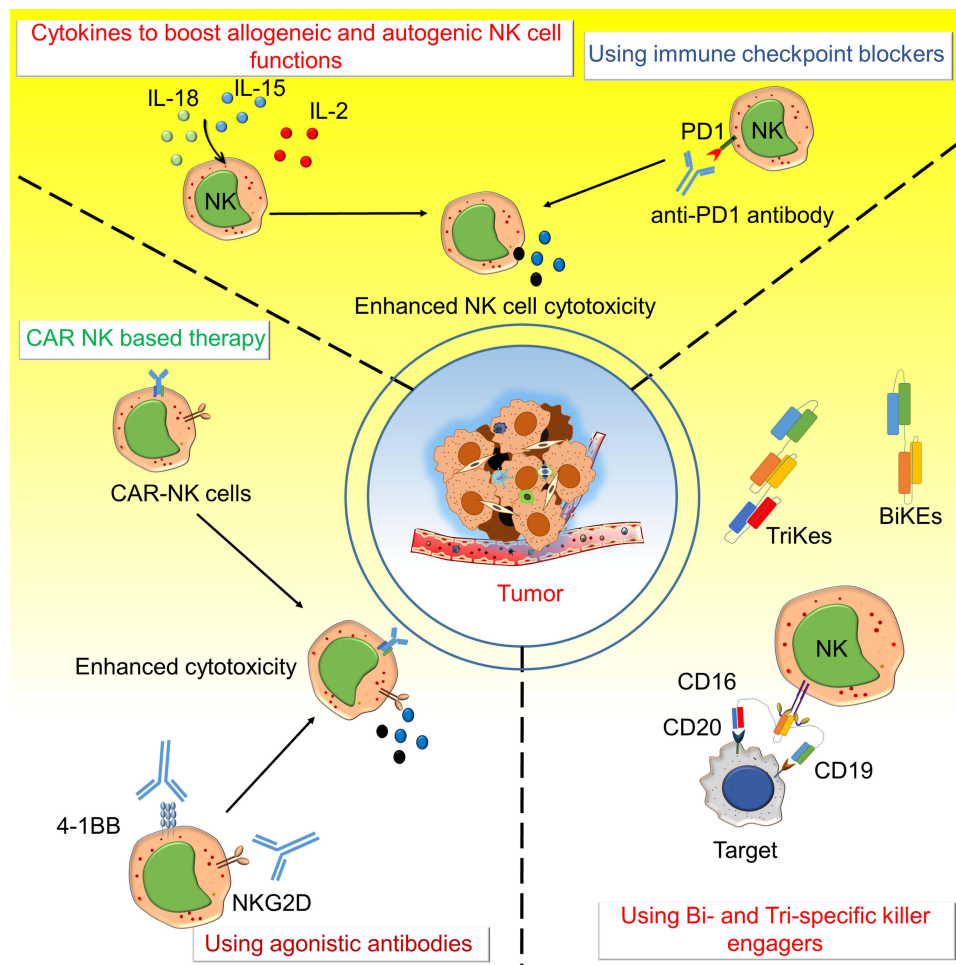
CD16 (FC $\gamma$ RIIIA) is an immunoglobulin superfamily of receptors expressed on NK cells and other immune cells that recognize Fc regions of antibody opsonized on target cells. This mechanism is called antibody-mediated cellular cytotoxicity (ADCC).<sup>62</sup> The NK cells then recognize and target to kill that antibody-coated target cell.<sup>81</sup> About 40% of total NK cells in the body also express CD32 (FC $\gamma$ RIIC). The expression of isoforms of CD32 (FC $\gamma$ RIIC) on NK cells varies among humans.<sup>82</sup> Among different isoforms, FC $\gamma$ RIIC-1 is more prominent among humans. Ernst et al showed that FC $\gamma$ RIIC-1 isoforms expressing CD32<sup>+</sup>NK cells show a potent cytotoxic

property than other isoforms expressing NK cells.<sup>83</sup> Scientists also tried to target -ADCC mechanism by divalent or trivalent antibodies (BiKEs and TriKEs) to enhance the antitumor response of NK cells. Either alone or combined with other receptors and costimulatory molecules, these activating receptors activate NK cell functions. As this receptor signaling promotes NK cell cytotoxicity, they act as a potential target to promote the antitumor response of NK cells.

## Inhibitory Receptors on NK Cells and Their Role in Tumor Immunity

NK cell-mediated cytotoxicity and release of proinflammatory cytokines can play an essential role in innate defense against viral infection and tumor cells. To restrain the over-activation of NK cells and protect normal healthy cells from NK cell-mediated cytolysis, scientists have promulgated the missing self-hypothesis.<sup>84,85</sup> This hypothesis states that cells without self-MHC class I were susceptible to NK cell-mediated lysis. This observation played a significant role in discovering NK cell inhibitory receptors and describing their regulatory function.

The ITIM motif in the cytoplasmic tail of the inhibitory receptors governs the inhibitory signaling in NK cells. There are mainly two inhibitory signaling pathways.<sup>86</sup> In this pathway, ITIM phosphorylation upon receptor–ligand interaction leads to recruitment of phosphatases such as Src homology-containing tyrosine phosphatase 1 (SHP-1), SHP-2, and lipid phosphatase SH2 domain-containing inositol-5-phosphatase (SHIP). SHP-1 and SHP-2 are involved in dephosphorylation of immunoreceptor tyrosine-based activation motif (ITAM)-containing Vav-1 and src family of tyrosine kinases such as Lck and Fyn (Figure 1). Early dephosphorylation of Vav-1 by SHP-1 inhibits actin-dependent NK cell activation signal blocking cytotoxicity.<sup>28</sup> Lck and Fyn belonging to the Src family of tyrosine kinase, are involved in the activation of the NK cells through the ITAM motif of activating receptor. Protein kinases ZAP70 and Syk act downstream of Lck tyrosine kinase. Engagement of NK cell inhibitory receptors inhibits tyrosine phosphorylation of Lck, ZAP70, and Syk via SHP-1 blocking NK cell activation.<sup>87,88</sup> In the other pathways, Abl-1 kinase phosphorylates the Crk adaptor, dissociating from signaling complexes formed upon NK cell activation. The signaling through inhibitory receptors can be independent and also involve several other receptors and co-receptors.<sup>86</sup>



**Figure 2** Various approaches to target NK cell activating and inhibitory receptors for anti-tumor immunity. The activating and inhibitory receptors on NK cells are targeted by several different strategies like Chimeric antigen receptors – natural killer (CAR-NK) cells, bi-specific killer engagers (BiKEs), and tri-specific killer engagers (TriKEs), agonistic antibody to NKG2D and 4-1BB, immune checkpoint blockers like an anti-PD1 antibody, and using various cytokines to boost allogeneic and autogenic or iPSC derived NK cells functions to boost anti-tumor immunity. CARs on NK cells were developed to target various molecules expressed on cancer cells to direct the NK cell-mediated killing of cancer cells. BiKEs and TriKEs mainly engage some tumor antigens with Fc receptors (CD16/CD32) of NK cells and enhance cytotoxic activity. Various agonistic antibodies target the activating receptors such as NKG2D and 4-1BB on NK cells and antagonistic antibodies of immune checkpoint molecules like anti-PD1 to enhance anti-tumor activity. Different cytokines (IL-2, IL-15 and IL-18) are also used to increase the NK cell survival and activity.

**Abbreviations:** NKG2D, natural killer group 2D member; PD-1, programmed cell death protein 1; iPSC, induced pluripotent stem cell; IL, interleukin.

Broadly, there are two types of inhibitory receptors regulating NK cell activation; HLA specific and non-HLA specific inhibitory receptors. The HLA-specific inhibitory receptors comprise KIR/CD158 family, CD94: NKG2A, LILRB1, and Ly49 inhibitory receptors.<sup>89</sup> The murine Ly49 inhibitory receptors are structurally different from human KIR but perform a similar function, ie, recognizing class I MHC and delivering inhibitory signals to NK cells. Human KIR recognizes polymorphic class I specific HLA molecules, such as HLA-A, HLA-B, and HLA-C, whereas NKG2A recognizes non-classical HLA-E molecule.<sup>90,91</sup> NKG2A is expressed during the early stages of NK cell development. KIR expression occurs later in mature NK cells.<sup>92</sup> Several highly aggressive

tumors (such as pancreas, lung, colon, liver, stomach, head, and neck) are found to overexpress the HLA-E ligand, which binds to NKG2A inhibitory receptor<sup>93,94</sup> and inactivates NK cells. In the tumor microenvironment, secretion of anti-inflammatory cytokine TGF- $\beta$  along with T cell exhaustion upregulated NKG2A expression on CD8<sup>+</sup> T cells.<sup>94-96</sup> The expression profile of inhibitory receptors on NK cells varies across different subsets with differentiation. CD56<sup>bright</sup> NK cells from peripheral human blood efficiently proliferate and secrete proinflammatory cytokines upon activation. However, these cells predominantly express the NKG2A receptor and are poorly cytotoxic. On the other hand, CD56<sup>dim</sup> NK cells are strongly cytotoxic and express NKG2A, KIR, or both.<sup>97</sup> There is



a progressive decline in the proliferation capacity with terminal differentiation and an increase in cytotoxicity of NK cells. In cytomegalovirus (CMV) infection, most mature NK cells in seropositive individuals comprise CD57<sup>+</sup>KIR<sup>+</sup>NKG2A<sup>-</sup> NK cells. These cells also activate NKG2C receptors and display adaptive immune characteristics such as recognizing HLA-E bound CMV peptides and show selective proliferation.<sup>98,99</sup> This pathway leads to the generation of immune cell memory allowing for NK cell-mediated prevention of CMV re-infection in the future. LIRB1 also called LIR1 inhibitory receptor, could recognize and interact with classical (HLA-A, HLA-B and HLA-C) and non-classical MHC class I (HLA-G) molecules.<sup>89,100,101</sup> LIRB1 also binds to UL18, a human CMV class I MHC homolog.<sup>102,103</sup> High expression of LIR-1 and KIRs on NKG2A<sup>+</sup>CD57<sup>+</sup> NK cells characterizes NK cell memory in CMV-positive patients.<sup>104</sup>

The non-HLA-specific inhibitory receptors on NK cells such as PD-1, SIGLEC-7, LAIR-1, and IRp60, along with HLA class I specific inhibitory receptors NKG2A, KIR, etc. play a significant role in regulating NK cell-mediated immune response. Several studies have shown the role of non-HLA-specific inhibitory receptors in regulating pyroptosis, inflammation, phagocytosis, immune cell proliferation, and response to viral infection.<sup>105–107</sup>

As the signaling through activating and inhibiting receptors governs NK cell cytotoxicity and modulates the killing of target cells, they are of interest to cancer immunologists. Some of the signaling from activating and inhibitory receptors are depicted in Figure 1. There are different ways to modulate receptors on NK cells and promote antitumor response, as shown in Figure 2. In the later section, we discussed several approaches used by scientists to manipulate the activating and inhibitory receptor functions to enhance antitumor functions of NK cells.

## Targeting the Stimulatory and Inhibitory Receptors of NK Cells

### Activating Receptors

#### Enhancement of Activation Receptor-Mediated Cytolytic Activity

In ADCC, the engagement of the CD16 receptor on NK cells with opsonized target cells through the Fc region of an antibody mediates NK cell cytotoxicity.<sup>108</sup> The CD16 receptor on NK cells can be activated using bispecific killer engagers (BiKEs) (Figure 2). BiKEs contain two single-chain variable antibody fragments that

simultaneously bind a tumor antigen and CD16 receptor to NK cells and show promising results against solid tumors and leukemia.<sup>109</sup> BiKEs are targeting epithelial cell adhesion molecules (EpCAMs), HER2, and EGFR for solid tumors or CD19, CD33, and CD20 for hematological malignancies. CD16 on NK cells showed a good response against both solid tumors and acute myeloid leukaemia.<sup>109</sup> NK cell activating receptor NKG2D is also targeted through BiKEs. A bispecific antibody CS1-NKG2D containing anti-CS1scFv and an anti-NKG2D scFv showed potently and dose-dependent cytotoxicity against multiple myeloma both in vitro and in vivo.<sup>110</sup> Scientists also developed another tetravalent BiKE AFM13 that contains tandem antibodies against CD16 and CD30. AFM13 has a higher affinity for the Fc receptor and has a longer half-life than other BiKEs and TriKEs. AFM13 also shows good tolerability and safety in relapsed/refractory Hodgkin's lymphoma patients, and 77% of patients had stable disease.<sup>29,111,112</sup> It is currently in Phase II monotherapy clinical trial in relapsed/refractory Hodgkin's lymphoma.<sup>113</sup> Tri-specific Killer engagers (TriKEs) (Figure 2) have also been developed, which link CD16 to CD19 and CD22.<sup>114,115</sup> TriKEs were modified to deliver IL-15 in the vicinity of action and also improved the durability of NK cell response.<sup>116</sup> Vallera et al showed that CD33/IL-15/CD16 TriKEs has a potent anti-cancer response and prolonged in vivo persistence due to IL-15.<sup>116</sup> This TriKE strategy (GTB-3550) is currently in a clinical trial to treat high-risk hematological malignancies (NCT03214666).<sup>116</sup> 1615EpCAM TriKE (EpCAM/IL-15/CD16), also developed that targets EpCAM on many cancers of epithelial origin, significantly showed enhanced NK cell proliferation, cytolytic properties, and cytokine secretion in vitro.<sup>117</sup> Gauthier et al also reported using engagers in improved NK cell response when linking tumor antigens with both CD16 and NKp46 in NK cells.<sup>118</sup>

Chester et al reported that anti-4-1BB agonistic antibodies have potent antitumor responses in mice.<sup>119</sup> However, treatment with anti-4-1BB monoclonal antibody urelumab in patients with advanced-stage solid tumors increases the level of interferon-induced cytokines and transaminitis at a higher dose.<sup>120</sup> However, another anti-4-1BB monoclonal antibody, utomilumab, is well tolerated in 55 patients. With this treatment, two out of 15 patients with advanced Merkel cell carcinoma showed a durable response.<sup>121</sup> Researchers also tried to target NKG2D function to stimulate NK cell activity. In tumors expressing

a high level of NKG2D ligand, MICA/B can down-regulate NKG2D expression on NK cells and CD8<sup>+</sup> T cells. Antibody targeting MICA/B, IPH4301 potentiates the cytotoxic properties of NK cells in vitro and in vivo and prevents NKG2D down regulation.<sup>12</sup> Ferrari et al showed that antibody targeting  $\alpha 3$  segment of MICA that prevent soluble MICA/B shedding enhance the antitumor activity in multiple murine and human tumor models, mainly by stimulating NK cell activity through NKG2D and CD16.<sup>122</sup>

### Use of Allogeneic NK Cells for Cell for Immunotherapy

NK cells from allogeneic donors are advantageous in cell-based therapy as they do not result in graft versus host disease (GVHD) as with allogeneic T cells. Alloreactive NK cells are delivered to the host either by adoptive cell transfer or thorough hematopoietic stem cell transfer (HSCT). Allogenic NK cells are prepared mainly from cell lines, adult donor lymphapheresis products, or umbilical cord blood cells. There are few benefits in using allogeneic NK cells over autologous cells. As these cells are educated in healthy donors than in tumor-bearing individual, they are more cytotoxic and possesses better antitumor activity.<sup>123</sup> Another study by Ruggeri et al showed that KIR ligand mismatches between donors and patients are associated with better outcomes in myeloid leukemia after T-cell-depleted haploidentical hematopoietic cell transplantation.<sup>124</sup> Allogeneic NK cells could be expanded in vitro using various doses of cytokines such as IL-2, IL-15, and IFN- $\gamma$  before administering to the patients.<sup>125</sup> Immunosuppression is a prerequisite for NK cell adoptive transfer or in HSCT. Combining NK cells adoptive transfer with a chemotherapeutic regimen with high doses of cyclophosphamide (Cy) and fludarabine (Flu) led to better in vivo expansion of NK cells.<sup>126</sup> This Hi-Cy/Flu regimen led to pancytopenia and induction of IL-15 secretion in vivo, which resulted in the expansion of allogeneic NK cells.<sup>127,128</sup> Geller et al also used this Hi-Cy/Flu regimen in NK cell-based therapy in metastatic recurrent breast cancer settings that already underwent phase II clinical trial.<sup>129</sup>

### CAR-Modified NK Cells in Cancer Immunotherapy

The recent development of CAR T cells widened the horizon of cancer immunotherapy. CAR-T cells targeting CD19 in B cell acute lymphoblastic leukemia (B-ALL) showed

promising outcomes in other relapsed non-Hodgkins lymphoma with 70–90% success rates. FDA has also approved this CAR-T cell therapy to treat ALL and diffuse large B cell lymphoma. Currently, scientists have developed various CAR-T cell approach to treat several hematological malignancies and solid tumor.<sup>79</sup> However, there are certain drawbacks to this therapy. The CAR-T sometimes develops GVHD, cytokine release syndrome, and neurotoxicity. From this perspective, CAR-NKs are much safer and more potent as their response is not MHC restricted and produces fewer side effects. Apart from CARs, CAR-NK cells also retain several ligands and receptors for stress signals, including CD226, NKp30, NKp46, NKG2D, and NKp44. NK cells express Fc $\gamma$ RIII (CD16) and stimulate an antitumor response in a CAR-independent and CAR-dependent manner.<sup>130,131</sup> The CAR-NK cells developed from the NK-92 cell line or through autologous or allogenic hosts show some unresponsiveness towards target cell killing. The activating signal incorporated in the CARs made these cells overcome this shortcoming and the potent inhibition posed by the NKG2A signaling.<sup>132</sup> Oei et al also showed that adaptive NK cells that express NKG2C, CD57, and self HLA specific KIRs possess a superior ability to kill CD19<sup>+</sup> HLA<sup>low</sup> target cells.<sup>132</sup> Zhuang et al reported that incorporating CD28 homolog protein (CD28H) in CARs triggers substantial lysis of B7H7<sup>+</sup>HLA-E<sup>+</sup> tumor cells and makes them overcome the potent inhibition triggered by the NKG2A signaling.<sup>133</sup>

To date, people have tried to develop different approaches to produce CAR-NK cells. However, the primary targets in hematological malignancies include CD33, CD22, and CD19 for relapsed and refractory leukemia and for lymphoma CD7 are used in several clinical trials (NCT03824951, NCT02742727, NCT02944162, NCT03692767, NCT03056339, NCT03579927, NCT02892695, NCT03690310, and NCT01974479).<sup>134</sup> In multiple myelomas, the B cell maturation antigen is used as a target (NCT03940833). In hematological malignancies, a few targets are also explored in several solid cancers currently under clinical trial. These targets are mucin1, NKG2D ligands, roundabout guidance receptor (ROBO1), mesothelin, and human epidermal growth factor receptor 1 (NCT03692637, NCT03383978, NCT03940820, NCT03692663, NCT03931720, NCT03415100, NCT03941457, NCT02839954).<sup>79</sup>

Scientists also used the piggyBac transposon system<sup>135</sup> to incorporate NKG2D in the CAR in NK-92 cells. These

non-viral engineered CAR-NK cells showed potent anti-tumor immunity against the xenograft model of human lung cancer in mice alone or combined with CD73 blockade.<sup>136</sup> First, NK cell-based CAR was designed to have NKG2D–DNAX activation protein 10 (DAP10)–CD3 $\zeta$  construct. This CAR-NK cell showed potent cytotoxic properties in both in vitro as well as in the mouse model of osteosarcoma.<sup>137</sup> This NKG2D-CAR-NK can recognize about 90% of human cancers that express ligands of NKG2D. Not only cancer cells, several immunosuppressor cells in the tumor microenvironment like MDSCs and Tregs also express ligands for NKG2D. These NKG2D-CAR NK cells can also eliminate these cells and promote antitumor activity.<sup>138</sup> Apart from NKG2D, other activating receptors of NK cells such as Nkp30 and DNAM-1 were used for making CARs to broaden the horizon of CAR-NK cell-mediated killing. For example, (PSCA)-DAP12 CAR is designed to target prostate stem cell antigen developed from primary NK cells and the YTS-NK cell line. These CAR-NK cells performed better cytotoxicity compared to PSCA-CD3 $\zeta$ -based CAR NK cells, even in the absence of other costimulatory ligands.<sup>70,71</sup> The optimal signaling through CARs in NK cell cytotoxicity CD3 $\zeta$  signaling domain, the intracellular 2B4 domain, and the transmembrane NKG2D are necessary. This knowledge was used to develop mesothelin-specific CAR-NK cells. These CARs showed potent antitumor action against murine ovarian cancer xenograft model.<sup>71</sup>

The inclusion of the IL-15 sequence in the CAR-NK sequence enhanced the efficacy and persistence of CAR-NK cells.<sup>139</sup> Depending upon the type of cancer and the antigen expressed, several tumor-specific targets of NK cells are used for CAR generation in the preclinical set up to increase the efficacy of CAR-NK cells. These targets include NKG2D ligand, glucosylceramide beta, epidermal growth factor receptor (EGFR), HER-2, EGFRvIII, epithelial cell adhesion molecules, glypican 3, guanine nucleotide-binding protein alpha-7, CD19, CD20, CD138, CD5, and CD2 subset 1(CS1).<sup>140–142</sup>

Though CAR-NK shows more significant potential to target and eliminates tumors, there are many challenges with CAR-NK cell therapy. First of all, NK cells are hard to transduce, so genetic modification is hard to perform. Even after their generation, allogeneic NK cells have a brief life span both in vivo and in vitro, limiting their durability of antitumor response.<sup>143</sup> The scientists' also used iPSC-derived NK cells to develop CAR-NK cells, and they hold

much promise in the future of “off-the-shelf” CAR-NK therapy. For example, mesothelin targeting CAR-NK cells developed from human iPSCs showed promising cytotoxicity potential against ovarian cancer.<sup>71,144–147</sup> Though there are challenges, this CAR-NK cell strategy holds great potential either alone or combined with other therapies (checkpoint blockade) in the future.<sup>148</sup>

### By “Priming” NK Cells to Perform Cytotoxicity

Although adoptive transfers of autologous and allogeneic or CAR-NK cells showed a favorable response, the major challenge of these therapies is the durability and apoptosis of these cells in the patients. Researchers also used different cytokines cocktails to “prime” the NK cell response in vivo. These primed NK cells show a more durable response in patients than NK cell.<sup>149</sup> Before injection, sometimes these NK cells are stimulated with either IL-2 or IL-15, or sometimes used along with NK cell infusions. ALT-803, a fusion protein of IL-15/IL-15a, has augmented NK cell activity and ADCC-mediated cytotoxicity both in vitro and in vivo. Recently, different groups showed NK cells primed with IL-12/IL-15, and IL-18 showed a potent and sustained anti-tumor response and showed the development of memory-like phenotype.<sup>150–152</sup>

Apart from cytokine-mediated priming, there are also other strategies used to prime NK cells for better cytotoxicity. Cichocki et al used GSK3 inhibitors to enhance NK cell activity. They showed that IL-15 primed human peripheral blood NK cells in the presence of GSK3 inhibitor ex vivo; they showed better cytotoxicity and antitumor activity. This strategy is used to develop FATE-NK100, which is currently undergoing Phase I clinical trial either alone or in combination with other monoclonal antibodies to treat the advanced stage of solid tumors (NCT03319459).<sup>153</sup> In another strategy, North et al reported overnight co-incubation of leukemia cell line CTV-1 or its lysate with NK cells without cytokines enhanced its cytotoxicity against non-responsive cancer cells in vitro.<sup>154</sup> Based on these preclinical results, CTV-1 lysate primed human NK cells (CNDO-109-NK cells) were evaluated for phase I clinical trial (NCT01520558) against high-risk AML patients after first complete remission.<sup>155</sup>

## Inhibitory Receptors

### Targeting Inhibitory Receptors on NK Cells to Promote Antitumor Response

During cancer progression, tumor cells sometimes down-regulate or lose their expression of MHC class

I molecule.<sup>156</sup> At the same time, transformed cells upregulate the ligands specific to NK cell activation receptors. This transformation results in increased NK cell-mediated cytotoxicity of transformed cells and a reduced tumor burden.<sup>89</sup>

## HLA-Specific NK Cell Inhibitory Receptor–Ligand Interaction and Therapeutic Application

Cancer cells have evolved different strategies to evade killing by NK cells and facilitate immune escape. These include over-expression of HLA class I (such as HLA-E, HLA-G) on cancer cells specific to inhibitory NK cell receptors and inducing inhibitory checkpoints (such as PD-1, TIM-3, Tactile, IRp60, etc.) on NK cells.

### NKG2A

In addition to the NK cells, NK T cells,  $\gamma\delta$  T cells, CD8<sup>+</sup> T cells, and subsets of innate lymphocytes also express CD94/NKG2A. Scientists also reported that head and neck squamous cell carcinoma (HNSCC) patients' NK cells show ~50% co-expression of CD94/NKG2A.<sup>157</sup> Blocking NKG2A with monoclonal antibody (mAb) monalizumab restores cytotoxicity of both NK cells and CD8<sup>+</sup> T cells, enhancing antitumor immunity.<sup>157,158</sup> Preliminary clinical studies of aggressive human tumors have shown favorable outcomes upon treatment with a monoclonal antibody (mAb) specific to NKG2A used alone or combined with mAb specific to a tumor antigen or checkpoint inhibitors. Therapeutic mAbs specific for PD-1/PD-L1 (durvalumab), when used in combination with NKG2A blocking mAbs, enhances the secretion of proinflammatory cytokine IFN- $\gamma$  and cytotoxicity of HLA-E<sup>+</sup> K562 cancer cells by NK and T cells.<sup>94</sup>

In patients with squamous cell carcinoma of the head and neck (SCCHN), the use of anti-NKG2A monalizumab along with anti-EGFR mAb cetuximab show improved clinical outcomes in phase II trials. Combination of monalizumab with cetuximab potentiated antibody-dependent cell cytotoxicity of SCCHN cell line.<sup>94</sup> Together, these studies suggest that blocking inhibitory receptors increases the NK cell cytotoxicity and ADCC of tumor cells in the presence of tumor antigen-specific antibodies.

### Lymphocyte Activating Gene 3 (LAG3)

LAG3 is known to bind to MHC II and interacts with galactin 3,  $\alpha$ -synuclein fibrils ( $\alpha$ -syn), and FGL-1 molecule.<sup>159</sup> Expression of LAG3 is reported on CD4 T cells, regulatory CD4 T cells,  $\gamma\delta$  T cells, NK cells,

invariant-NKT cells, B cells, and plasmacytoid dendritic cells.<sup>159</sup> One among the three splice variants of LAG3 exists in soluble form.<sup>159</sup> LAG-3 inhibitory receptor is associated with exhaustion of tumor-infiltrating T cells and immune suppression. The synergy between LAG3 and PD-1 inhibitory pathway facilitates the immune escape of tumor cells by regulating T cell function.<sup>160,161</sup> The effect of LAG3 expressed on NK cells on tumor progression is yet to be elucidated.

### HLA-G

HLA-G, expressed by trophoblast cells, is involved in the tolerance of the fetus during pregnancy.<sup>50,101</sup> HLA-G interacts with KIR2DL4 and ILT2 expressed in NK cells. Several tumors highly express HLA-G, which limits antitumor response by impairing NK cell and T cell-mediated cytotoxicity. HLA-G on tumors can also induce Treg formation and accumulation of MDSC.<sup>162</sup> Francois Anna et al have reported generating therapeutic CAR T cells specific to immunosuppressive HLA-G isoforms expressed on cancer cells.<sup>163</sup> CAR-NK and T cells specific for HLA-G might represent a novel strategy in the treatment of cancer.

### Killer Cell Immunoglobulin-Like Receptor (KIR)

KIR molecules recognize the motif of MHC I molecules and show an inhibitory response. KIR plays a significant role in leukemia treatment. KIR<sup>+</sup> alloreactive NK cells effectively treat acute myeloid leukemia upon hematopoietic stem cell transfer. The transfer of NK cells from haploidentical donors leads to KIR-KIR-L mismatch in the host resulting in alloreactive KIR<sup>+</sup> NK cells efficiently clearing leukaemia.<sup>164–168</sup> Immunotherapy targeting KIR by improving antitumor immune response is also currently in focus. IPH4102, an anti-KIR3DL2 monoclonal antibody, showed encouraging clinical outcomes in patients with cutaneous T cell lymphoma in Phase I trials.<sup>169</sup> The genotype of KIR and its associated HLA ligands play an important role in influencing tumor progression and efficacy of immunotherapy in some cancers across different individuals. In high-risk neuroblastoma, patients without one or more HLA ligands for NK cell inhibitory KIRs had a higher survival rate than patients with all inhibitory KIR ligands following autologous hematopoietic stem cell transplantation.<sup>170</sup> Anti-disialoganglioside (GD2) effectiveness in mAb therapy was significantly improved in patients with NK cells lacking self HLA Class I ligand for inhibitory KIRs.<sup>171</sup> Patients having inhibitory KIR2DL2 with its ligand HLA C1 and KIR3DL1 with

its ligand HLA-Bw4 showed improved outcomes to immunotherapy in both neuroblastoma and follicular lymphoma.<sup>172,173</sup> Therefore determining patient KIR genotype might help design a better anti-tumoral regimen to improve survival in high-risk cancer.

## Non-HLA Specific Inhibitory NK Cell Receptors and Therapeutic Applications

Tumor cells induce the expression of non-HLA-specific inhibitory receptors along with HLA-specific inhibitory receptors on NK cells to evade immunosurveillance and cytotoxicity.

### Programmed Cell Death Protein 1 (PD-1)

PD-1 (also known as CD279), belonging to the CD28/CTLA4 subfamily of inhibitory checkpoint regulators, are expressed on T cells, B cells, myeloid cells, and NK cells.<sup>174</sup> High expression of PD-1 in NK cells was reported in gastric cancer, multiple myeloma, Kaposi sarcoma, malignant mesothelioma, and adenocarcinoma,<sup>107</sup> which also correlates with poor survival in oesophageal and liver cancer.<sup>175</sup> PD-1 expression on NK cells leads to functional impairment and NK cell exhaustion and promoting tumor escape. Blocking PD-1 augmented pro-inflammatory cytokine production, decreased apoptosis of NK cells, and increased cytotoxicity of PD-L1<sup>+</sup> tumor cells without affecting surrounding healthy cells.<sup>175–177</sup> Analysis of factors in the tumor microenvironment (TME) promotes upregulation of PD-1 on NK cells revealed the vital role of glucocorticoids and cytokines IL-12, IL-15, and IL-18 in increasing PD-1 expression on NK cells.<sup>178</sup> Immunotherapy involving monoclonal antibodies blocking PD-1/PD-L1 is currently an effective therapeutic regimen for cancer treatment.<sup>179,180</sup> Inhibition of PD-1 leads to activation of both cytotoxic CD8<sup>+</sup> T cell and NK cells, generating a potent antitumor immune response. However, response to PD-1 immunotherapy varies from patient to patient and across different cancers because of tumor heterogeneity, genetic polymorphism, and response to different antibody clones.<sup>181,182</sup>

### T Cell Immunoglobulin- and Mucin-Domain-Containing Molecule-3 (TIM-3)

TIM3, also known as Hepatitis A virus cellular receptor 2 (HAVCR2), is expressed on Th1, Th17, Treg, DCs, and mature NK cells. Increased TIM3 expression was observed in tumor-infiltrating leukocytes in melanoma, lung cancer, lymphoma, gastric, head, and neck cancer. TIM3

expression in NK cells correlated with poor prognosis and shorter survival in patients with metastatic melanoma and lung adenocarcinoma.<sup>183</sup> Blockade of TIM3 reverses the NK cell exhaustion and enhances the cytotoxicity and secretion of proinflammatory cytokines by NK cells.<sup>184,185</sup> TIM-3 is also suggested as a potential prognostic biomarker for patients with solid tumors.<sup>186</sup>

### T Cell Immunoreceptor with Immunoglobulin and ITIM Domain (TIGIT)

TIGIT (also known as WUCAM, Vstm3, VSIG9), an inhibitory receptor expressed in T cells and NK cells, interacts with two ligands, CD155 (PVR) and CD112 (PVRL2, nectin-2) expressed on DCs, macrophages, etc., resulting in immune tolerance.<sup>187</sup> TIGIT contributes to immune suppression by inhibiting both T cell and NK cell-mediated immune responses. High TIGIT expression is associated with poor prognosis and tumor progression in different cancer.<sup>187</sup> TIGIT deficiency in vivo led to reduced tumor growth and increased secretion of pro-inflammatory cytokine IFN- $\gamma$  by CD8<sup>+</sup> T cells and NK cells in a colorectal cancer model.<sup>188</sup> Antibody-mediated inhibition of TIGIT/PVR interaction restored CD8<sup>+</sup> T cell activation and decreased tumor burden. Blockade of TIGIT impedes NK cell exhaustion, augments NK cell memory response to tumor re-challenge, and promotes NK cell-dependent adaptive immunity to tumor cells in a mouse model of melanoma and colon cancer.<sup>188,189</sup> Inhibition of TIGIT enhances the antitumor efficacy of PD-1/PD-L1 immunotherapy. Indeed, combinatorial therapy involving blockade of both TIGIT and PD-1 leads to improve survival and generation of a potent antitumor immune response to glioblastoma.<sup>190</sup>

### Sialic Acid-Binding Ig-Like Lectin 7 (Siglec 7)

Siglec 7 is a member of the sialic acid-binding immunoglobulin-like lectins (Siglecs) family, expressed mainly on NK cells and monocytes. Siglec-7<sup>+</sup> NK cells show increased expression of activating receptors CD38, CD16, DNAM1, NKp30, NKp46, and low expression of NKG2A and CD158a molecules.<sup>191</sup> Overexpression of sialyltransferase in tumor cells and dysregulation of sialidase leads to increase expression of sialylated glycan on the surface of tumor cells. Engagement of Siglec 7 and other Siglec receptors, such as Siglec 9 with sialylated glycans in malignant cells of different types of cancer, was found to attenuate NK cell-mediated antitumor immune response.<sup>192</sup> Powerful therapeutic strategies

targeting immune suppression through inhibitory receptors include desialylation of cancer cells and inhibiting hypersialylation of cancer cells. A complex of sialidase with human epidermal growth factor receptor 2 (HER2) targeting therapeutic mAb trastuzumab abrogated ligands for inhibitory Siglec receptors (Siglec 7 and Siglec 9), potentiating NK cell-mediated ADCC.<sup>193</sup> Sialic acid blockade by sialyltransferase inhibitor 3Fax-Peracetyl Neu5Ac reduces melanoma tumor growth, suppresses lung metastases, and increases intratumoral infiltration of NK cells and effector CD8<sup>+</sup> T cells.<sup>194,195</sup> CAR-T cells have great potential in the treatment of hematological malignancies. Siglec-7/9-based CAR-T cells display potent cytotoxicity against hypersialylated targets and reduced tumor growth.<sup>196</sup>

### Interleukin-1 Receptor 8 (IL-1R8)

IL-1R8, also known as single immunoglobulin IL-1R-related receptor (SIGIRR, or TIR8), is a member of the IL-1R family. IL1R8 negatively regulates ILR and TLR downstream signaling pathways, preventing pathology due to excessive inflammation upon pathogenic infection.<sup>197</sup> IL-1R8 is known to act as a checkpoint for NK cell maturation and effector function and regulates the antitumor and anti-viral activity of NK cells.<sup>198</sup> Enhanced maturation and upregulation of activation receptors Ly49H, NKG2D, and DNAM-1 were observed on NK cells deficient for IL-1R8. The absence of inhibitory IL-1R8 receptor on NK cells was found to be associated with inhibition of liver carcinogenesis and metastasis.<sup>198</sup>

### Leukocyte-Associated Immunoglobulin-Like Receptor-1 (LAIR-1)

LAIR-1, also known as CD305, is a widely expressing inhibitory receptor. Differentiation and activation of immune cells are associated with the downregulation of LAIR-1 expression.<sup>199–201</sup> The high expression of LAIR-1 in hematopoietic cancers, AML, and CLL correlate with poor prognosis.<sup>202</sup> The interaction between the inhibitory LAIR1 receptor and its ligand, extracellular matrix collagen, was associated with resistance to anti-PD-1 immunotherapy and CD8<sup>+</sup> T cell exhaustion in melanoma.<sup>203</sup> Cross-linking of LAIR-1 with monoclonal antibody leads to inhibition of NK cell cytotoxicity.<sup>204</sup> Thus blocking LAIR-1 can be a potential therapeutic strategy to enhance NK cell activation and kill cancer cells. High expression of LAIR-1 is reported to be clinically associated with poor outcomes in breast carcinoma.<sup>205</sup>

### Killer Cell Lectin-Like Receptor Subfamily G Member 1 (KLRG1)

KLRG1, an inhibitory C-type lectin receptor, is expressed in humans and mice by memory T cells and NK cells. The biological ligand of the KLRG1 receptor is E-cadherin and N-cadherin with similar affinities (7–12  $\mu$ M).<sup>206,207</sup> Blocking of KLRG1 receptor leads to decreased tumor growth and metastasis in melanoma, breast cancer, and colon cancer.<sup>208,209</sup> KLRG1 inhibition increases the maturation and accumulation of NK cells along with effector CD8<sup>+</sup> T cells in the tumor microenvironment. The antitumor efficacy of PD-1 therapy enhances by synergistic blockade of KLRG1 in melanoma and breast cancer,<sup>209,210</sup> suggesting its role as an essential checkpoint inhibitor in cancer immunotherapy.

### CD300a

CD300a (also known as IRp60) inhibitory receptor, a member of the CD300 family, is expressed in several lymphoid and myeloid cells. CD300a is involved in the regulation of several physiological processes, including phagocytosis and inflammation.<sup>107</sup> CD300a can inhibit NK cell-mediated cytotoxicity.<sup>211</sup> CD300a recognize and interact with phosphatidylserine (PS) and phosphatidylethanolamine expressed on the surface of infected, apoptotic, and tumor cells.<sup>212–214</sup> The interaction of PS on tumor cells with CD300a on NK cells prevents NK cell-mediated cytotoxicity of tumor cells.<sup>213</sup> High expression of CD300a correlates with poor prognosis in AML and diffuse large B cell lymphoma (DLBCL).<sup>215,216</sup> Suppression of CD300a expression leads to reduced tumor growth in an in vivo DLBCL xenograft model.<sup>216</sup>

## Conclusion and Future Perspective

The cytotoxic capacity of NK cells ultimately depends on activating and inhibiting receptors on them with their cognate ligands on the target cells. Understanding the biology of NK cell activating and inhibitory receptors paves the way to modulate them for better antitumor functions. Both antibody-mediated targeting and CAR-NK approach show a promising response against various hematological malignancies and solid tumors. Few clinical trials are up and running to test the efficacy of these therapies in the tumor (Table 1). Though some therapies showed great promise in vitro and preclinical settings, they failed in different phases of clinical trials. Not only as a monotherapy but NK cell-mediated therapy can also be

explored in combination with existing cancer therapies. In traditional chemotherapy or radiotherapy, most of the time, resistance is generated. In this setting, the use of NK cell therapy in synergy may overcome that resistance. To date, many activating and inhibitory receptors have been identified, and some of them are also targeted. Still, there are many mysteries about these KIR receptors that are unexplored. Their in-depth knowledge of signaling in NK cells and their targets can give a powerful tool to potentiate the clinical benefits in cancer patients.

## Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; NK, natural killers; HLA, human leukocyte antigen; MHC, major histocompatibility complex; CD, cluster of differentiation; ITAM, immunoreceptor tyrosine-based activation motif; DNAM-1, DNAX accessory molecule-1; NKG2D/A/C, natural killer group 2D/2A/2C; DAP10/12, DNAX activating protein 10/12; TRAIL, tumour necrosis factor (TNF)-related apoptosis-inducing ligand; ITIM, immunoreceptor tyrosine-based inhibitory motif; TIGIT, T cell immunoglobulin and ITIM-domain; TIM-3, T cell immunoglobulin and mucin-domain containing 3; PD1, programmed cell death protein 1; ILR, interleukin-1 receptor; TLR, toll-like receptor; Epcam, epithelial cell adhesion molecule; CAR, chimeric antigen receptors; LAG-3, lymphocyte-activation gene 3; KLRG1, killer cell lectin-like receptor G1; KIR, killer Ig-like receptor.

## Funding

GL received grants from the Department of Biotechnology (Grants numbers, BT/PR15533/MED/30/1616/2015; BT/PR14156/BRB/10/1515/2016), Swarna Jayanti Fellowship (DST/SJF/LSA-01/2017-18), from Department of Science and Technology, and Science and Engineering Research Board (EMR/2016/007108), Ministry of Science and Technology, Government of India. SK received a Senior Research Fellowship from the Council of Scientific and Industrial Research (CSIR), Government of India. PP received a Senior Research Fellowship from the Department of Biotechnology (DBT), Government of India.

## Disclosure

The authors declare no competing interests.

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