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NK cell receptor engineering: A New Hope for cancer immunotherapy

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Abstract: In recent years, Natural killer cells have become a rising alternative for cancer immunotherapeutic purposes. Their exploitation for adoptive cell transfer (ACT) offers a safer and broader application compared to T cell-focused therapies due to significantly less toxicity and a wide variety of autologous and allogeneic NK cell sources. However, certain limitations such as *in vivo* persistence, the tumor immune escape mechanism of the cancer cells and the ineffective solid tumor treatment still remain to be addressed. NK cell surface receptors are majorly affected by the aforementioned challenges and the hostile tumor microenvironment (TME), leading to the diminishing of NK cell cytotoxicity against tumor cells. Hence, a promising approach appears to be the modification of NK cell receptors in order to reinvigorate their activating mechanisms, block the inhibiting effect of certain immune checkpoints or boost the tumor infiltration via chemotaxis. To this direction, this review focuses on the preclinical and current clinical studies regarding the engineering of the activating, inhibitory and chemotactic NK cell receptors for the overall efforts to improve their infiltrating cytotoxicity against hematological malignancies and solid tumors.

Key words: Cancer Immunotherapy, adoptive cell transfer (ACT), NK cells, NK cell receptors, tumor microenvironment (TME), receptor engineering, activating receptors, inhibitory receptors, chemotactic receptors.

1. Introduction

The field of cancer immunotherapy has shown an admittedly exponential growth over the last decades. There are several promising existing approaches based on immunotherapy, such as cancer vaccines, oncolytic virus therapies, cytokine therapies, immune checkpoint inhibitors (ICIs) and adoptive cell transfer therapy (ACT) [1]. Specifically, ACT introduces the *ex vivo* expansion of immune effector cells and their subsequent infusion in cancer patients [2], [3]. Immune cells used for ACT are mostly autologous (deriving directly from the patient) but other types of immune cells, such as γδ T cells and Natural Killer (NK) cells can also be allogeneic (provided by donors) [1], [4]. Recent developments of cancer immunotherapy with T cells include T cell receptor engineering and the clinically tested and Food and Drug Administration (FDA) approved revolutionary concept of the chimeric antigen receptor (CAR) T cells technology [4], [5]. CAR-T cells have already been applied for the treatment of B-cell malignancies, multiple myeloma (MM), acute lymphocytic leukemia (ALL) and others [5]. However, there are certain limitations for T cell therapies regarding their toxicity due to the possible induction of the cytokine release syndrome (CRS) or the immune effector cell-associated neurotoxicity syndrome (ICANS) [6]. Moreover, the usage of allogeneic T cells poses the risk of inducing graft versus host disease (GvHD) [6]. Thus, the focus has lately been shifted towards the seemingly safer and more efficient utilization of NK cells as an alternative for cancer immunotherapeutic purposes [2], [6].

In contrast to T cells, NK cells offer a broader application for anti-tumor immunotherapy, mainly due to their enhanced cytotoxic killing ability [7], [8]. This is achieved by the degranulation process, meaning the release of the cytolytic granules (perforin, granzyme and

granulysin), the antibody-dependent cellular cytotoxicity (ADCC), ignited by the CD16 receptor-mediated identification of antibody-coated target cells and the induction of tumor cell apoptosis by expressing tumor necrosis factor α (TNF-α), FasL or TNF-related apoptosis-inducing ligand (TRAIL) [3], [7]–[11]. Additionally, NK cells eliminate tumor cells without the need of pre-activation nor Major Histocompatibility Complex (MHC) class I-dependent antigen presentation by the targeted cells [3], [12]. In addition, allogeneic NK cell transplantation is safer compared to allogeneic T cells, due to the lack of NK cells expressing the rearranged T cell receptors (TCR) and thus avoiding the risk of inducing neurotoxicity, GvHD, and CRS caused by the proinflammatory cytokines produced by T cells [2], [13]. Cytokines secreted by NK cells, like IFN-γ and GM-CSF, are considered safer compared to those produced by T cells, such as TNF-α and IL-6 [14]. Finally yet importantly, the short life span of allogeneic NK cells is limiting the possibility of off-target *in vivo* events in contrast to the longer survival of autologous T cells [8], [15].

NK cells are defined as large granular lymphoid $CD56^{+}CD3^{-}$ cells in the first line of defense of the innate immune system, targeting cancerous and virally infected cells [8], [16], [17]. They belong to the group 1 of Innate Lymphoid Cells (ILCs) due to their production of IFN-γ and the absence of receptor rearrangement [16]. Constituting almost the 10% of the peripheral blood mononuclear cells (PBMC), NK cells are distinguished in two major subpopulations, based on the expression of the low-affinity Fc gamma receptor 3A (FcγRIIIa) also known as CD16 and the adhesion molecule CD56 (NCAM); the $CD56^{bright}CD16^{dim}$ and the $CD56^{dim}CD16^{bright}$ cells. The $CD56^{bright}CD16^{dim}$ subpopulation (10% of the total PBMCs) consists of immunomodulatory cytokine producing NK cells, while the $CD56^{dim}CD16^{bright}$ cells (90% of the total PBMCs) appear to have stronger cytotoxicity [8], [11], [18].

NK cells' cytotoxicity depends on their capability of identifying malignant or infected cells through their wide variety of activating and inhibitory surface receptors [16], [18]. The spontaneous cytolytic killing activity of the NK cells is ignited by their activating receptors, such as NKG2D, CD16 and the natural cytotoxicity receptors (NCR), that empower the NK cells to recognize cells with increased levels of ligands induced by stress, a state named "induced self" [13], [17]. In parallel, NK cells can distinguish the healthy cells from the malignant ones depending on the deficiency of MHC complex I , also known as human leukocytes antigen (HLA) molecules, on the target cell surface [7]. This "missing-self" state is an escape mechanism of tumor cells against T cell mediated killing [7]. However, it is inducing the exact opposite outcome against the HLA-binding NK cell inhibitory receptors, such as KIR and NKG2A/CD94, resulting in the subsequent NK cell activation and killing of the cancer cells [7], [13], [17]. Additionally, NK cells are equipped with a variety of chemotactic surface receptors which are responsible for their migration to chemokine-expressing tumor sites [10], [19].

NK cells can be obtained from several sources, either autologous or allogeneic [2], [4]. Primary NK cells deriving from peripheral blood (PB-NK), umbilical cord blood (UCB-NK), the bone marrow or the placenta (by the method of apheresis) are the most studied sources with many applications [4], [11], [20]. In addition, hematopoietic stem/progenitor cells, human embryonic stem cells and induced pluripotent stem cells (iPSCs) offer an off-the-self solution, since they do not require donor-patient HLA matching [3], [21], [22]. A subcategory of NK cells available for adoptive cell therapy are the allogeneic cytokine-induced memory-like (CIML) NK cells, which are PB-NK cells incubated *in vitro* with the cytokines IL-12, IL-15 and IL-18 prior to their infusion in the patient, to boost their activity and persistence for weeks to months [3], [11], [19], [22]. However, the dose limitations of allogeneic NK cells due to the possible induction of GvHD by alloreactive T cells, indicate the importance of prior thorough purification of the cells [20]. Besides primary NK cells, NK cell lines offer an alternative NK cell source [12]. These cell lines are the NK-92, NK-YS, KHYG-1, NKG, IMC-1, NKL, NK3.3 [11], [12]. The immortalized NK-92 cell line, deriving from a large granular lymphocyte (LGL) lymphoma patient, is the only cell line used in human immunotherapy due to its indefinite proliferation resulting in cell abundancy and easier manipulation for therapeutic purposes [11], [21], [23]. The other existing NK cell lines

do not appear to have the same cytotoxic potential as the NK-92 cell line [11], [12].

The NK-92 cells express only the NKG2A inhibitory receptor and not KIR receptors, besides being CD16$^-$ and thus unable to induce the ADCC pathway [13], [21]. Furthermore, irradiation of the NK-92 cells is required due to their tumor origin in order to avoid oncogenic adverse events in the patients, a process that might lead to the need of multiple infusions [8], [11], [13]. However, the IL-2 dependency of all the available cell lines raises toxicity issues from the possible repeated IL-2 administration [8]. For this reason, the NK-92MI cell line can be a very promising alternative, since they emerge from the non-viral transfection of NK-92 cells to produce their own IL-2 that eliminates the necessity for additional cytokine injections [11].

All of the above NK cell sources are usually primed and stimulated via cytokines such as IL-2, IL-12, IL15, IL-18, IL-21 and type 1 interferons like IFN-α [24]. Nevertheless, the administration of those cytokines, especially IL-2, for a prolonged period and in high doses can result in toxicity and the expansion of unwanted immunosuppressive cells, such as T regulatory cells (Tregs) [6], [8]. An alternative strategy for *ex vivo* NK cell expansion is the use of feeder cell lines, like the modified K562 leukemia cells that express IL-15 or the most recent improved version expressing IL-21 and 4-1BBL, which are already thought to be safe for clinical application [19], [25].

The tumor cell targeted cytotoxicity of NK cells is dependent on their surface receptor repertoire, other inhibitory immune checkpoint molecules, metabolic and transcriptional factors, and the tumor microenvironment (TME) [1], [10], [17], [19]. The progress of various cancer types enables the so-called tumor immune escape, disrupting the NK cell's receptor balance and diminishing the expression of the activating receptors while boosting the expression of inhibitory molecules [26]. To overcome these challenges, NK cells can be engineered *ex vivo*, enhancing their post-infusion persistence and cytotoxic potential [4]. One example is the recent application of CAR technology on NK cells (CAR-NK), leading to a more potent lysis of tumor cells compared to CAR-T cells, via both the CAR-dependent and CAR-independent cell lysis pathways [3], [14]. In general, NK cells have been successfully genetically manipulated via viral and non-viral methods, with a lot of studies having already reached the stage of clinical trials [12]. Viral transduction of NK cells is possible with the usage of retroviruses or lentiviruses, with safety concerns surrounding the permanent expression of viral DNA [11], [27]. On the contrary, transfection of NK cells via mRNA electroporation results in transient gene expression that lasts only for a few days, a feature that could be positively considered for safety issues [17], [28], [29]. DNA transposon delivery is a rising alternative for a more persisting DNA expression of longer DNA sequences [2], [12], [30]. Other methods of NK cell transfection such as nucleofection, lipofection, mechanoporation, trogocytosis and polymer or lipid-based nanoparticles (LNPs) have also been introduced [8], [27], [29], [31]. Genetic engineering of NK cells is also feasible with gene editing techniques, like the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 complex, zinc finger nucleases (ZFNs) or transcriptional activator-like effector nucleases (TALENs) [8]. Moreover, the production of specific monoclonal antibodies (mAbs) known as immune checkpoint inhibitors (ICIs), destined to block the inhibitory receptors' functions via the obstruction of the receptor-ligand inhibitory effect, have been clinically evaluated [1].

This review presents a thorough description of the most recent modifications performed on NK cell receptors, inhibitory checkpoints or other pivotal factors for monotherapy or combination applications in cancer immunotherapy.

2. NK cell receptor engineering

In the following section the ongoing preclinical and clinical studies of NK cell activating, inhibitory and chemotactic receptors' engineering will be discussed. Table 1 presents the mostly studied NK cell receptors to date with their respective ligands and Figure 1 schematically summarizes the mostly studied NK cell receptor modification approaches. Finally, table 2 highlights the current clinical studies regarding NK cell receptors' engineering for anti-tumor purposes.

Activating NK cell Receptors

CD16 is probably the most important activating receptor of the NK cell's surface receptor repertoire [16], [17]. NKG2D, NKG2C and the natural cytotoxic receptors (NCR) such as NKp44, NKp46, and NKp30 constitute the rest of the crucial activating receptor list, with recent reports also referring to DNAM-1 (CD226) and CD137 as promising options for gene therapy [16], [30]. These receptors are usually downregulated or blocked by the TME and thus restoring or even increasing their expression leads to higher sensitivity to their respective ligands presented by the tumor cells [17]. There are different ways to achieve that, either by simply enhancing the expression of those receptors or by introducing a CAR construct in the NK cell taking advantage of the aforementioned transfection techniques.

Table 1: List of the most studied NK cell receptors for cancer immunotherapy and their ligands.

Receptor/Molecule	Ligand/Mode of action	Reference
Activating Receptors		
CD16 (FcγRIII)	IgG-ADCC	[23]
NKG2D	MHC-I, MICA, MICB, ULBPs	[16], [32]
NKG2C	HLA-E	[33]
NKp46 (NCR1, CD335)	Viral hemagglutinins	[34]
NKp44 (NCR2, CD336)	Viral hemagglutinins, Nidogen-1, PCNA, 21spe MML5	[34]–[38]
NKp30 (NCR3, CD337)	B7-H6, BAT3, pp65	[34], [39]
Inhibiting Receptors		
KIR family	HLA-A,B,C	[9], [22]
NKG2A/CD94	HLA-E	[40]
TIGIT	PVR (CD155), Nectin-2 (CD112)	[11], [34], [41]
TIM3	Galectin-9	[25], [42], [43]
PD-1	PD-L1, PD-L2	[26], [34]
CTLA-4	B7-1 (CD80), B7-2 (CD86)	[44]
CD96	PVR (CD155)	[34], [45]
Chemotactic Receptors		
CXCR1	CXCL6, CXCL8	[46]
CXCR2	CXCL1-CXCL7	[6], [46]
CXCR3	CXCL9, CXCL10, CXCL11	[21], [47]
CXCR4	CXCL12 (or SDF-1a)	[48], [49]
CCR7	CCL19, CCL21	[50]
CX3CR1	CX3CL1	[51]

CD16

The CD16a isoform of the CD16 receptor, expressed by NK cells and other types of immune cells has the most important activating role, since it induces the ADCC pathway by the Fc-binding to the respective IgG on the surface of tumor cells [23], [52]. Its expression is regulated by the A disintegrin and metalloprotease 17 (ADAM17), which acts when the receptor is activated in the tumor environment by cleaving the receptor [23], [53]. Even though this shedding of CD16a, leading to the detachment of NK cells, might be an advantage for their survival and targeted-cell engagement, it also reduces their cytotoxic activity [54].

It is known that the NK-92 cell line is CD16 deficient and therefore incapable of performing ADCC [8]. For this reason, the successful transduction or electroporation of the CD16a gene in NK-92 cells has been already achieved by different groups [10], [23]. The group of Jochems et al. engineered NK-92 cells via electroporation with a designed plasmid DNA delivering both the genes of the high-affinity variant of CD16 (CD16-158V) and IL-2 for self-production, leading to increased cytotoxicity against breast and lung tumors [15], [55]. CRISPR/Cas9 technology has been

applied by Pomeroy et al. to knock-out the ADAM17 cleavage sequence in PB-NK cells, that led to upregulation of IFN-γ production and ADCC in a PD-1 knock-out combined therapy [56], [57]. Moreover, iPSCs have been engineered to be ADAM17-cleavage resistant, resulting in more potent ADCC towards different types of tumors. These studies have reached the stage of clinical trials [15], [23], [53].

Zhu et al. recently engineered iPSCs with the high-affinity non-cleavable variant of CD16a (hnCD16), after showing that ADAM17-cleavage can be prevented with a point mutation of CD16a [53]. This modification led to the production of human induced pluripotent stem cell–derived NK (hnCD16-iNK) cells, which were more effective against both hematological malignancies and solid tumors [53]. Combination therapies with anti-tumor mAbs, like anti-CD20 mAb or anti-HER2 mAb also produced promising results [53]. Daratumumab, the manufactured monoclonal antibody for CD38 (a receptor highly expressed by myeloma cells), has been reported to reduce the NK cell active anti-tumor population because of the fratricide killing of CD38-expressing NK cells [21]. Thus, FT538 cells deriving from an iPSC line, were genetically modified to knock-out the CD38 receptor and knock-in the hnCD16, to induce ADCC in antibody-combined therapies [21]. In another study conducted by Toffoli et al. a bispecific single domain antibody (VHH), combining the C21 for CD16 targeting and 7D12 for epidermal growth factor receptor (EGFR) targeting was constructed [52]. The application of this engager resulted in *in vitro* and *ex vivo* augmented activation of patient PBMC NK cells and lysis of EGFR expressing tumor cell lines and metastatic colorectal patient-derived cancer cells [52]. The construction of another bispecific killer cell engager antibody construct (BiKE antibody), which acts through CD16 while also targeting the myeloid differentiation antigen CD33, could induce the degranulation and lytic ability of NK cells towards acute myeloid leukemia (AML) cells *in vitro* [14], [58]. In another study by Sarhan et al. on myelodysplastic cells, a trispecific antibody construct (TriKE) was produced including a IL-15 linker sequence connecting the CD16 and CD33 scFv domains [59]. This led to NK activation via the self-production of this cytokine [59]–[61]. Another group designed and produced a TriKE with the combination of an Fc fragment to induce ADCC by CD16, a tumor-associated antigen and fragments of the mAb for the activating receptors NKp46, resulting in decreasing cancer growth [22], [62]. Finally, Laskowski et al. successfully targeted CD30+ tumor cells with an AFM13 BiKE construct utilized to complex CD30 expressed by lymphoma or leukemia cells with $CD16^{+}$CB-NK cells [22].

NKG2D

The natural killer group 2 member D protein (NKG2D) activating receptor, belongs to the C-lectin family and can recognize ligands associated with viral or bacterial infected cells but most notably with tumor transformed cells [16], [63]. NKG2D plays an important role in the anti-tumor activity of NK cells, due to its ability to bind to MHC class I chain-related molecules, such as MICA, MICB and the UL16-binding proteins (ULBPs) [16], [18], [32]. In mice, retinoic acid early inducible-1 gene (RAE-1) and UL16-binding protein-like (MULT)-1 have also been reported as NKG2D ligands [16]. These NKG2D ligands are overexpressed by tumor transformed cells and infected cells and thus distinguish them from healthy cells [32], [63]. NKG2D induces NK cell cytotoxic activity and cytokine secretion via signaling through the DNAX-activating protein of 10kDa (DAP10) in human and DAP12 in mice [32], [63].

Malignant cells escape immune cell targeting through secreting soluble forms of the NKG2D ligands in order to disorientate the NK cell receptor [63]. Analyzing the specific molecular pathways of NKG2D downregulation in tumors, Xing et al. reported that both soluble and surface forms of MICA and MICB cause the desensitization of NK cells [64]. Other studies have shown that histone deacetylases (HDACs) inhibitors can induce higher levels of MICA/MICB expression on tumor cells, thus promoting NK cell tumor activity [64]. MICA gene-specific transcriptional activation and overexpression by tumor cells using CRISPR/Cas9 technology was studied by Sekiba et al., and resulted in increased NKG2D-mediated clearance of the targeted cells [65], [66]. Chitosan-based nanoparticles were used by Tan et al. for the successful delivery of a

plasmid encoding NKG2D and IL-21 (dsNKG2D-IL-21) into solid tumor cells, inducing the augmented secretion of the ligand and the cytokines [67]. Subsequently this led not only to NK cell but also to T cell stimulation and migration to the tumor tissue [67], [68]. In another study by Youness et al. the insulin-like growth factor-1 receptor (IGF-1R), which is critical for hepatocellular carcinoma, was targeted through lipofection of primary NK cells with one of its regulators named miR-486-5p [2]. The outcome of this technique was the amelioration of the NK cell cytotoxicity via augmented expression of NKG2D and perforin [2].

The knock-out of NKG2D has also been studied by several groups, with findings that prove its importance in cancer immunotherapy. Inhibition of NKG2D in early NK cell developmental stages has been associated with the hyperactivity of the NKp46 (NCR1) activating receptor and targeting of NKp46-ligand expressing tumors [69]. Wang et al. co-incubated the Kasumi-1 AML cancer cell line with NK92MI cells and the anti-NKG2D antibody, showing the importance of the NKG2D cytotoxicity potential [63]. In another study, a BiKE construct was produced with the combination of an anti-CS1 scFv domain and an anti-NKG2D scFv domain [14]. The employment of this engager in an *in vitro* human multiple myeloma (MM) model, proved the dose-dependency of IL-2 primed PBMC derived NK cells' cytotoxicity and cytokine secretion in the presence of the NKG2D receptor [14], [70]. Similarly, a BiKE construct including Fab fragments for the binding of NKG2D and HER2, a tumor-expressed antigen, could stimulate NK cell cytotoxicity *in vitro* [70]. Novel strategies with bi-specific immunoconjugate constructs are focused on simultaneous targeting of NKG2D ligands, like MICA or ULBP1/2, and tumor expressed antigens such as BCMA, CD19, VEGFR2 [70]. *In vivo* preclinical studies confirmed the efficiency of such molecules in inducing NKG2D activation of NK cells, with subsequent enhancement of NK cell cytotoxic activity towards the respective antigen-presenting tumor cells [70]. Finally, Calabrese et al. pointed out that knocking-out the NKG2D resulted in the reduction of primary graft dysfunction (PGD) occurrence in a pulmonary ischemia-reperfusion injury (IRI) study [71]

NKG2C

The C-type lectin CD94/NKG2C activating receptor is highly expressed by the adaptive CD56^{dim} NK cells, usually post-stimulation by a cytomegalovirus (CMV) infection and very potent for ADCC induction and IFN-γ secretion [33], [72]. The NKG2C ligand, HLA-E, is also associated with the NK cell inhibitory receptor NKG2A/CD94 [33]. A well-established technique in ACT is the incubation of allogeneic NK cells with feeder cells and IL-15 for the induction of NKG2C^{+} adaptive NK cells and the exploiting of their cytotoxic potency [40]. Haroun-Izquierdo et al. produced adaptive single self-KIR^{+}NKG2C^{+} NK cells, named ADAPT-NK cells [33]. These cells appeared to have a higher proliferation rate compared to common adaptive NK cells, both in *in vitro* and *in vivo* AML tumor cell models [33]. The improved expansion and activity of these cells was caused by the single self-KIR expression providing higher alloreactivity, the targeting of HLA-E and the induction of CD16-facilitated ADCC [33]. NKG2C^{+}CD57^{+} NK cells, deriving from CMV-seropositive donors, highly expressed those molecules after CMV reactivation post hematopoietic stem cell transplantation (HSCT), depicting a potent activity of cytolysis and a "memory-like" behavior [73]. The same memory NK cell subtype was utilized on bone marrow-transplanted patients with leukemia for the reduction of the relapse rate [74], [75]. Finally, an anti-NKG2C/IL-15/anti-CD33 tri-specific killer engager antibody (TriKE) construct was produced by Chiu et al. in order to target CD33^{+} AML cells with NKG2C^{+} CMV-reactivated patient derived PB-NK cells but also NKG2C-engineered iPSC-derived NK cells [76]. The outcome of this study was an increase in NKG2C+ NK cell proliferation, cytotoxicity, degranulation, IFN-γ production and efficient AML tumor cell elimination [76].

NCRs: NKp46, NKp44 and NKp30

The most important Natural Cytotoxic Receptors (NCRs) of the NK cells are the NKp46 (NCR1, CD335), the NKp44 (NCR2) and the NKp30 (NCR3) [18]. Those receptors recognize tumor cells by identifying very specific tumor-associated ligands [34]. They

also induce NK cell cytotoxicity and cytokine secretion, especially of IFN-γ [77].

NKp46

NKp46 is a crucial activating receptor of the NK cells responsible for stimulating their cytolytic activity and cytokine secretion [62]. NKp46 signals through post-engagement phosphorylation of CD3ζ and FcR-γ, two immunoreceptor tyrosine-based motif (ITAM)-bearing molecules [62]. Moreover, NKp46 is used for the identification of $CD3^-$ NK cells in mice [20]. As mentioned earlier, NK cell TriKEs combining a NKp46 scFv binding domain, a CD16 Fc binding domain and a tumor-associated antigen have been manufactured, leading to augmented ADCC and NK cell cytotoxicity against mice cancer [60], [62], [70]. Another available multi-specific killer engager called FLEX-NK, developed by Cytovia Tharapeutics in 2021, targets NKp46 and GPC3, a glycoprotein expressed by solid tumors, and CD38 to simultaneously eliminate solid tumors and multiple myeloma (MM) [78]. Finally, Berhani et al. produced a novel NKp46 monoclonal antibody (hNKp46.02) that results in NKp46 lysosomal degradation to eliminate malignancies associated with NKp46 and NK cells [79].

NKp44

NKp44 is only found in human activated NK cells and other types of immune cells and it has been associated with the identification of transformed cells, signaling via DAP12 [80]. Several NKp44 ligands have been mentioned in literature with the most dominant being Nidogen-1, the proliferating cell nuclear antigen (PCNA), the mixed-lineage leukemia-5 protein (21spe MML5) and viral hemagglutinins [35]–[38]. Barrow et al. reposted that the platelet-derived growth factor isoform PDGF-DD produced by tumor cells is recognized by NKp44 [80]. This results in IFN-γ and TNF-a secretion by the NK cells, as showed by the NCR2 (the gene that expresses NKp44)-transgenic mice experiments [80]. Finally, in another study on MM cells, the inhibitory binding of cancer cells with NKp44 through PCNA, was blocked with the mAb 14-25-9, leading to increased anti-tumor activity, IFN-γ production and degranulation of NK cells [77].

NKp30

NKp30 (NCR3, CD337) belongs to the immunoglobulin superfamily and is a type I transmembrane NK cell receptor, signaling via linking with the ITAM-associated molecules CD3ζ and FcRγ [70]. The activation of NK cells through recognition of the NKp30 ligands, such as the surface molecule B7-H6 and the nuclear factor HLA-B-associated transcript 3 (BAT3)/ Bcl2–associated athanogene 6 (BAG6), stimulates the NK cell cytotoxicity and cytokine secretion [39], [70]. NK cell killer engager molecules, also known as immunoligands, have been produced for NKp30 and EGFR targeting, with the bi-specific construct consisted of a humanized Fab variant from the Ab cetuximab and affinity-optimized variants of the N-terminal Ig-like V-type domain of B7-H6 [39]. The implication of these BiKEs was proven sufficient to stimulate fundamentally increased EGFR-positive tumor cell killing and IFN-γ and TNF-α secretion by NK cells [39], [70]. A follow-up study targeting again NKp30 on NK cells and EGFR on tumor cells used a BiKe construct combining NKp30-specific single-domain Abs of Camelidae origin (VHH) and the EGFR-specific humanized Fab of cetuximab [81]. This bi-specific killer engager approach had an increased NK cell-mediated tumor killing effect, when compared to the previous B7-H6 construct or plain cetuximab treatment [81]. Finally, another available antibody produced by Compass Therapeutics is CTX-8573, a multi-specific construct, combining anti-NKp30 Fab fragments and the C-terminus of the B-cell maturation antigen (BCMA) [70]. It has been used to engage with CD16 and NKp30 NK cell receptors, both in *in vitro* and *in vivo* studies [25], [70].

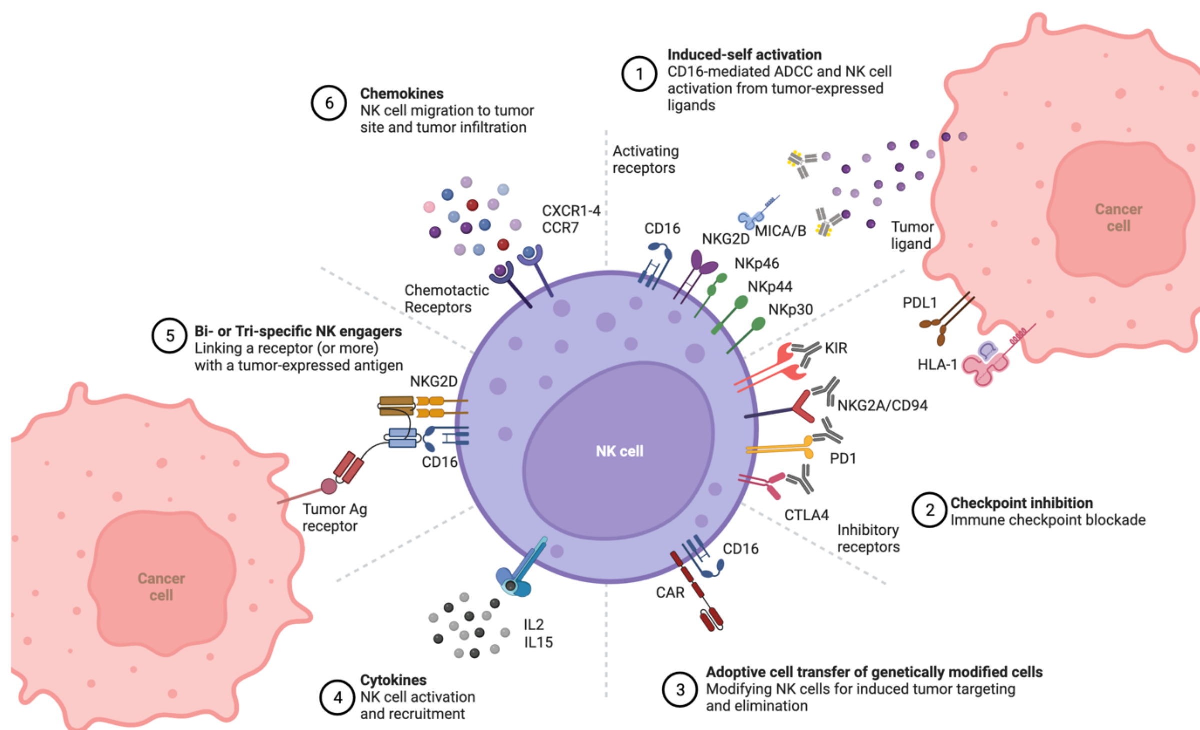

Figure 1: Schematic representation of the mostly studied NK cell approaches for cancer immunotherapy. 1) CD16-mediated ADCC and activation by tumor-expressed ligands through other activating receptors 2) Monoclonal antibodies for the blockade of inhibitory checkpoint molecules' interaction with their ligands in order to ignite NK cell cytotoxicity 3) ACT of modified NK cells with CAR constructs or knock-in enhancement of receptor repertoire 4) Administration of cytokines for the NK cell stimulation 5) Use of BiKEs or TriKEs for the simultaneous targeting/linking of NK cell receptors and tumor-associated antigens 6) NK cell migration to tumor site and subsequent infiltration after NK cell chemotactic receptor activation. (Created with BioRender)

Cytokine genes manipulations

As it has already been mentioned, the administration of exogenous cytokines is the default method for the expansion of infused NK cells *in vivo* [6]. Several studies on the genetic manipulation of the cytokine expression by the NK cells have been conducted in order to avoid multiple toxic doses of the related cytokines [6]. To this extent, Nagashima et al. retrovirally transduced the IL-2 gene into NK-92 cells, expanding their *in vitro* persistence for up to 5 months, without the need of additional cytokine administration [28], [82]. Primary NK and NK-92 cells have been transduced with retroviral vectors for the expression of IL-2 or IL-15, augmenting the NK cell expansion and perseverance *in vivo* in mice [83]. CB-NK cells have also been retrovirally transduced for IL-15 expression in a CAR construct produced for a xenograft Raji lymphoma murine model [65], [84]. The IL-15 transgene has been incorporated in a CAR construct by Daher et al., increasing the NK cell proliferation and sustainment *in vivo* [6]. However, it is shown that IL-15 is negatively regulated by the cytokine-inducible SH2-containing protein, also known as CIS regulatory element, which is encoded by the CISH gene [15], [21]. Blocking CIS activity leads to a major reduction in the NK cell activation threshold [15]. The importance of the CISH gene knock-out for the NK cell metabolic activity in murine *in vivo* studies against several cancer types such as prostate, melanoma and breast cancer, has been proved by Delconte et al. [17], [85], [86]. The absence of CISH regulation of IL-15 resulted in increased levels of IL-15, surpassing this inhibitory obstacle against the NK cell perseverance and cytotoxicity [23], [85]. CRISPR/Cas9 technology was also utilized by the Kaufman group for the CISH gene deletion in iPSC derived cells before their differentiation

into NK cells, providing NK cells with elevated killing potential and survival in AML *in vitro* and *in vivo* studies [21], [23], [87].

Inhibitory NK cell Receptors and Checkpoint Molecules

Besides inducing the NK cell cytotoxicity through modifications on the activating receptors, downregulating the expression of inhibitory receptors and checkpoint molecules can also shift the NK signaling balance towards their effective anti-tumor activation [17]. Those receptors and molecules can be divided in two subgroups based on their HLA specificity.

HLA specific Inhibitory Receptors

KIR

Killer cell immunoglobulin-like receptor (KIR) family consists of polymorphic activating and inhibitory transmembrane proteins, critical for the NK cell encounter with the major MHC-I molecules, especially the MHCIa [22], [40]. Out of the fourteen KIR receptors, seven of them are inhibitory (KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR3DL2, KIR3DL3) and six of them are activating receptors (KIR2DS1-2DS5, KIR3DS1), depending on the presence of an intracellular tail on the activating domain [19], [40]. The remaining KIR2DL4 has both types of activating and inhibitory features [19], [30]. KIR inhibitory receptors are important for the "missing-self" state recognition by the NK cells, facilitating the distinguishing between healthy and malignant cells in the absence of MHC-I on the tumor cell surface [9], [40].

Anti-KIR antibodies to block the KIR association with HLA-C molecules and the subsequent inhibition of NK cell cytotoxic activity have recently reached the stage of clinical trials reporting limited side effects [88]. The humanized IgG4 mAb 1-7F9 (IPH2101, anti-KIR2D) was produced for the blockage of KIR2DL1/L2/L3 and KIR2DS1/S2 receptors' binding to HLA- class I ligands [9], [14]. In preclinical studies, this antibody induced longer NK cell survival against AML cells, and when tested in AML and MM patients, successful KIR2D binding increased also NK cell cytotoxicity [9], [25], [89]–[91]. IPH2101 was also combined with lenalidomide, an agent that increases the NK cell activity by augmenting the NK cell activating receptor ligands, in phase I/II clinical trials with AML and MM patients [45], [91], [92]. Nevertheless, further studies were suspended since lower NK cell degranulation and cytokine secretion was observed when the antibody was tested for *ex vivo* treatment. This can be explained by the removal of KIR2D from the NK cell surface by phagocytes via FcγRI-mediated trogocytosis [19]. Lirilumab (IPH2102) is a recombinant version of IPH2101 and is the first fully clinically tested anti-KIR monoclonal antibody which was created for the inhibition of the KIR-HLA class I ligand interaction in the autologous NK cell ACT applications [9], [11]. The effectiveness of this anti-KIR2DL1/2/3 antibody was firstly confirmed by Sola et al. in an HLA-C expressing B cell lymphoma xenograft model on RAG-1 deficient mice [93]. Yet, the clinical efficacy of lirilumab monotherapy remains debatable [22], [60], [90]. For this reason, lirilumab has also been applied in combination therapy with the immunosuppressing drug lenalidomide, inducing ADCC [9]. Ongoing clinical trials have shown encouraging outcomes with KIR inhibition strategies using various combinations of anti-PD-1, anti-CD20, anti-SLAMF7, and 5-azacytidine. [88], [90]. The combination of lirilumab and rituximab (anti-CD20) has also been tested in a human lymphoma *in vitro* model using NK-92 cells, leading to elevated NK cell cytotoxic activity [89], [94]. Moreover, clinical studies on advanced or metastatic solid tumor patients are currently ongoing with the combination of lirilumab with nivolumab (targeting PD-1) and ipilimumab (targeting CTLA-4) [19], [93], [95]. A recent addition of the anti-KIR antibodies group is IPH4102 (lacutamab), a humanized IgG1 mAb targeting KIR3DL2 [89], [90]. This antibody has already entered the phase of clinical studies, has shown a good safety profile on relapsed/refractory cutaneous T-cell lymphoma, and it has been used in combination with chemotherapy [89], [90], [95]. Finally, in *in vivo* studies performed by Wei et al, the HHLA2+ human lung cancer cell HCC827 was used to challenge immunodeficient mice, thus proving that KIR3DL3 inhibition can enhance the NK cell activity [96].

NKG2A/CD94

Considered as the major inhibitory receptor of NK cells, NKG2A forms a heterodimer with CD94 and forms a complex which recognizes the tumor cell expressed non-classical MHCIb molecules HLA-E in humans or the Qa-1 molecule in mice [40], [60]. The interaction of the NKG2A/CD94-HLA-E ligation results in the induction of inhibitory signals via the phosphorylation of the tyrosine residue in the ITIM domain [40]. The interaction of NKG2A/CD94 is also important for the "missing-self" recognition ability of the NK cells [40]. NKG2A/CD94 facilitates the migration and subsequently the possible tumor cell killing by the NK cells [40]. NKG2A/CD94 and HLA-E have been both mentioned in literature as potential therapeutic targets, due to their non-polymorphic nature, in order to increase NK cell anti-tumor killing efficacy [92]. In the past, silencing of NKG2A with shRNA technology was used for the modification of $NKG2A^{+}$ cells [28]. Furthermore, PB-NK cells were transduced with lentiviral vectors for the RNAi knock-out of the NKG2A gene by Figueiredo et al, resulting in enhanced NK cell activity in *in vitro* HLA-E expressing B-lymphoblastoid cell line experiments [10], [97]. Similar results were also observed on AML-derived HLA-E negative K562 cells, due to the possible stimulation by the increased levels of NKp30 AR in the NKG2A deficient cells [10].In another study, NKG2A/CD94 was targeted with the use of protein expression blockers (PEBLs) [17], [98]. Such constructs include an anti-NKG2A antibody single-chain variable fragment connected with endoplasmic reticulum-retention domains, which block the NKG2A transport from the endoplasm to the cell membrane [17]. Kamiya et al. transduced PB-NK cells with retroviral vectors to produce NKG2A deficient NK cells [98]. These cells had improved cytotoxic functionality in targeting HLA-E-expressing and HLA-E deficient cancer cells deriving from Ewing's sarcoma, osteosarcoma and AML [10], [98]. They also successfully interfered with the *de novo* expression of the NKG2A/CD94, induced via IL-12 incubation without any side effects on the NK cell proliferation [17], [98]. These results from *in vitro* cultures were certified in xenograft models as well [98].

Most recently, the inhibition of NKG2A/CD94 has been achieved with the production of the humanized anti-NKG2A/CD94 mAb monalizumab (IPH2201), which has been already tested in both *in vitro* and *in vivo* studies [90]. Monalizumab has been proven to stimulate not only NK cell activity but $CD8^{+}$ T effector cell anti-tumor functionality as well, both in mice and in human [45], [99]. The use of monalizumab for cancer treatment against hematological malignancies and solid tumors, such as gynecological cancer is thought to fulfil the safety and efficacy requirements [45], [90]. NK cells showed recovery of their cytotoxic activity in preclinical studies with monalizumab on chronic lymphoid leukemia (CLL) patients [45], [92]. Besides the ongoing clinical trials as a monotherapy, monalizumab has also been applied in combinatory therapies with other therapeutic mAbs [14], [25], [34]. More specifically, the co-administration of monalizumab with the anti-EGFR antibody cetuximab in patients with head and neck squamous cell carcinoma (HNSCC) resulted in the induction of anti-tumor memory [25], [34]. The co-administration with the anti-PD-L1 mAb durvalumab had similar effect, for a variety of tumor types, such as mouse lymphoma and colorectal cancer (CRC) patients.[45], [61], [99].

Non-HLA specific checkpoint molecules

Apart from the HLA-specific inhibitory receptors mentioned above, there are several identified inhibitory checkpoint molecules that have been considered as possible targets for cancer immunotherapy. Most of these molecules are only stimulated upon interaction of NK cells with tumor cells or CMV infection [34].

TIGIT

T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) is a non-MHC class I-specific CD16+ NK cell receptor that blocks their cytotoxic activity via competing with the DNAM-1 receptor for the binding of its ligands, poliovirus receptor PVR (CD155) and Nectin-2 (CD112) [11], [34], [41], [45], [100]. TIGIT is expressed by both T cells and NK cells and its expression is observed in advancing tumor

cases and is linked to NK cell exhaustion and suppression of IFN-γ production [45], [100]. Interestingly, Jia et al. proposed in their functional experiment studies in AML patients that TIGIT expression by the NK cells might be correlated with increased secretion of IFN-γ and TNF-a cytokines, and granzyme B, supporting the NK anti-tumor activity in AML [92].

Recent studies have mostly focused on the effect of TIGIT inhibition on $CD8^+$ T and Treg cells and its competitive correlation with DNAM-1 (CD226) either as a monotherapy or in combination of anti-TIGIT mAbs with anti-PD-1and anti-PD-L1 [60], [101]. Currently there are various clinical trials based on TIGIT targeting with the use of monoclonal antibodies [102]. TIGIT blocking has also been reported to enhance NK cell cytotoxic activity towards HCC cell lines [103]. Colon cancer inhibition was achieved via TIGIT antibody inhibition which led to NK cell IFN-γ production and anti-tumor activity [45]. The blockade of TIGIT could be a promising alternative to enhance anti-tumor immunity. Nevertheless, the exact mechanism of action behind the NK cell interaction with $CD8^+$ T cells upon TIGIT inhibition is yet to be fully defined [100], [104]. Moreover, genetic modifications have been conducted for TIGIT, to eliminate its inhibitory functions and enhance NK cell activity [6]. TIGIT knock-out effect was studied by Zhang et al, demonstrating positive outcomes regarding the NK cell protection and tumor immunity in mouse models [6], [14], [100]. More specifically, an anti-TIGIT mouse mAb, called 13G6, was produced and used to block TIGIT activity in a CT26 colon cancer, 4T1 mammary cancer or methylocholanthrene (MCA)-induced fibrocarcinoma mouse model [100]–[102]. Interestingly, the use of this antibody resulted in the inhibition of the tumor growth via tumor-inflitrating NK cell protection and in the increase of $CD8^+$ T cell responses and elimination of tumor cells[101], [102], [104]. In a more recent mouse study, B16F10 and LWT1 metastatic melanoma tumor types were eradicated by combination of anti-TIGIT mAb and IL-15 induced NK cell cytotoxic activity [101], [105]. The same combination approach was selected for a B16 melanoma mouse model, where NK cell-specific TIGIT-deficiency resulted in increased expression of DNAM-1 (CD226) from tumor-infiltrating NK cells, with similar results and inhibition of tumor growth in CD155 deficient mice [103]. In a study on TIGIT expressing NK cells, the inhibition of this receptor resulted in less immunosuppression of the NK cytotoxic activity by the myeloid derived suppressor cells (MDSCs) [102].

CD96

CD96 has been recently indicated as another inhibitory checkpoint of NK cell activity, although it is a type I transmembrane Ig glycoprotein, mostly expressed by T cells [104]. CD96 interacts with PVR (CD155) expressed by tumor cells, in a similar competitive way TIGIT acts versus the DNAM-1 AR [34], [45]. This ligation leads to the decrease of IFN-γ production by the NK cells [45]. Higher $CD96^+$ NK cells levels with distinctive exhaustion and cytokine secretion have been found in intra-tumoral sites of hepatocellular carcinoma (HCC) patients or ovarian cancer ascites cases [34], [45], [96]. On human NK cells, treatment with IL-15 or TGF-β resulted in enhanced CD96 expression [96]. It has been reported in mice models that CD96 knock-out increased NK cell cytokine production and lung metastasis control [45]. Anti-CD96 mAb has also been produced for CD96 inhibition, showing higher efficacy when combined with anti-CTLA-4 or anti-PD-1 mAbs [106]. The effect of this CD96 blocking was not immense in terms of cytokine production and degranulation [96]. The precise mechanism of action of CD96 on NK cells, either inhibitory or activating, is yet to be studied [104], [106].

CD112R

Recently identified as an inhibitory immune checkpoint and lymphocyte receptor, CD112R (or PVRIG) has the ability to ligate with CD112 with a higher affinity compared to TIGIT or DNAM-1 [96], [107]. It is expressed in low levels on mouse but both on CD16-positive and CD16-negative NK cells [96]. It has also been found to be highly expressed by intra-tumor NK cells in prostate and endometrial cancer patients [96]. Anti-CD11R Abs for human NK cell experiments on a CD112 and CD155 human breast cancer cell line induced enhanced cytokine production and degranulation of NK

cells, confirming the inhibitory functions of CD112R [96], [108]. In *in vivo* studies by Li et al., subcutaneous MC38 tumor-bearing mice were treated with anti-CD112R mAbs while also confirming the aforementioned effects [96], [109]. Combination of TIGIT and CD112R inhibition by Xu et al., led to enhanced NK cell-mediated ADCC against breast cancer cells that were coated with trastuzumab *in vitro* [96], [107], [108], [110]. Until now, the effect of CD112R has been mostly studied on T cells [96], [107].

CTLA-4

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) is expressed in very low levels by NK cells, compared to its expression by T cells and T reg for immunomodulatory purposes [42], [44]. CTLA-4 binds with high affinity to B7-1 (CD80) and B7-2 (CD86) [44]. CTLA-4 expression by mouse NK cells is observed after treatment with IL-2 [96]. An anti-CTLA-4 monoclonal antibody, ipilimumab, has already been characterized for its effect on T cells and has reached the stage of clinical trials, either as a monotherapy or in combination with anti-PD-1 mAb nivolumab [44]. Treatment with ipilimumab has stimulated ADCC and TNF-α secretion by NK cells [42]. CTLA-4 inhibition via treatment with ipilimumab and tremelimumab, demonstrated promising results in the survival rate and the NK cell enhancement in melanoma and malignant pleural mesothelioma (MPM) [111]. The low expression of CTLA-4 by NK cells might be a negative indication regarding its targeting for therapeutic reasons [42].

PD-1

Programmed death protein 1 (PD-1), also known as CD279, is another inhibitory checkpoint molecule which is upregulated upon CMV infection or interaction of NK cells with tumor cells [26], [34]. PD-1 ligates with PD-Ll and PD-L2 in order to facilitate immune cell inactivation [26], [34]. While the abundance of NK cell-expressed PD-1 still remains hindered, it has been mentioned in literature that PD-1 could not be solely endogenously expressed by the NK cells but also provided from the tumor microenvironment [34], [112]. Reports have mentioned the presence of PD-1$^+$ NK cells in ovarian carcinoma, Hodgkin lymphoma, intestinal adenocarcinoma, Kaposi sarcoma, bladder carcinoma, lung, breast and uterine cancer patients [26], [34].

In a promising gene editing approach, CRISPR/Cas9 technology was used by the group of Pomeroy et al. on PBNK cells to knock-out the inhibitory genes of PD-1 and ADAM17, performing the targeted integration through homology-directed repair using a recombinant adeno-associated virus (rAAV) as a donor [2], [57]. The outcome of these studies was augmented cytotoxic killing by the NK cells via non-ADCC related pathways [57].

In preclinical studies of MM it was shown that the blocking of PD-1 augmented the NK cell killing activity [92]. Hsu et al. demonstrated within their *in vivo* mice studies that the inhibition of PD-1 or PD-L1 can be able to stimulate NK cell activity [34], [113]. Moreover, blocking the PD-1/PD-L1 seems to be very crucial for HLA negative cancer types [45]. For example, inhibition of PD-1/PD-L1 in MHC class I deficient tumor cells, like most Hodgkin's lymphomas which express higher levels of PD-L1, resulted in a better response of such patients and a higher NK cell activity [45]. Maskowska et al. have confirmed the role of IFN-β in inducing PD-L1 expression by nasopharyngeal carcinoma cells (NPC) and PD-1 expression by NK cells [114], [115]. Most importantly, those studies showed that the stimulation of NK cell anti-tumor cytotoxicity by chemotherapy could be supported by PD-1/PD-L1 inhibition with anti-PD-1 antibody treatment of NPC patients [114], [115]. In mouse models, the combination of anti-PD-1 and anti-KIR showed the pivotal role of NK cells against lymphoma tumor targeting [116].

Several anti-PD-1 antibodies have been designed, such as nivolumab, pidilizumab and pembrolizumab and their effect has been mostly studied on clinical stage for canceling the T cell immunosuppression by PD-1 [14]. However, the anti-PD-1 mAb nivolumab has been used in combination therapies for solid tumors to successfully restore the NK cell activity [42]. Finally, anti-PD-1 and/or anti-LAG-3 mAbs have been used in combination with IL-12 treatment, inducing the stimulation of NK cells for targeting metastatic breast cancer [45].

TIM-3

T cell immunoglobulin mucin receptor 3 (TIM-3), also known as hepatitis A virus cellular receptor 2 is another checkpoint molecule of the CD16^{high} NK cells, having an important role in their maturation process [19], [43]. The association of Tim-3 with galectin-9, expressed in various metastatic cancer types, has been reported to induce either IFN-γ production or suppression of NK cell cytotoxic activity [25], [42], [43]. Xu et al. verified with their *in vitro* studies the stimulation of CXCR1 and CXCR3 chemotactic NK cell receptor expression after activation of the Tim-3 pathway [43]. Thus, this NK cell receptor might have both activating and inhibiting effect on the NK cell anti-tumor activity [117]. Other ligands of Tim-3 reported in literature are phosphatidylserine, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) and high-mobility group protein B1 (HMGB1) [117]. The expression of Tim-3 has been shown to be upregulated in the presence of TNF-a [45]. Tim-3+ NK cells have been identified in lung cancer and advanced-stage melanoma, depicting it as a target for non-small cell lung cancer and other types of anti-tumor treatment [19], [111]. *In vitro* Tim-3 inhibition has resulted in IFN-γ and TNF-a restoration and revitalization of NK cell cytotoxic activity in liver, lung, and melanoma cancer [45], [96], [111]. Several other preclinical studies on tumor-bearing mice models have proven the efficacy of Tim-3 knock-out or antibody blocking on the NK cell contribution in inhibiting sarcoma, colon carcinoma or prostate tumor growth [45], [92]. Combination of antibody blocking of Tim-3 and PD-1 in *in vivo* models of melanoma, fibrosarcoma, colon cancer and leukemia proved to be very effective [45]. In another study on glioblastoma by Morimoto et al., the knock-out of Tim-3 with the use of CRISPR/Cas9 technology was proven beneficial to increase NK cell inhibition of glioblastoma tumor cell growth, not interfering with the expression of other NK cell checkpoint receptors [117]. Anti-Tim-3 antibodies have reached the stage of clinical trials as monotherapy or in combination therapies with anti-PD-1 and anti-LAG-3 antibodies, mostly focusing on their effect on T cells [NCT03744468, NCT03708328] [111]. In clinical trials of patients with solid tumors, the use of a Tim-3/PD-1 bi-specific antibody is also under examination [NCT03708328] [111].

Chemotactic Receptors

Several NK cell receptors are in charge of their chemotaxis in response to chemokines secreted by the tumor cells [10]. The most important chemotactic receptors of the NK cells are CXCR4, CCR7, CXCR3, CXCR2, CXCR1, CX3CR1 and CCR3-CCR5 [19], [118]. Following association with their respective ligands, these receptors facilitate the efficient migration of the NK cells to the tumor site and the subsequent infiltration of the tumor site by the NK cells, a process that is crucial for cancer clearance [10].

CXCR4

The importance of CXCR4 ligation with CXCL12 (or SDF-1a) for the homing of NK cells to the bone marrow (BM) has been depicted via inhibition of the CXCR4-SDF-1a binding [48]. Similarly, Levy et al suggested within their *in vitro* studies that mRNA transfected NK cells via electroporation for the expression of a CXCR4 receptor variant might be a promising approach to tackle BM sited tumors like myeloma and leukemia [48]. In studies by Yang et al, upregulation of IL18 expression and subsequent NK cell activation was observed after CXCR4 knock-out via tissue-specific LysM-Cre-mediated recombination gene editing [49]. This method resulted in the total remission and higher survival rate of mice in models [49]. Furthermore, NK-YT cells have been lentivirally transduced with an anti-EGFRvIII CAR construct and CXCR4, with augmented chemotaxis towards glioblastoma cells and cancer survival in xenograft mouse models [10].

CCR7

Other studies have been focusing on CCR7, a receptor responsible for the lymph node homing of the NK cells, aiming to improve tumor targeting by the NK cells [119]. NK cell migration and binding with the lymph node-associated chemokines CCL19 and CCL21 was improved through increased CCR7 expression on the NK cell surface [120]. Moreover, this

specific homing of NK cells has been enhanced by Carlsten et al. with mRNA electroporation of primary NK cells, in combination with CD16, in order to induce ADCC and NK cell-mediated killing of lymphoma cells [2], [6], [10], [121]. Moreover, in another study K562 feeder cells were used for the transfer of CCR7 to NK cells via trogocytosis leading to increased NK cell migration to the lymph nodes [6]. Finally, genetically modified NK-92 cells via lentiviral transduction, with the chemokine receptors CXCR4 and CCR7, showed increased migration to human colon and ameliorated tumor prognosis in mice xenograft models [49].

CXCR3

Another important chemotactic NK cell receptor is CXCR3, which ligates with the IFN-γ induced and tumor cell secreted CXCL9, CXCL10 and CXCL11 chemokines [21], [122]. CXCR3 is crucial for NK cell solid tumor infiltration [122]. In a study using irradiated EBV-LCL feeder cells with IL-2, the augmented CXCR3 expression by the NK cells led to improved homing and anti-tumor activity against CXCL10-transfected melanoma xenograft mice [6]. Nevertheless, observations regarding CXCR3-mediated accumulation of NK cells in the blood and mediocre survival of MM patients underline that further studies are required to establish the CXCR3 therapeutic role [122].

CXCR1-CXCR2

CXCR1 and CXCR2 are expressed at high levels from the $CD56^{dim}$ NK cells and facilitate their tumor infiltration [65], [118]. Their ligands are the cancer cell expressed chemokines CXCL6 and CXCL8 for CXCR1 and CXCL1-CXCL7 for CXCR2 [46]. In a renal cell carcinoma (RCC) mouse study, primary NK cells were retrovirally transduced with CXCR2, resulting in enhanced migration to the tumor sites [3], [6], [10], [65], [123]. Interestingly, the inhibition of CXCR2 in a melanoma mice study reduced the tumor infiltration by NK cells and augmented the survival rate of melanoma bearing mice [124]. Moreover, the importance of CXCR1 receptor was observed in ovarian cancer xenograft models, when its expression enhanced the migration, infiltration and efficacy of NKG2D-engineered CAR-NK cells [3], [20], [125].

Finally, numerous studies have shown the importance of fractalkine (CX3CL1) receptor CX3CR1 for the recruitment of NK cells as well as of other immune cells like monocytes and dendritic cells [51]. Interestingly, NK cell migration was substantially decreased via CX3CR1 antagonism in a esophagogastric adenocarcinomas (EAC) study [126].

Table 2: Current clinical studies related to NK cell receptors and their modification

NK Cell Receptor	Product/Study	Malignancy	NK Cell source	Sponsor	Status	Clinical Phase	ClinicalTrials.gov Identifier
Activating Receptors							
CD16	AFM13	Hodgkin Lymphoma	Intravenous infusion	Affimed GmbH	Completed	Phase 1	NCT01221571
CD16	AFM24 SNK01	Squamous Cell Carcinoma of Head and Neck Carcinoma, Non-Small-Cell Lung Colorectal Neoplasms Advanced Solid Tumor Refractory Tumor Metastatic Tumor	Autologous SNK01	NKGen Biotech, Inc.	Recruiting	Phase 1 Phase 2	NCT05099549
CD16	AFM24	Advanced Solid Tumor	Intravenous infusion	Affimed GmbH	Recruiting	Phase 1 Phase 2	NCT04259450
CD16	haNK Avelumab N-803	Merkel Cell Carcinoma	NK-92	ImmunityBio, Inc	**Terminated**	Phase 2	NCT03853317
NKG2D	NAKIP-AML	Acute Myeloid Leukemia	Haploidentical human allogeneic NK cells	German Cancer Research Center	Not yet recruiting	Phase 1 Phase 2	NCT05319249
NKG2C and PD-1	Dasatinib	Chronic Myeloid Leukemia	CMV-activated NKG2C+NK	Nanfang Hospital of Southern Medical University	Recruiting	Not posted	NCT04991532
Inhibitory Receptors							
KIR	IPH2101	Multiple Myeloma Myeloma Smoldering Multiple Myeloma	Intravenous infusion	National Cancer Institute (NCI)	**Terminated**	Phase 2	NCT01248455

CTLA4	Ipilimumab CIML NK cells N-803	Squamous Cell Carcinoma of the Head and NeckRecurrent Head and Neck Squamous Cell Carcinoma	CIML NK	Dana-Farber Cancer Institute	Recruiting	Phase 1	NCT04290546
PD-1	SMT-NK Pembrolizumab	Biliary Tract Cancer	Allogeneic SMT-NK	SMT bio Co., Ltd	Completed	Phase 1/ Phase 2	NCT03937895
PD-1	NK cells Sintilimab	Non-small Cell Lung Cancer	Autologous PBMCs	The First Hospital of Jilin University	Unknown	Phase 2	NCT03958097
PD-1	Pembrolizumab DC-NK cells	Solid Tumors	Intravenous infusion	Allife Medical Science and Technology Co., Ltd	Unknown	Early Phase 1	NCT03815084
PD-1	NK and DC cells Pembrolizumab, Nivolumab, Sintilimab, Toripalimab, Camrelizumab, Tislelizumab	Digestive Carcinoma, Gastrointestinal Tumors	Autologous NK cells	China Medical University, China	Not yet recruiting	Phase 2	NCT05461235
PD-1	COH06 Azetolizumab	Several types of Non-Small cell Lung carcinoma	CB-NK	City of Hope Medical Center	Recruiting	Phase 1	NCT05334329
PD-1	D-CIK cells Axitinib	Renal Metastatic Cancer	PBMCs	Sun Yat-sen University	Unknown	Phase 2	NCT03736330

PD-1	CCICC-002b CIK cells Sintilimab	Non-small cell lung cancer	Autologous CIK cells	Tianjin Medical University Cancer Institute and Hospital	Not yet Recruiting	Phase 2	NCT04836728
PD-1	D-CIK anti-PD-1	Refractory Solid Tumors	PBMCs	Sun Yat-sen University	Unknown	Phase 1 Phase 2	NCT02886897
PD-1	D-CIK and Pembrolizumab	Lung cancer neoplasms	Autologous PBMCs	Capital Medical University	Unknown	Phase 1 Phase 2	NCT03360630
PD-1	Anti-PD-1 P-GEMOX	High-risk Extranodal NK/T-cell lymphoma	Intravenous infusion	Cancer Institute and Hospital, Chinese Academy of Medical Sciences	Recruiting	Phase 2	NCT05254899
PD-1	Pembrolizumab	NK/T cell lymphoma	Intravenous infusion	The University of Hong Kong	Unknown	Phase 2	NCT03021057
PD-1	Merck NK-IIT Pembrolizumab	Melanoma	Intravenous infusion	Nina Bhardwaj	**Terminated**	Phase 2	NCT03241927
PD-1	SHR-1210 CIK cells	Renal Cell Carcinoma	Autologous CIK cells	Tianjin Medical University Cancer Institute and Hospital	Unknown	Phase 2	NCT03987698
PD-1	Toripalimab	Extranodal NK/T-cell lymphoma	Intravenous infusion	Beijing Tongren Hospital	Not yet recruiting	Phase 2	NCT04338282

PD-1	Anti-PD-1 Chidamide Lenalidomide Etoposide	Relapsed or refractory NK/T-cell lymphoma	Intravenous infusion	Mingzhi Zhang	Unknown	Phase 4	NCT04038411
PD-1	Anti-PD-1 Pegaspargase	Extranodal NK/T-cell lymphoma	Intravenous infusion	Ruijin Hospital	Unknown	Phase 2	NCT04096690
PD-1	SHR1210 Apatinib	NK/T-cell lymphoma	Intravenous infusion	Peking University	Unknown	Phase 2	NCT03701022
PD-1	Toripalimab Chemoradiotherapy	NK/T-cell lymphoma	Intravenous infusion	Sun Yat-sen University	Recruiting	Phase 3	NCT04365036
PD-1	CAR2BRAIN NK-92/5.28.z Ezabenlimab	Glioblastoma	NK-92	Johann Wolfgang Goethe University Hospital	Recruiting	Phase 1	NCT03383978
PD-L1	QUILT-3.060 NANT ha-NK	Pancreatic Cancer	NK-92	ImmunityBio, Inc.	Unknown	Phase 1 Phase 2	NCT03329248
PD-L1	QUILT-3.064 PD-L1 t-haNK	Advanced or metastatic solid tumors	NK-92	ImmunityBio, Inc.	Active, not recruiting	Phase 1	NCT04050709
PD-L1	Sacituzumab PD-L1 t-haNK N-803	Advanced Triple Negative Breast Cancer	NK-92	ImmunityBio, Inc.	Active, not recruiting	Phase 1 Phase 2	NCT04927884

PD-L1	PD-L1 t-haNK N-803	Pancreatic Cancer	NK-92	ImmunityBio, Inc.	Recruiting	Phase 2	NCT04390399
PD-L1	QUILT-3.063 Avelumab haNK	Merkel Cell Carcinoma	NK-92	ImmunityBio, Inc.	**Terminated**	Phase 2	NCT03853317
PD-1/PD-L1	QUILT-3.055 Anti-PD-1 Anti-PD-L1 PD-L1 t-haNK	Multiple	NK-92	ImmunityBio, Inc.	Active, not recruiting	Phase 2	NCT03228667
CXCR4	Revolution CXCR4 antagonists in combination with Nivolumab	RCC Metastatic Renal Cell Carcinoma	PBMCs	National Cancer Institute, Naples	Unknown	Phase 1	NCT03891485

CAR-NK

The chimeric antigen receptor (CAR) approach has been established over the past few decades as a breakthrough cell manipulation technology for tumor targeting, with CAR-T constructs already receiving FDA and EMA approval [5] [22]. Similarly, NK cells can also be reprogrammed to target any specific tumor antigen expressing type of cancer [22]. A CAR is a synthetic protein construct, usually deriving from the combination of 4 different domains; an extracellular antigen binding domain, a hinge region, a transmembrane domain and an intracellular signaling domain (see Figure 2) [127]–[129]. The target binding specificity is determined by the antigen-binding domain, which in most cases includes a single-chain fragment variable (scFv), deriving from antibodies and more rarely a native protein or a peptide [127], [130]. NK cell receptors have also been applied as an extracellular binding domain [131]. For instance, CD19 has been undoubtedly the major CAR target for hematological malignancies [20]. In addition, CAR-NKs have been developed for solid tumor elimination in order to target EGFRvIII, Her2 and mesothelin as these molecules are present in various cancer types, such as glioblastoma, colorectal, ovarian and breast cancer [20]. Other types of tumor antigens have been targeted as well with recent CAR-NK applications [129]. The hinge region of the CAR construct, usually deriving from CD8, CD28 or IgG4 is responsible for exposing the antigen-binding domain on the cell surface [127], [131]. The transmembrane domain's role is docking the CAR construct on the immune cell membrane and is also connected with the intracellular signaling domain [127], [130].

The intracellular signaling domain stimulates the NK cell cytotoxic activity and is the most thoroughly studied domain in the CAR engineering field [22], [127], [129]. CARs have evolved in 4 different so called generations, based on that domain of the construct [127]. Conventional first generation CAR-NKs, similarly to CAR-Ts, include only CD3ζ as a signaling domain while second and third generation CAR-NKs derive from the addition of one or two co-stimulatory domains respectively, mostly CD28 and 4-1BB (CD137) for CAR-T cells [127], [130]. These co-stimulatory domains were also successfully applied for CAR-NKs [130], [132]. Other co-stimulatory domains such as OX40, CD27 and inducible T cell co-stimulator (ICOS) have also been applied in preclinical trials mostly on CAR-T cells [127], [130]. Simultaneously, novel CAR-NKs have been recently developed with the inclusion of DNAX-activation proteins DAP12 and DAP10, which are responsible for the stimulation of NKp44, activating KIR receptors (KIR2DS and KIR3DS) and NKG2C [22], [132], [133]. Moreover, NKG2D ligands are expressed by several tumor types, making this activating receptor a promising tool that is already implemented in CAR-NK clinical applications targeting hematological malignancies and solid tumors [128], [131], [133], [134]. Besides inducing CAR-NK cytotoxicity towards antigens expressed by various tumor types, NKG2D-CAR-NKs have been designed by Parihar et al. to ameliorate NK cell persistence within the immunosuppressive TME, by eradicating the myeloid-derived suppressor cells (MDSCs) and M2 tumor-associated macrophages (TAMs) [20].

Finally, fourth generation CARs consist of engineered NK cells to self-produce IL-2, IL-15 or IL-12 to enhance their proliferation and self-sustainment [128], [130], [133]. The addition of other features to the original CAR structure, such as chemotaxis via co-expression of chemotactic NK cell receptors, is thought to be another promising direction of this approach [127]. All of the aforementioned available methods of introducing new genetic material to the NK cells for the expression of the fusion CAR-NK receptor have been utilized by various research groups in preclinical studies [20]. Table 3 summarizes the CAR-NK studies which are currently in the stage of clinical trials.

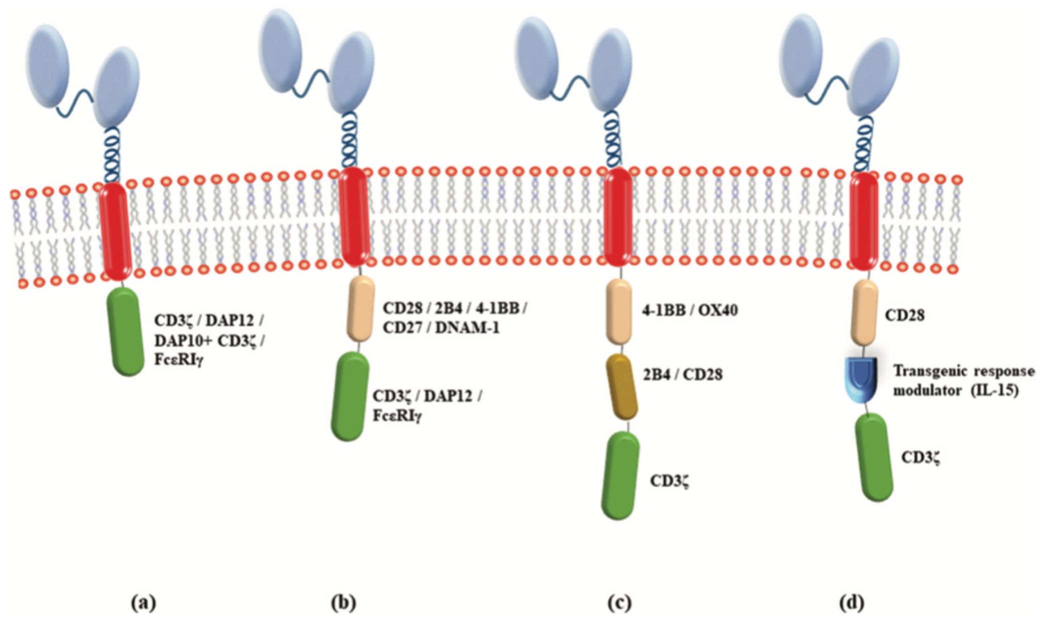

Figure 2: Illustration of the different CAR-NK generations. a) First generation CAR-NKs with CD3ζ/ DAP12/DAP10/FceRIγ b) Second generation CAR-NKs with the addition of CD28/2B4/4-1BB/CD27/DNAM-1 as co-stimulatory domains c) Third generation CAR-NKs with the addition of second stimulatory domain d) Fourth generation CAR-NKs with the inclusion of a transgenic response modulator for the expression of IL-15 [31].

In conclusion, tumor targeting can be accomplished through CAR-dependent and receptor-dependent manners by the engineered NK cells, even for the non-antigen expressing tumor types [133]. Finally, CAR technology has also been expanded to other types of immune cells like NKT cells, γδT cells and macrophages, with ongoing preclinical and clinical studies [130].

Table 3: Current CAR-NK clinical trials including major NK cell receptors in the CAR construct.

NK Cell Receptor	Product Name	Malignancy	NK Cell source	Sponsor	Status	Clinical Phase	ClinicalTrials.gov Identifier
NKG2D	NKG2D-CAR-NK92 cells	Relapsed/Refractory Solid Tumors	NK-92	Xinxiang medical university	Recruiting	Phase 1	NCT05528341
NKG2D	NKG2D CAR-NK Cell Therapy	Relapsed or Refractory Acute Myeloid Leukemia	Intravenous infusion	Hangzhou Cheetah Cell Therapeutics Co., Ltd	**Terminated**	Not Applicable	NCT05247957
NKG2D	CAR-NK cells targeting NKG2D ligands	Metastatic Solid Tumors	PBMCs	The Third Affiliated Hospital of Guangzhou Medical University	Unknown	Phase 1	NCT03415100
NKG2D	NKG2D CAR-NK	Refractory Metastatic Colorectal Cancer	-	Zhejiang University	Recruiting	Phase 1	NCT05213195
NKG2D	NKX101	Relapsed/Refractory AML AML, MDS, Refractory Myelodysplastic Syndromes	Allogeneic CAR-NK	Nkarta Inc	Recruiting	Phase 1	NCT04623944
PD-L1	PD-L1 CAR-NK Pembrolizumab N-803	Gastroesophageal Junction (GEJ) Cancers Advanced HNSCC	t-haNK	National Cancer Institute (NCI)	Recruiting	Phase 2	NCT04847466

3. Conclusion

This review presents the mostly studied and clinically tested cases of engineered NK cells in cancer immunotherapy. These studies highlight the versatile anti-tumor capability of NK cells while underlining the necessity of such modifications, in order to overcome certain safety and efficacy barriers for anti-tumor *in vivo* applications.

Viral transduction and mRNA electroporation are the most well-established delivery methods for NK cell receptor modifications that have advanced to the point of clinical trials. However, in order to avoid all the toxicity and potential mutation-causing issues of these methods, there has been a recent shift in the field's interest towards the promising use of lipid-based nanoparticles (LNPs) for the transfection of immune cells. The use of LNPs in a clinically fully approved pharmaceutical product was successfully introduced with the production of mRNA vaccines against COVID-19 [135]. Moreover, LNP applications related to T cells for cancer immunotherapy are already increasing [136], [137]. Although there are currently no NK cell receptor-related clinical trials reporting the use of LNPs, it seems like there is an ongoing trend for their application through a number of preclinical studies. For instance, Nakamura et al. successfully transfected NK-92 cells with siRNA-loaded CL1H6-LNPs [135]. In another study performed by the same group, the formulation and utilization of STING-loaded YSK12-C4-LNPs in a B16-F10 lung metastasis model led to the stimulation of IFN-γ production by activated NK cells and the subsequent expression of PD-L1 by tumor cells [138]. The results of this investigation provide a potential way of overcoming anti-PD-1 therapy resistance [138]. Additionally, interesting findings have also been depicted by Liu et al. in their studies on the effect of intratumoral administration of the cytokines IL-12 and IL-27 mRNA-loaded LNPs [139]. The outcome of these studies was the induction of tumor infiltration by NK cells and CD8+ T cells in a B16F10 mouse melanoma model [139]. Based on the fact that LNP-based applications focused on RNA delivery for anti-tumor therapies are rapidly expanding nowadays, it is more than evident that this mode of delivery will be widely applied in the field of cancer immunotherapy in the near future [140]–[143].

CD16 and NKG2D appear to be the most clinically studied activating receptors, with the latter being included in many CAR-NK constructs with already promising clinical results. Moreover, the involvement of more NK receptor domains in CAR-NK cells, either as extracellular binding domains or through intracellular signaling domains could be a crucially beneficial alternative to investigate. The already applied concept of dual CAR-Ts for the simultaneous targeting of two different tumor-expressed antigens, resulting in increased resistance to immunosuppression overcoming the tumor immune escape should also be considered for the case of CAR-NKs [22], [118]. The advance of successful *in vivo* manipulation of other activating receptors, such as the NCRs, possibly with BiKE and TriKE constructs, is imminently closing into the clinical stage. Additionally, genetic knock-out of the CISH gene, coding the CIS regulatory element of IL-15, is an approach that could reduce the possible toxicity of exogenous cytokine administrations while boosting the NK cell *in vivo* persistence and cytotoxicity.

KIR and PD-1 inhibitory receptors have undergone extensive research and have been targeted with knock-out or ICI techniques (lirilumab and nivolumab, respectively). Several other inhibitory receptors are targeted with specified mAbs, such as monalizumab for NKG2A/CD94 or anti-TIM-3 antibodies, as a monotherapy or in combination therapies, although their clinical efficacy could be re-evaluated. Other receptors, such as CTLA-4, TIM-3, TIGIT or CD96, should be studied more thoroughly for their possible clinical targeting for cancer immunotherapies. Moreover, combination therapies have already been applied in preclinical studies but their clinical translation has mostly been focused on their effect on T cells. Hereby we suggest that those techniques should also be studied in depth for the amelioration of NK cell cytotoxicity against tumor cells.

The set of NK cell chemotactic receptors appears to be significantly crucial for improving the NK cell migration and tumor infiltration, especially for solid tumor cases. In this direction, the engineering studies regarding the enhanced anti-tumor chemotaxis of NK cells might be an interesting approach to look

into. Finally, the novel breakthrough gene editing technique of CRISPR/Cas9 has already been applied in several studies that could be proven essential in future studies for the *in vivo* genetic manipulation of cancer patients' NK cells.
All things considered, it is certain that modified NK cell receptors is an effective and promising approach in the field of cancer immunotherapy and will be thoroughly researched and clinically tested in the years to come.

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