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# Targeting natural killer cells in cancer immunotherapy

Camille Guillerey[1,2], Nicholas D Huntington[3–5] & Mark J Smyth[1,2,5]

**Alteration in the expression of cell-surface proteins is a common consequence of malignant transformation. Natural killer (NK) cells use an array of germline-encoded activating and inhibitory receptors that scan for altered protein-expression patterns, but tumor evasion of detection by the immune system is now recognized as one of the hallmarks of cancer. NK cells display rapid and potent immunity to metastasis or hematological cancers, and major efforts are now being undertaken to fully exploit NK cell anti-tumor properties in the clinic. Diverse approaches encompass the development of large-scale NK cell–expansion protocols for adoptive transfer, the establishment of a microenvironment favorable to NK cell activity, the redirection of NK cell activity against tumor cells and the release of inhibitory signals that limit NK cell function. In this Review we detail recent advances in NK cell–based immunotherapies and discuss the advantages and limitations of these strategies.**



As their name suggests, natural killer (NK) cells spontaneously kill cells deemed to be dangerous to the host (cancer, foreign or virus-infected cells) and thus are presumed to be key effectors in cancer immunosurveillance, transplantation rejection and early viral immunity[1]. NK cells are usually defined as $CD3^{-}CD56^{+}$ cells in humans and $CD3^{-}NK1.1^{+}$ or $CD3^{-}NKp46^{+}$ cells in mice. They represent 5–15% of circulating lymphocytes in humans and can be categorized into subpopulations with different maturation statuses and functional specificities. $CD56^{lo}CD16^{+}$ NK cells with high cytotoxic potential are predominant in human blood, while the immunomodulatory $CD56^{hi}CD16^{-}$ subset is more predominant in lymph nodes[2]. Similarly, different subpopulations categorized on the basis of their expression of CD11b, CD27, KLRG1 and CD226 (DNAM-1) have been described in mice[3,4], with no equivalence established so far between mouse NK cell subsets and human NK cell subsets. In addition, tissue-resident NK cells that differ from conventional NK cells in terms of their origin, development and/or function have been observed in the mouse thymus, liver, skin, uterus and salivary glands[5,6], and putative human counterparts of these have also been described[7]. The emergence of NK cell diversity and the recently identified innate lymphoid cells has led to a new nomenclature that assigns conventional NK cells to group 1 innate lymphoid cells[8]. Of note, conventional NK cells are distinguished from other subsets of innate lymphoid cells by their intrinsic cytotoxic ability and their dependence on interleukin 15 (IL-15) and the transcription factor Eomes[8].

[1]Immunology of Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute, Herston, Australia. [2]School of Medicine, University of Queensland, Herston, Australia. [3]The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia. [4]Department of Medical Biology, The University of Melbourne, Melbourne, Australia. [5]These authors contributed equally to this work. Correspondence should be addressed to M.J.S. (mark.smyth@qimrberghofer.edu.au).

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NK cells have developed several mechanisms for distinguishing healthy cells from target cells. These mechanisms form the basis of NK cell activation and cannot be considered in isolation but instead must be considered as complex integration of signals from an array of receptors. NK cells express inhibitory receptors for molecules of major histocompatibility complex (MHC) class I, which are Ly49 receptors in mice, killer immunoglobulin-like receptors (KIRs) in humans, and the CD94-NKG2A heterodimer in both species. Binding of self MHC class I is proposed as a major mechanism for the tolerance of NK cells to self tissue, and engagement of self MHC class I by developing NK cells allows their 'licensing'[9]. Cells undergoing malignant transformation often downregulate their expression of MHC class I molecules, and the absence of inhibitory signaling on NK cells permits their function. However, for NK cell activation to result, strong stimulatory signals that overcome inhibitory signals are required. NK cells express activating receptors that recognize stress-induced ligands on the surface of the target cell; these receptors include NKG2D, NKp46, NKp30, NKp44 and CD226. Moreover, most NK cells express the low-affinity activating receptor FcγRIIIa (CD16) that binds the Fc portion of immunoglobulin G1 (IgG1) and mediates antibody-dependent cellular cytotoxicity (ADCC). Finally, soluble factors such as cytokines[10] and Toll-like receptor ligands[11] also modulate NK cell activity.

Granzyme B and perforin are the core molecules required for NK cell–mediated tumor killing[12], although death-receptor pathways (involving FasL and TRAIL) are sometimes used. Furthermore, NK cells secrete pro-inflammatory cytokines and chemokines (such as IFN-γ, TNF, IL-6, GM-CSF and CCL5) that might exert direct anti-tumor activity in addition to promoting innate and adaptive responses. Thus, NK cells are not only killers but also immunoregulatory cells that can positively or negatively influence anti-cancer responses by modulating the responses of dendritic cells (DCs) and T cells[13]. Another mechanism linked to the

## Box 1 Sources and preparation of NK cells for adoptive-transfer therapy

Autologous or allogeneic NK cells are usually obtained from the peripheral blood. They can also be derived from the bone marrow or umbilical-cord blood, and human embryonic stem cells or induced pluripotent stem cells are now under investigation as alternative sources of therapeutic NK cells[36]. Mature NK cells or NK cell progenitors can be inoculated together with other cells as part of HSCT or can be transferred alone after a pre-enrichment process. Enrichment for NK cells is generally achieved by magnetic depletion of T cells and/or selection of $CD56^+$ NK cells. Then, NK cell expansion is achieved by 1–3 weeks of culture in the presence of IL-2 with or without feeder cells engineered to express cytokines and/or co-stimulatory molecules. Depending on the protocol, the final NK cell product shows variable purity and might include a substantial proportion of contaminating T cells as well as B cells and monocytes. Residual monocytes might be beneficial, but reactivation of Epstein-Barr virus in residual B cells can cause lymphoma, and T cells must be carefully removed from allogeneic products to prevent GVHD[51]. Given the donor-dependent variability of the NK cell yield, NK cell lines represent an easy and attractive tool for adoptive cellular therapy. The human NK cell line NK-92 is highly cytotoxic against a broad spectrum of malignant cells, and infusions of NK-92 cells are safe and well tolerated in patients with cancer[35].

immunosurveillance of cancer by NK cells involves the elimination of senescent cells. Indeed, the cytokines and chemokines associated with the 'senescence-associated secretory phenotype' mobilize effective NK cell responses[14].

### Targeting NK cell function in the tumor microenvironment

Changes in NK cell receptor repertoire and ligand expression at the sites of primary tumor or metastases might result in decreased NK cell activity and thus lead to increased invasion and metastasis of tumors[15]. Tumor cells, tumor-associated fibroblasts and tumor-induced aberrant infiltrates (i.e., tolerogenic or suppressive macrophages, DCs and T cells) can either secrete immunosuppressive products or interfere with the complex receptor array that regulates the activation and anti-tumor activity of NK cells. At the site of primary tumor development and in the periphery, regulatory T cells ($T_{reg}$ cells) and myeloid-derived suppressor cells inhibit the activation and function of NK cells by a range of mechanisms[16]. Additionally, activated platelets can directly inhibit NK cells, and platelet-cloak formation seems to be critical for the inhibition of NK cell–mediated killing of circulating tumor cells[17]. Cytokines and metabolites reported to directly suppress the maturation, proliferation and functional activities of NK cells include TGF-β[18,19], adenosine[20,21], $PGE_2$, IDO and others. Of note, despite being categorized as an immunosuppressive cytokine, IL-10 does not inhibit NK cell function[22,23]. In contrast, TGF-β signaling affects the number and anti-metastatic function of NK cells[24].

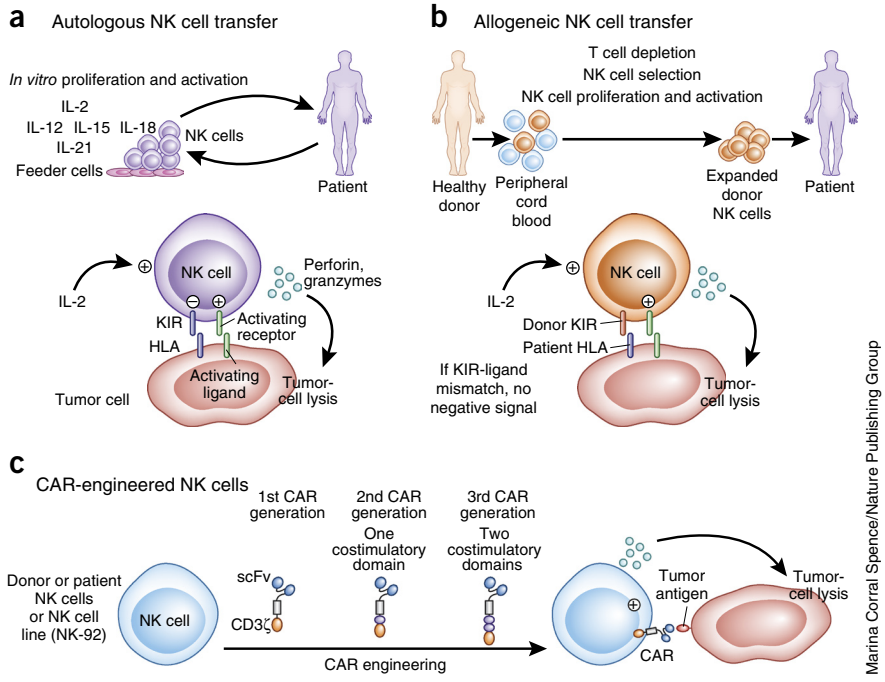


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**Figure 1** Various approaches to therapy with the adoptive transfer of NK cells. NK cells can be obtained from either the patient or from a donor. (**a**) In autologous transfer, NK cells from the patient are activated and expanded *in vitro* in the presence of cytokines. Historically, IL-2 has been used for this purpose, but findings now suggest that the combination of IL-12, IL-15 and IL-18 might generate NK cells that are more functional and have memory properties. Feeder cells can be added to the culture. Irradiated human lymphoblastic K562 cells are often used as feeder cells and can be engineered to express cytokines (such as IL-15 and IL-21) and/or co-stimulatory molecules. The expanded and activated NK cells are then transferred back into the patient, who generally receives cytokine administration (IL-2, in most cases) to sustain the expansion and function of the infused NK cells. Although autologous NK cells might recognize activating signals such as stress molecules on cancer cells, their anti-tumor activity is limited by the inhibitory signal transmitted by self HLA molecules. (**b**) In allogeneic transfer, NK cells can be obtained from HLA-matched or haploidentical (partially matched) donors. NK cells are expanded through processes similar to those used for autologous transfer, but T cells should be removed to avoid GVHD. In this setting, the best responses are obtained when haploidentical donors do not express KIRs that recognize the patient's HLA molecules, because donor NK cells do not receive an inhibitory signal from the patient's cancer cells. (**c**) CARs can be engineered in autologous or allogeneic NK cells or in NK cell lines such as NK-92. CARs are designed by the fusion an antigen-binding domain (derived from a mAb scFv of known specificity) with a hinge region, a transmembrane domain and one or more stimulatory molecules. Each CAR has the CD3ζ chain (or sometimes the FcRγ chain) as its main signaling domain. Additionally, one or two co-stimulatory domain(s), usually from CD28 or CD137, can be added to the CAR construct; this leads to increased persistence and superior functionality. CARs from the first generation have no stimulatory domain, whereas CARs from the second generation and third generation have one co-stimulatory domain or two co-stimulatory domains, respectively. CAR engineering endows NK cells with antigen specificity. The binding of a CAR to the tumor antigen delivers a potent activating signal that triggers NK cell cytotoxicity, which results in elimination of the cancer cell.

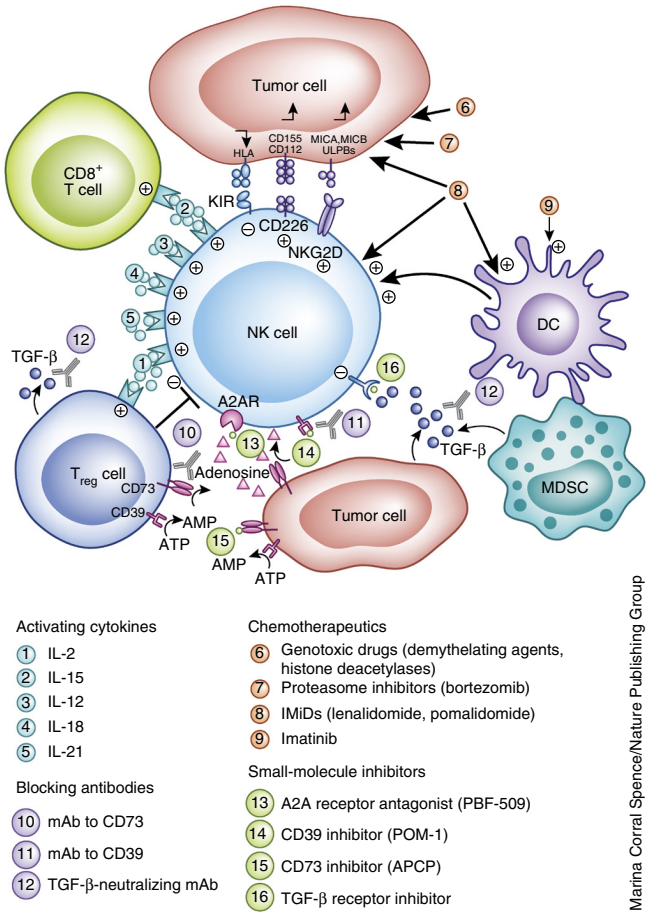


**Figure 2** Targeting the tumor microenvironment to improve NK cell responses. Various strategies can be adopted to create a microenvironment favorable to NK cells to improve the efficacy of NK cell–based therapies. Cytokines are generally administered to expand adoptively transferred NK cells, with IL-2 (1) being the most widely used in the clinic. However, IL-2 also activates $T_{reg}$ cells that hamper NK cell activity partly through production of the immunosuppressive cytokine TGF-β. In contrast, IL-15 (2) does not activate $T_{reg}$ cells and provides the additional advantage of stimulating both NK cells and cytotoxic $CD8^+$ T cells. Other cytokines such as IL-12 (3), IL-18 (4) and IL-21 (5) can potentiate NK cell responses. Moreover, some anti-tumor agents have NK cell–modulating properties beyond their direct toxicity toward cancer cells. Genotoxic drugs (6), proteasome inhibitors (7) or immunomodulatory drug (IMiDs) (8) sensitize tumor cells to NK cell–mediated killing by altering their expression of surface molecules (downregulation of HLA molecules and upregulation of stress-induced ligands of activating receptors on NK cells). Moreover, immunomodulatory drugs (8) and the tyrosine-kinase inhibitor imatinib (9) stimulate NK cell function either directly or indirectly through the activation of other immune-cell subsets such as DCs. CD39 and CD73 are two enzymes expressed in the tumor microenvironment that contribute to production of the immunosuppressive metabolite adenosine. Blockade of these two enzymes via mAbs (10 and 11) or small-molecule inhibitors (14 and 15) restores NK cell function. Alternatively, blockade of the high-affinity adenosine receptor $A_{2A}$ (13) might prevent the direct suppressive effect of adenosine on NK cells. Finally, the immunosuppressive cytokine TGF-β substantially represses NK cell activity, and this pathway can be blocked by neutralizing this cytokine (12) or its receptor (16). MDSC, myeloid-derived suppressor cell.

Overcoming the immunosuppressive tumor microenvironment to restore NK cell function is an attractive therapeutic option. So far, treatments that involve T cells, including immunological checkpoint blockade and adoptive cellular therapy, have been the main focus of most immunotherapies. However, NK cells are receiving renewed interest, as they present the considerable advantage of not relying on antigen specificity. Still, the great potential of NK cells depends on the type of malignancy. The poor ability of NK cells to infiltrate solid tumors[25] might explain the superior efficacy of these cells against metastases or hematological cancers such as acute myeloid leukemia (AML)[26] or multiple myeloma[27]. Targeting NK cells also holds potential in the context of minimal residual disease. Indeed, studies have highlighted the ability of human NK cells to 'preferentially' kill cancer stem-like cells, a quiescent subpopulation described in most cancers that displays enhanced tumorigenic potential and is resistant to conventional therapies[28]. Moreover, several studies have successfully exploited NK cell functions directed against neuroblastoma[29,30] or glioblastoma[31–33], and encouraging data have also been reported for lung cancer[34,35]. Conversely, solid tumors such as breast cancer or colorectal carcinoma might be resistant to NK cell–based therapy. In the following sections, we briefly discuss the methods of targeting NK cells in cancer.

## Adoptive NK cell therapy

NK cells can be derived from various sources (**Box 1**) and can be obtained from the patient (autologous setting) or from a healthy donor (allogeneic setting) (**Fig. 1**). Few NK cells circulate in human blood, and naive NK cells exhibit limited cytotoxic activity. For these reasons, several protocols have been designed to expand large numbers of NK cells with full anti-tumor functions. These methods have been discussed before[36,37].

A major approach to adoptive NK cell therapy is the transfer of unmodified autologous or allogeneic NK cells. In patients with hematological cancer who are receiving autologous hematopoietic stem cell transplantation (HSCT), blood NK cell numbers recover very early after the transplant; the anti-tumor potential of NK cells is shown by the fact that in these patients, a large number of NK cells correlates with a positive outcome[38,39]. Nevertheless, the transfer of expanded autologous NK cells in patients with metastatic melanoma, renal-cell carcinoma or advanced gastrointestinal cancer does not translate into clinical responses[40,41]. In fact, adoptively transferred NK cells that persist in the circulation are unable to lyse tumor cells unless re-stimulated *in vitro*[41]. This observation emphasizes the need for combination strategies to fully exploit the potential of autologous NK cells. Conversely, adoptive transfer of allogeneic NK cells from KIR-mismatched donors has shown impressive results in patients with AML. In such settings, tumor cells lack the appropriate MHC class I ligands to engage inhibitory KIRs and are thus eliminated by the alloreactive NK cells. A seminal study has identified KIR–ligand incompatibility as the only factor that can be used to predict the outcome of patients with AML who have received hematopoietic transplants from HLA-mismatched donors[26]. Subsequent clinical trials investigating the transfer of haploidentical NK cells for AML treatment have reported encouraging complete remissions in patients with a poor prognosis or elderly patients[42,43] and have reported 100% event-free survival at 18 months in a pediatric cohort[44]. Another therapeutic advantage of alloreactive NK cells is their ability to prevent graft-versus-host disease (GVHD) by eliminating host antigen-presenting cells[26]. However, such a protective effect has been questioned by a study suggesting that allogeneic HSCT followed by the transfer of donor NK cells activated by IL-15 and CD137L (the ligand for the costimulatory receptor CD137) exacerbates acute GVHD by augmenting underlying T cell alloreactivity[45]. Differences in the source and preparation of the transferred NK cells might explain these opposite results. In fact, insufficient depletion of T cells in KIR-mismatched

## Box 2 The IL-15 signaling pathway

The cytokine IL-15 is known to have an essential role in most aspects of NK cell biology, including development and cell activation[139]. IL-15 is trans-presented on the receptor chain IL-15Rα expressed by various immune cells (including DCs) or non-hematopoietic cells. IL-15 binds to heterodimers of IL-15Rβ and the common γ-chain ($\gamma_c$) on the surface of NK cells to induce activation of the β-chain- and $\gamma_c$-associated tyrosine kinases JAK1 and JAK3, recruitment and activation of the transcription factor STAT5, and transcription of STAT5 target genes encoding products required for differentiation, survival, proliferation, priming and cytotoxicity. In addition, mTOR is a metabolic regulator newly identified as an essential component of this pathway that regulates responses to high concentrations of IL-15 (ref. 74). Deletion of IL-15, its receptor or downstream signaling proteins (JAK3 or STAT5) results in NK cell lymphopenia. Id2, a transcription factor essential for NK cell development, has been found to regulate signaling via IL-15 receptor[140]. Deletion of *Id2* in peripheral NK cells results in the rapid loss of NK cells from all organs. *In vitro*, $Id2^{-/-}$ NK cells have diminished phosphorylation of JAK1-STAT5, poor metabolism, minimal proliferation and enhanced apoptosis in response to IL-15. A major breakthrough in the understanding of IL-15 regulation has been made by studies identifying CIS ('cytokine induced SH2-containing protein') as the key negative regulator of IL-15 signaling in NK cells. The gene encoding CIS (*Cish*) is a target of STAT5 that is induced by IL-15, and CIS has been found to inhibit JAK1 activity directly and to target JAK1 for proteasomal degradation. As a result, $Cish^{-/-}$ NK cells are hyper-responsive to IL-15 and have substantially enhanced anti-tumor function *in vivo*[135].

grafts might result in severe GVHD and abolish the clinical benefits of NK cell alloreactivity[46,47]. In addition, while they are protective in AML, KIR–ligand mismatches do not influence transplantation outcome in every hematological cancer[26]. Therefore, future studies need to investigate the specific conditions and cancer types that would benefit from the infusion of allogeneic NK cells.

In an alternative adoptive-transfer approach, NK cell activity can be redirected against a specific tumor antigen through recombinant chimeric antigen receptor (CAR) engineering. Although T cells expressing a CAR directed against the signal-transduction receptor CD19 (anti-CD19 CAR) were declared a 'breakthrough therapy' in 2014 (ref. 48), CAR-expressing NK cells remain mostly at the preclinical stage. NK cells modified to express CARs that recognize a variety of tumor antigens have been tested *in vitro* or in animal models[49,50], but their clinical development is limited to two trials (NCT00995137 and NCT01974479) investigating NK cells expressing anti-CD19 CAR for the treatment of B lineage acute lymphoblastic leukemia. However, NK cells engineered to express CARs present several advantages over their T cell counterparts[49,50]. The long-term persistence of cells engineered to express CARs increases the risk of autoimmunity or malignant transformation. Because NK cells are short lived, mature NK cells engineered to express CARs are expected to disappear rapidly after mediating their anti-cancer effects and should not require a 'suicide system' for clearance of the cells. Furthermore, evasion of the immune system by cancer cells through down-modulation of tumor-antigen expression would render CAR-expressing T cells useless when NK cells would still be effective. Notably, the cytokine-release syndrome that results from the activation of CAR-expressing T cells may be extremely severe and even fatal. In this context, the NK cell cytokine-production profile is considered safer, as it consists mainly of IFN-γ and GM-CSF. Despite such advantages, manufacturing CAR-expressing NK cells is hampered by the difficulty of isolating and expanding large numbers of NK cells from peripheral blood, the sensitivity of NK cells to cryopreservation, and the low transfection efficiency of these cells[51]. For these reasons, particular interest has been accorded to NK-92, a transformed cell line that consists of 100% activated NK cells, is easy to transfect and can be expanded under good-manufacturing-practice conditions. Unmodified NK-92 cells have been successfully infused into patients with advanced treatment-resistant malignancies, with encouraging responses observed for patients with lung cancer[35]. The possibility of redirecting NK-92 cells to specifically recognize tumor-associated surface antigens is gaining interest. NK-92 cells expressing anti-CD19 CAR overcome the resistance of acute lymphoblastic leukemia cells to NK cell–mediated cytolytic activity[52], and NK-92 cells specifically targeting the receptor tyrosine kinase ErbB2 (HER2) or the epidermal growth factor receptor protect mice against glioblastoma[31–33]. Finally, because NK-92 cells should be irradiated before infusion into patients, as a safety measure, a study has assessed the effects of such irradiation; this demonstrated that NK-92 cells expressing a CAR directed against ErbB2 retain high and specific cytotoxic activity and protect mice against experimental lung metastasis even after irradiation and thus seem to be suitable for clinical development[53].

### The tumor microenvironment and immunosuppression

In patients with AML who have received haploidentical NK cells, large numbers of circulating NK cells correlate with disease remission[43]. Therefore, treatments that create a microenvironment favorable to NK cell expansion should be explored in combination with NK cell adoptive therapy (**Fig. 2**).

Various cytokines, including IL-2, IL-12, IL-15, IL-18, IL-21 and type I interferons, can be used for the *in vitro* expansion and activation of NK cells before adoptive transfer. The combination of IL-12 with IL-15 and IL-18 is particularly attractive because of its ability to induce a population of memory-like NK cells that further proliferate in immunodeficient mice exposed to exogenous IL-2 (ref. 54). Patients given transfusion of NK cells are often given IL-2 for the promotion of *in vivo* expansion. Repeated injections of IL-2 at low dose are generally well tolerated, but no clinical advantage of IL-2 therapy was detected in a matched-pairs analysis[55]. $T_{reg}$ cells might be responsible for this lack of efficacy, as they express the high-affinity IL-2 receptor α-chain (CD25) and hamper NK cell proliferation by competing for IL-2. This inhibition can be overcome by depleting mice of $T_{reg}$ cells with an IL-2–diphtheria toxin fusion protein, a strategy that has led to improved NK cell proliferation and complete remission following infusion of haploidentical NK cells into patients with AML[56]. Cytokines that activate NK cells but not $T_{reg}$ cells are also being explored[10]. The anti-tumor effects of IL-12 and IL-18 as single agents are rather limited. In contrast, IL-21 holds promise, especially when combined with tumor-targeting monoclonal antibodies (mAbs)[57]. However, overall, IL-15 is probably the cytokine that brings the greatest hope[58], with progress being made in delineating the IL-15 signaling pathway in NK cells (**Box 2**). In non-Hodgkin's lymphomas, high concentrations of serum IL-15 following autologous HSCT are associated with better survival[59]. A phase I clinical trial of patients with metastatic malignancies has reported that daily

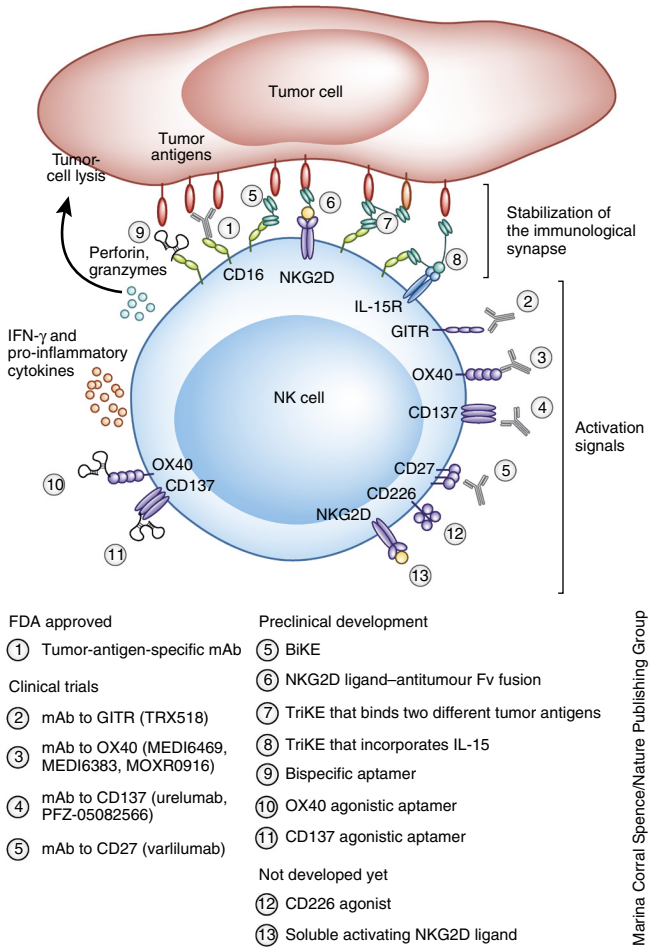


**Figure 3** Therapeutic approaches that engage activating receptors on NK cells. mAbs that target tumor-specific antigens and have been approved by the US Food and Drug Administration (FDA) (1) are widely used in the clinic. Their anti-tumor activity is attributed in part to their ability to trigger CD16 (the low-affinity receptor for IgG) on NK cells and induce ADCC. Agonistic mAbs to GITR (2), OX40 (3), CD137 (4) and CD27 (5) are currently being tested in clinical trials. These mAbs have been developed with the primary aim of stimulating T cells, but they might also positively influence NK cell functions. Enthusiasm has been growing for bispecific killer-cell engagers (BiKE) or trispecific killer-cell engagers (TriKE) that link activating receptors on NK cells to tumor antigens. Most of the bispecific engagers (5) trigger CD16, but fusion proteins that bridge NKG2D to tumor antigens (6) have also been designed. Trispecific engagers present three binding sites, and this provides the opportunity of targeting two different tumor antigens (7). The incorporation of IL-15 into a trispecific construct (8) further enhances the activation of NK cells. Alternatively, aptamers can also redirect NK cell activity toward tumor antigens (9) or can stimulate co-stimulatory molecules (10 and 11) to amplify the activation of NK cells. Finally, agonists of the activating receptor CD226 (12) have not been developed yet, but several pieces of evidence indicate that they might improve the anti-tumor activity of NK cells.

infusion of IL-15 induces NK cell proliferation and substantially increases the number of NK cells[60]. Although no objective response was observed in this study, some patients manifested a decrease of their marker lesions. Furthermore, IL-15-stimulated NK cells induced a clinical response in four of six pediatric patients with solid refractory tumors[61]. Administration of IL-15 is currently being tested with adoptive transfer of NK cells for the treatment of both solid cancers and hematologic cancers (clinical trials NCT01385423 and NCT01875601). The superagonist IL-15–IL-15Rα–Sushi-Fc fusion complex (ALT-803) exhibits greater biological activity than that of native IL-15, and this potent stimulator of NK cell anti-metastatic functions has entered clinical trial (NCT02099539)[62]. Finally, genetic engineering for the ectopic expression of IL-15 represents another possibility for enhancing NK cell function[63,64].

As mentioned above, inhibitory factors in the tumor microenvironment can hinder NK cell function, with TGF-β being a major suppressor of NK cell responses[18,24]. Several pharmaceutical inhibitors of TGF-β signaling have been developed, and the immunomodulatory activity of one of these (galunisertib) is currently being tested in cancer patients (clinical trial NCT02304419). Alternatively, approaches that target the immunosuppressive adenosinergic pathway deserve particular attention, as NK cells express the high-affinity adenosine receptor $A_{2A}$ (ref. 21). Antagonists directed against this receptor are already in use for the treatment of Parkinson's disease and might benefit patients with cancer by promoting NK cell–mediated anti-metastatic responses.

New classes of drugs with both direct anti-tumor effects and immunomodulatory activity can regulate NK cell function[65]. Thalidomide analogs called 'immunomodulatory drugs' are of special interest because of their ability to increase NK cell–mediated cytotoxicity[66]. Conversely, tyrosine-kinase inhibitors mostly abolish NK cell function[65], the exception being for imatinib, which indirectly activates NK cells through DC stimulation[67]. Small-molecule inhibitors of the serine-threorine kinase GSK3 have direct anti-cancer properties and can enhance NK cell activity in a humanized mouse model of AML[68]. Demethylating agents, histone deacetylases and proteasome inhibitors have dual effects; these drugs favor the recognition of cancer cells by NK cells through the modulation of the expression of ligands for NK cell receptors on the surface of the cancer cell, but they also exert direct inhibitory effects on NK cells by downregulating the expression of activating receptors and increasing apoptosis[69–72]. Interestingly, the proteasome inhibitor bortezomib enhances the anti-tumor effect of the infusion of autologous NK cells in mice[73]. In contrast, rapamycin and other inhibitors of the serine-threonine kinase mTOR inhibit NK cell function by antagonizing the IL-15 signaling pathway[74]. Hence, anti-cancer agents should be carefully selected for combination with NK cell–based therapy.

## Engagement of activating receptors on NK cells

NK cells express a wide array of activating and co-stimulatory receptors that can be targeted by means of antibodies and soluble ligands to improve NK cell activity *in vivo* (**Fig. 3**). In its simplest form, this involves ligation of CD16 expressed by NK cells with the Fc region of antibodies to allow ADCC. Tumor-targeting mAbs such as trastuzumab (mAb to ErbB2 (HER2)), cetuximab (mAb to epidermal growth factor receptor) or rituximab (mAb to the B cell–specific surface antigen CD20) have demonstrated success in the treatment of various malignancies and are now widely used in the clinic[75]. Their therapeutic efficacy relies on two main mechanisms of action mediated by the two domains of the mAb: the Fab fragment binds signaling receptors on the tumor cell surface and thereby directly promotes apoptosis or growth arrest, while the Fc portion recruits immunological effector cells, which leads to tumor-cell elimination[76]. Several pieces of evidence suggest that Fc-dependent mechanisms, including ADCC, are pivotal to the clinical effect of these mAbs. Polymorphisms in genes encoding Fc receptors have been found to correlate with clinical responses to tumor-targeting mAbs[77,78], and mAb-mediated

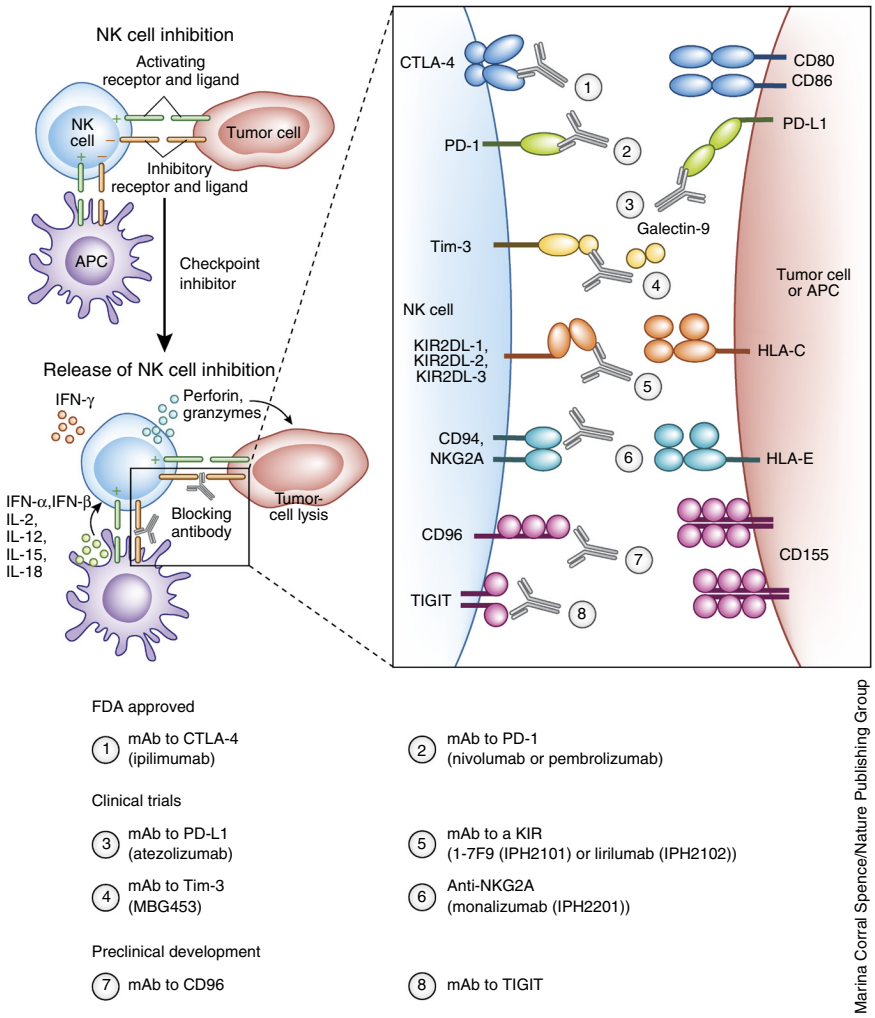


**Figure 4** Checkpoint inhibitors that 'release' NK cell functions. The balance between activating signals and inhibitory signals received by receptors on NK cells determines the activation of NK cells. Inhibitory ligands expressed on the surface of target tumor cells or on antigen-presenting cells (APC) prevent the activation of NK cells, and abolishing these inhibitory signals by the means of blocking mAbs would restore full NK cell activity. Blocking mAbs directed against CTLA-4 (1), PD-1 (2) or PD-L1 (3) constitute one of the major advances of the past decade in terms of cancer immunotherapy. Even if T cells are considered the key mediators of the impressive efficacy of these checkpoint inhibitors, blockade of the CTLA-4 or PD-1 pathway might also enhance the anti-tumor responses of NK cells. Tim-3 is another checkpoint molecule shared by T cells and NK cells, and mAbs to Tim-3 (4) are currently being tested in cancer patients. In addition, mAbs that specifically block NK cell inhibitory receptors for MHC molecules have entered clinical trials. By blocking KIRs (5) or NKG2A (6), these mAbs release a major brake on the activation of NK cells. Finally, signaling via CD96 and TIGIT can restrain NK cell activity ,and blocking mAbs to CD96 (7) or TIGIT (8) have great anti-cancer potential.

protection is lost in mice that lack activating Fc receptors[79]. Even if macrophages and neutrophils contribute substantially to this effect[76], it is clear that high NK cell activity benefits mAb therapy. Indeed, in a phase I clinical trial evaluating rituximab combined with injection of IL-2, the best responses were seen in patients with increased NK cell counts and ADCC activity in the peripheral blood[80]. Many efforts are being deployed to improve NK cell–mediated ADCC. A major issue is the shedding of CD16 mediated by the metalloproteinase ADAM17 following the activation of NK cells[81]; this can be overcome through the use of metalloproteinase inhibitors that lead to increased and sustained CD16-mediated signaling and promote human NK cell polyfunctionality *in vitro*[82]. Other approaches involve manipulation of the Fc portion of cancer-specific mAbs[83,84], fusion with IL-2 (ref. 30), and combination with NK cell–activating treatments such as cytokines[85], Toll-like receptor ligands[86] or immunomodulatory drugs[87]. Of interest, adoptively transferred autologous NK cells retain ADCC activity and might boost the efficacy of anti-cancer mAbs[41].

Bispecific or trispecific killer-cell engagers represent an alternative strategy for the efficient engagement of CD16 and the induction of ADCC-like responses[88]. Killer-cell engagers are designed by the fusion of Fv domains that recognize tumor-cell antigens with Fv domains that bind CD16. Bispecific mAbs that bridge NK cells to tumor cells were tested in clinical trials two decades ago and yielded promising results in patients with Hodgkin's lymphoma[89]. Over time, various constructs have been engineered. Improved *in vitro* results have been reported with the addition of third Fv domain that recognizes the same or a different tumor antigen[88,90] or by the combination of killer-cell engagers with a metalloproteinase inhibitor[91]. A new bispecific antibody directed against the stem-cell antigen CD133 presents the advantage of targeting the cancer stem-cell population that is drug resistant and is probably responsible for tumor relapse in some malignancies[92]. A very interesting trispecific construct incorporates IL-15 together with CD33- and CD16-binding Fv domains[93]. This trispecific antibody induced much more cytokine release and ADCC activity by NK cells than did the bispecific antibody without IL-15, and in a humanized mouse model, it showed tumor control, superior to that of the bispecific antibody without IL-15 associated with larger numbers of circulating NK cells. Finally, aptamers are structured single-stranded oligonucleotides that represent an alternative to tumor-specific mAbs. Bispecific aptamers that bind simultaneously to CD16 and the receptor tyrosine kinase c-Met are able to induce ADCC against c-Met expressing tumors[94].

NK cells express activating receptors and co-stimulatory molecules, but so far receptors other than CD16 have received less therapeutic interest. Still, bispecific proteins consisting of a NKG2D ligand fused with an Fv fragment that recognizes a tumor antigen have been shown to induce NK cell–mediated killing of primary lymphoma cells *in vitro*[95] and have demonstrated protective activity in a xenograft model of multiple myeloma[96]. While tumor-membrane-bound NKG2D ligands (for example, the MHC class I–related chains MICA and MICB, and ULBPs, in humans) might stimulate protective anti-tumor immunity, native NKG2D soluble ligands have long been considered immunosuppressive[97]. However, it has been shown that the mouse soluble NKG2D ligand MULT1, whose expression is commonly upregulated in primary tumors, promotes NK cell activation and tumor rejection

## Box 3 NK cell receptors that bind to nectin and nectin-like proteins

Special attention has been accorded to a group of receptors on NK cells that compete for the binding of molecules of the nectin and nectin-like family[118]. These receptors are CD226 (DNAM-1), TIGIT and CD96, all of which share CD155 (PVR) as a ligand. Moreover, CD226 and CD96 bind to CD112 (nectin-2), while TIGIT also binds to CD113 (nectin-3) and CD96 also binds to CD111 (nectin-1). In addition to regulating NK cell–DC cross-talk, interactions with nectins and nectin-like molecules modulate the anti-tumor responses of NK cells. CD155 expression is often upregulated in transformed cells, and the importance of the CD226-CD155 interaction in cancer immunosurveillance has been demonstrated in several mouse models. In contrast, TIGIT limits the cytotoxicity and IFN-γ production of NK cells and is thought to prevent the killing of healthy self cells by human and mouse NK cells[141,142]. Likewise, CD96 has been shown to downregulate CD226-dependent anti-tumor NK cell responses in mice[119], and blockade of CD96 has been proposed as a strategy to increase NK cells' control of metastasis[120], although some differences might exist between mouse CD96 and human CD96. Indeed, despite the presence of cytoplasmic immunoreceptor-tyrosine-based-inhibitory-motif–like motifs, human CD96 can promote NK cell function, mainly by facilitating the adhesion of NK cells to CD155-expressing cells[143]. Interestingly, CD226 expression on mouse NK cells distinguishes two functional subsets, with $CD226^+$ NK cells being potent producers of IFN-γ, IL-6 and GM-CSF, and $CD226^-$ NK cells arising from $CD226^+$ NK cells and secreting mainly cytokines of the MIP family[4]. Most human NK cells express CD226; whether CD226 also delineates two functional NK cell subsets in humans has not yet been studied.

in mice[98]. Such opposing effects of soluble NKG2D ligands might be due to the differences in their affinities, which might lead to different signaling and systemic effects: MULT1 is a high-affinity ligand, while MICA and MICB are low-affinity ligands. The biology of NKG2D ligands and their role in cancer have been reviewed extensively elsewhere[99,100]. Furthermore, various isoforms of the receptor NKp30 have been described, with NKp30a and NKp30b being immunostimulatory, and NKp30c being immunosuppressive. The nature of NKp30 isoforms can be used to predict the clinical outcome of gastrointestinal sarcoma and neuroblastoma[101,102]. Notably, depletion of the immunosuppressive isoform by means of specific small interfering RNA restores the release of T helper type 1 cytokines by NK cells and might open up new potential therapeutic avenues[102]. Of interest, phase I and II clinical trials have demonstrated the ability of DC-derived exosomes harboring the cytokine-receptor chain IL-15Rα and ligands of NKG2D or NKp30 to restore NK cell function in patients with advanced melanoma or non-small-cell lung cancer, associated with longer progression-free survival[34,103]. Finally, the co-receptor CD226 is required for the anti-tumor function of NK cells[104,105], and stimulating CD226 represents an alternative strategy for the therapeutic activation of NK cells; however, no agonistic drug has been developed so far, and CD226 expression on platelets might be of concern.

NK cells share with T cells the expression of various co-stimulatory molecules that can be targeted by agonistic mAbs. For example, mAbs to the integral membrane protein CD137 display NK cell–dependent efficacy in various mouse tumor models and have now reached the clinic[106]. While ligation of CD137 promotes the function of mouse NK cells, its effect on human NK cells remains controversial. One study has reported that CD137L–CD137 interactions impair the activity of human NK cells[107], whereas another has found that an agonistic mAb to CD137 enhances human NK cell–mediated ADCC[108,109]. Ongoing clinical trials combining mAb to CD137 with rituximab (NCT02420938, NCR01775631 and NCT01307267) or cetuximab (NCT02110082) should shed light on the therapeutic benefit of stimulation via CD137. OX40, GITR and CD27 represent other receptors of the tumor-necrosis-factor family that are able to potentiate NK cell responses[110]. Agonists directed against these receptors have been developed with the main purpose of stimulating T cells, and their effect on NK cell function is still unknown.

### Releasing NK cell inhibition

Signaling through inhibitory receptors and immunological checkpoints limit NK cell function, and blocking these pathways should unleash the anti-tumor potential of NK cells (**Fig. 4**). The first strategy of this type blocks HLA–inhibitory receptor interactions. Downregulation of MHC class I is common in cancer but is not observed in every tumor. When tumor cells have high expression of HLA class I, the engagement of inhibitory receptors constitutes a major brake on NK cell activation. IPH2101 (1-7F9) is a fully human IgG4 mAb that binds with high affinity to the inhibitory receptors KIR2DL-1, KIR2DL-2 and KIR2DL-3 and thereby blocks inhibitory KIR signaling mediated by HLA-C molecules of both group 1 and 2 allotypes[111]. Phase I clinical trials have established that IPH2101 is safe and is well tolerated by patients with AML or multiple myeloma[112,113]. In these trials, NK cell activation and enhanced *ex vivo* cytotoxic activity have been observed following administration of IPH2101. However, a subsequent phase II trial has failed to demonstrate any clinical efficacy of IPH2101 in patients with smoldering multiple myeloma[114]. Despite its limited clinical benefits as a single agent, IPH2101 might still be effective in combination with other agents. Pre-clinical studies support the combination of KIR blockade with lenalidomide[27] or with rituximab[115] for the treatment of multiple myeloma or lymphoma, respectively. Lirilumab (IPH2102; BMS-986015), a recombinant version of IPH2101 with a stabilized hinge[115], is currently being tested in combination in eight clinical trials of patients with hematological or solid cancer.

In addition to the KIRs, NKG2A-CD94 is another inhibitory receptor that has received great interest. The heterodimer NKG2A-CD94 binds to the non-classical HLA class I molecule HLA-E, which is often upregulated on cancer cells[116]. Antibodies that block NKG2A-HLAE interactions increase the cytotoxic activity of NK cells *in vitro*[117]. Five clinical trials are now being conducted to investigate the safety and anti-cancer activity of monalizumab (IPH2201), a blocking mAb to NKG2A. Monalizumab is being tested as a single agent or in combination with other agents for the treatment of various cancers, including chronic lymphocytic leukemia and carcinomas of various origins.

Another family of inhibitory receptors now emerging are those that bind nectin and nectin-like molecules; TIGIT and CD96 (TACTILE) are two receptors of the immunoglobulin family that negatively regulate NK cell function[118]. By interacting with nectin and nectin-like molecules, TIGIT and CD96 counterbalance the CD226-mediated activation of NK cells (**Box 3**). Although TIGIT deficiency has no effect on NK cell–mediated control of experimental metastasis, mice genetically deficient in CD96 are more resistant to both methylcholanthrene-induced fibrosarcomas and experimental lung metastasis[119]. Thus, neutralizing CD96 signaling might benefit patients with cancer.

That idea is supported by a study that established the protective effect of CD96 blockade on both experimental metastasis and spontaneous metastasis in mice[120]. This work demonstrated that mAbs to CD96 not only are efficient as a monotherapy but also act in synergy with chemotherapy and with mAbs that inhibit the T cell checkpoint molecules PD-1 and CTLA-4. Another interesting finding of this study is the enhanced anti-tumor effect of a mAb to CD96 in TIGIT-deficient mice relative to that in TIGIT-sufficient mice. This observation provides a rationale for the combination of the blockade of TIGIT and CD96.

NK cells also express immunological checkpoint molecules commonly associated with T cells, such as CTLA-4 and PD-1. Blocking antibodies directed against CTLA-4 or PD-1 have shown impressive results in patients with advanced cancer[121]. The efficacy of these checkpoint inhibitors is thought to rely on the reversion of T cell exhaustion, and very little attention has been accorded to NK cells in

**Table 1 The most promising approaches for targeting NK cells**

| Therapy | | Development stage | Advantages | Drawbacks | Ref. | Trial |
|---|---|---|---|---|---|---|
| Adoptive transfer | Autologous NK cells | Clinical trial (or FDA approved if part of HSCT) | Safe | Low efficacy | 40,41 | UMIN000007527 |
| | Allogeneic NK cells | Clinical trial (or FDA approved if part of HSCT) | Highly effective against some malignancies if KIR-ligand mismatch | Substantial depletion of T cells needed to avoid GVHD | 26,43 | NCT00187096 |
| | NK cell lines | Clinical trials | Easy to expand; transfer safe after irradiation | Low efficacy if not modified | 35 | NCT00900809; NCT02465957 |
| | CAR NK cells | Clinical trials | Redirect NK cell activity against a specific tumor antigen | Difficulty to manufacture large numbers of CAR NK cells | 49,50 | NCT0099513; NCT01974479 |
| Cytokines | IL-2 | FDA approved | Repeated injections well tolerated at low doses | Toxicity at high doses (capillary leak syndrome); activation of $T_{reg}$ cells | 55 | |
| | IL-15 | Clinical trials | Activates both NK cells and $CD8^+$ T cells without activating $T_{reg}$ cells | Potential toxicities: fever, thrombocytopenia and hypotension | 60 | NCT01385423; NCT01875601 |
| | IL-15SA–IL-15Rα–Su-Fc (ALT-803) | Clinical trial | More potent NK cell activator than IL-15 | Potential toxicities remain to be evaluated | 62 | NCT02099539 |
| Anti-cancer agents | IMiDs and imatinib mesylate | FDA approved | Direct anti-tumor effect combined with immunostimulatory effects | | 65 | |
| | Bortezomib and genotoxic agents | FDA approved | Direct cytotoxicity toward tumor cells, Sensitize tumor cell to NK cell killing | Might have immunosuppressive effects and inhibit NK cell function | 65 | |
| | GSK3 inhibitors | Clinical trial | Direct anti-tumor effect; enhances NK cell killing activity | Might promote $T_{reg}$ cell function | 68 | NCT01632306 NCT01287520 NCT01214603 |
| Targeting immune-suppressive pathways | Depletion of $T_{reg}$ cells (IL2DT) | Clinical trial | Improves efficacy of haploidentical NK cell transfer, Safe (short half-life of IL2DT) | | 56 | NCT01106950 |
| | Inhibitors of the TGF-β pathway | Clinical trial and FDA approved (not for cancer) | Broad effect on immune responses not restricted to NK cells | Effects of TGF-β blockade on tumor progression not well defined | 24 | NCT02304419 |
| | Inhibitors of the adenosinergic pathway | Clinical trial | Safe; broad effect on immune responses not restricted to NK cells | | 138 | NCT02403193 |
| Agonists of NK cell activating receptors | Tumor-targeting mAbs | FDA approved | Redirect NK cell activity against a specific tumor antigen | Possible on-target, off-tumor effect | 80 | |
| | BiKEs and TriKEs | Pre-clinical development | Re-direct NK cell activity against a specific tumor antigen | Possible on-target, off-tumor effect | 93 | |
| | mAbs to CD137 | Clinical trial | Stimulate NK cell ADCC; also stimulate T cells | Conflicting reports on the stimulation of human NK cells; severe liver toxicity at high doses | 108 | NCT02420938; NCR01775631; NCT01307267; NCT02110082 |
| Checkpoint inhibition | mAbs to KIRs (IPH2101 (1-7F9) and lirilumab (IPH2102, BMS-986015)) | Clinical trials | Safe; can be used in any patient without KIR genotyping | Low efficacy; needs combination | 112,113 | NCT02399917; NCT01592370; NCT02252263; NCT02599649; NCT02481297; NCT01687387; NCT01714739; NCT01750580 |
| | mAbs: to NKG2A (monalizumab (IPH2201)) | Clinical trials | Can be used in any patient without KIR genotyping | | 117 | NCT02459301; NCT02331875; NCT02557516; NCT02643550; NCT02671435 |

this context. Still, checkpoint inhibition probably modifies the tumor microenvironment in a way that would facilitate NK cell activity and might also directly target NK cells. For example, CTLA-4 negatively regulates IFN-γ production by mouse NK cells in response to stimulation by DCs[122]. Furthermore, the transfer of IL-2-activated NK cells pre-incubated with a PD-1-blocking antibody resulted in improved survival in a mouse glioma model[123]. The great potential of suppressing the pathway of PD-1 and its ligand PD-L1, in combination with NK cell–based therapies, is supported by the encouraging results of a phase II trial combining pidilizumab with rituximab in patients with relapsed follicular lymphoma[124]. Finally, expression of the glycoprotein Tim-3 has been found to be upregulated on the surface of NK cells from patients with metastatic melanoma, lung adenocarcinoma or gastric cancer[125–127]. Notably, higher expression of Tim-3 seems to correlate with poorer prognosis, while blockade of Tim-3 has been shown to reverse the exhausted phenotype of NK cells from patients with melanoma[125].

## Conclusions and future directions

Despite the discovery of the 'natural killing' character of NK cells more than 40 years ago, NK cell function is yet to be successfully and fully exploited in the clinic. This delay might be explained by the lack of appropriate models that recapitulate the complexity of human NK cell biology. Indeed, NK cells are a heterogeneous population comprising cytotoxic, immunoregulatory, memory and licensed-unlicensed subsets. Choosing the right subset to target is not an easy task, as this heterogeneity is not fully understood. The still incompletely resolved question of NK cell 'licensing' is of considerable importance not only in the context of HSCT[128] and hematological cancers[37] but also for the *in vivo* targeting of NK cells. Indeed, in contrast to such function of licensed NK cells, the ADCC function of the unlicensed subset is not inhibited by self MHC, and the presence of unlicensed NK cells has been found to correlate with better prognosis for patients with neuroblastoma treated with anti-GD2 (ref. 129). Substantial interspecies differences constitute a major obstacle to clinical translation; these include the absence of clear equivalence for NK cell subsets in mice and humans, as well as differences in surface receptors (for example, KIRs, NKp30 and NKp44 are not expressed by mice). Several models have been used to investigate NK cells[130]. Humanized mice are perhaps the most useful clinical model, even though the homeostasis of human NK cells in these mice is achieved only through injection of human IL-15 (refs. 131,132). Despite all that, several approaches targeting NK cells are currently in clinical or preclinical development (**Table 1**), and most of these have a good safety profile. Indeed, no toxicity has been observed following the adoptive transfer of autologous NK cells, which is a considerable advantage over adoptive T cell therapy, but the efficacy such cell transfer is rather modest. However, KIR-mismatched allogeneic NK cells that show strong anti-tumor effects, at least against some hematological cancers[26], can have toxic effects in some cases[26,45]. Moreover, the difficulties in manufacturing large numbers of NK cells, together with the low persistence and diminished activity of these cells in patients with cancer, are the main barriers to overcome[36,51]. In this context, passive approaches for mobilizing NK cells in the host with antibodies, small-molecule drugs, cytokines and adjuvants might be effective alternative strategies. In fact, strategies that combine the adoptive transfer of NK cells together with the modulation of signals received by receptors on NK cells hold great promise. Patients with cancer usually undergo substantial conventional therapy before they receive immunotherapy, and future work should focus on understanding the influence of chemotherapeutics and conditioning agents on NK cell activity for selection of the correct clinical settings for NK cell–based therapies. In this context, the immunomodulatory drugs imatinib and bortezomib offer the possibility of directly targeting the tumor while potentiating NK cell responses. Moreover, sequenced therapy targeting first NK cells and then T cells should be considered, as tumor-cell killing and IFN-γ production by NK cells would probably foster a microenvironment conducive to the development of antigen-specific T cell responses. The majority of the approaches described in this Review have considered external signals sensed by NK cells, such as ligands or soluble factors present in the microenvironment. However, progress in understanding NK cell biology should encourage the development of small-molecule inhibitors that directly target intracellular signaling cascades, such as the Cbl-b pathway[133], CDK8 pathway[134], GSK3 pathway[68] or CIS pathway[135]. Finally, NK cells might be more effective against hematological cancers, residual minimal disease and nascent metastasis, possibly because of their early role, cross-talk with other leukocytes and inability to vigorously proliferate, in contrast to T cells. Crucial knowledge about NK cell–homing capacities[136,137] and their poor infiltration of solid tumors is still lacking. Deeper understanding of the heterogeneity of NK cells and their activity *in vivo* will allow researchers and clinicians to take full advantage of these cells. All things considered, it is an exciting time for the NK cell field; the tremendous anti-tumor capacities of NK cells are finally being brought to the clinic, and the results from the many ongoing trials are eagerly awaited.

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The authors declare competing financial interests: details are available in the online version of the paper.



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