NK cells for cancer immunotherapy

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Abstract | Natural killer (NK) cells can swiftly kill multiple adjacent cells if these show surface markers associated with oncogenic transformation. This property, which is unique among immune cells, and their capacity to enhance antibody and T cell responses support a role for NK cells as anticancer agents. Although tumours may develop several mechanisms to resist attacks from endogenous NK cells, ex vivo activation, expansion and genetic modification of NK cells can greatly increase their antitumour activity and equip them to overcome resistance. Some of these methods have been translated into clinical-grade platforms and support clinical trials of NK cell infusions in patients with haematological malignancies or solid tumours, which have yielded encouraging results so far. The next generation of NK cell products will be engineered to enhance activating signals and proliferation, suppress inhibitory signals and promote their homing to tumours. These modifications promise to significantly increase their clinical activity. Finally, there is emerging evidence of increased NK cell-mediated tumour cell killing in the context of molecularly targeted therapies. These observations, in addition to the capacity of NK cells to magnify immune responses, suggest that NK cells are poised to become key components of multipronged therapeutic strategies for cancer.

Graft-versus-host disease (GVHD). A condition resulting from the systemic attack of allogenic T cells on the tissues of an immunosuppressed recipient, which, in its most severe grade, is fatal. It can occur after allogeneic haematopoietic stem cell transplantation or after infusion of allogeneic T cells.

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The spectacular success of autologous chimeric antigen receptor (CAR) T cells in patients with leukaemia and lymphoma[1] has raised considerable interest in using immune cells as a cancer treatment modality. The results with CAR T cells have energized efforts to explore the clinical utility of other immune cell types, such as natural killer (NK) cells, which might circumvent some of the limitations of CAR T cells. Issues of particular interest include developing ways to target tumour cells that lack suitable surface targets, generating safe and robust allogeneic products and mitigating toxic effects associated with CAR T cell infusions.

Naturally occurring lymphoid non-T cells that can rapidly kill virally infected cells and tumour cells were discovered more than four decades ago[2,3]. NK cells can recognize tumour cells by unique mechanisms, which rely on a set of stimulatory and inhibitory receptors[4–6]. These receptors can sense whether a proximal cell expresses a profile of corresponding ligands associated with oncogenic transformation: a tumour-associated profile triggers NK cell activation and targeted cell killing. This capacity to recognize and rapidly kill tumour cells together with limited reactivity against healthy tissues suggests potential for NK cells as anticancer 'living drugs'. Unlike T cells, NK cells lack surface T cell receptors (TCRs) and do not cause graft-versus-host disease (GVHD)[7–10]. Therefore, they hold promise as an 'off-the-shelf' cell therapy product, which can be prepared in advance, optimized and administered on demand to multiple patients. This prospect has been bolstered by the development of clinical-grade methods that can generate large numbers of NK cells from multiple sources, including peripheral blood, umbilical cord blood and induced pluripotent stem cells (iPSCs). These platforms allow the clinical exploration of a variety of approaches that can activate NK cells, increase their proliferation in vivo and increase their capacity to recognize tumour cells. In this Review, we discuss the biological bases of these approaches, results of clinical trials and prospects for including NK cells in the arsenal of cancer immunotherapies.

An overview of NK cell biology

Classification, development and memory

NK cells belong to the innate lymphoid cell family[6,11]. In human peripheral blood, bone marrow and tissues, they can be identified by the absence of surface TCR and associated CD3 molecules, and by expression of neural cell adhesion molecule (NCAM; also known as CD56)[12]. Natural cytotoxicity triggering receptor 1 (NCR1; also known as NKp46 or CD335) can be used to specifically identify NK cells in circulation as well as in formalin-fixed, paraffin-embedded tissue specimens[13,14].

Human NK cells derive from multipotent $CD34^+$ haematopoietic progenitors in the bone marrow[15]. NK cell maturation occurs in the bone marrow as well as in the lymphoid organs and, unlike for T cells, does not require the thymus[15–17]. NK cells can persist in peripheral blood even if differentiation from progenitor cells is impaired, which suggests that there is homeostatic maintenance

in the periphery[18,19]. The turnover of human NK cells in blood takes around 2 weeks[20]. The estimated NK cell doubling time in vivo is 13.5 days[21], and continuous stimulation of adult peripheral blood NK cells in vitro could achieve a median of 16 (range 11–30) population doublings before they enter senescence, a proliferative potential in the lower range of that measured in T cells[22]. Overexpression of telomerase reverse transcriptase (TERT) allowed at least 130 additional doublings, pointing to telomere shortening as a major limiting factor of NK cell lifespan[22].

It was believed that innate immune responses exerted by NK cells were not amplified by subsequent exposure to the same target and were maximal at the first encounter. There is, however, increasing evidence that NK cells can acquire 'memory-like' functional features characterized by elevated functional activity and can generate specific recall responses[23]. Thus, repeated exposure of mouse NK cells to cytomegalovirus (CMV) results in increasingly more vigorous responses[23,24]. NK cell memory-like reactivity to viruses has also been observed in macaques and humans[23,25–27]. CMV infection in humans can be associated with an increased in the proportion of NK cells that express the activating receptor NKG2C, which can differentially recognize polymorphic CMV peptides[28].

Licensing and activation

NK cell activation is managed by a suite of activating, co-stimulatory and inhibitory receptors[4–6] (FIG. 1). The joint signals from these receptors determine whether an adjacent cell is targeted for killing and they also regulate cytokine secretion.

A fundamental function of NK cells is the elimination of cells with diminished or absent expression of major histocompatibility complex (MHC; also known as human leukocyte antigen (HLA)) class I molecules[29]. MHC class I molecules bind a set of inhibitory killer cell immunoglobulin-like receptors (KIRs), which suppress NK cell function and minimize the destruction of healthy self-cells[4–6]. During NK cell development, the interaction between KIRs and self-MHC molecules provides essential signals for NK cell maturation and contributes to the acquisition of functional competency, a process termed 'licensing'[30,31]. The number and type of MHC class I alleles quantitatively determine NK cell functionality[32]. Potential mechanisms underlying this gain in functionality involve distinct compartmentalization of activating and inhibitory receptors in licensed versus unlicensed NK cells[33,34] and lysosomal remodelling[35]. NK cells that are chronically stimulated by putative self-ligands might also become anergic if stimulation is not mitigated by inhibitory receptors engaged with self-MHC[30,31,36]. Once functional competency has been achieved, mature NK cells are suppressed by ligation of intact self-MHC, whereas suppression is released if MHC is altered or downregulated, which may occur in tumour cells[30,31]. There is plasticity in the responsiveness of mature NK cells, as this can be reset in an environment with different MHC expression[32,37], and unlicensed NK cells can nevertheless stimulate adaptive immune responses[38]. In mature NK cells, KIR-mediated inhibition is not absolute and can be overcome by strong activating stimuli. For example, NK cells expressing an anti-CD19 CAR from patients with acute lymphoblastic leukaemia (ALL) can readily kill autologous ALL cells that are resistant to NK cells lacking CARs[39].

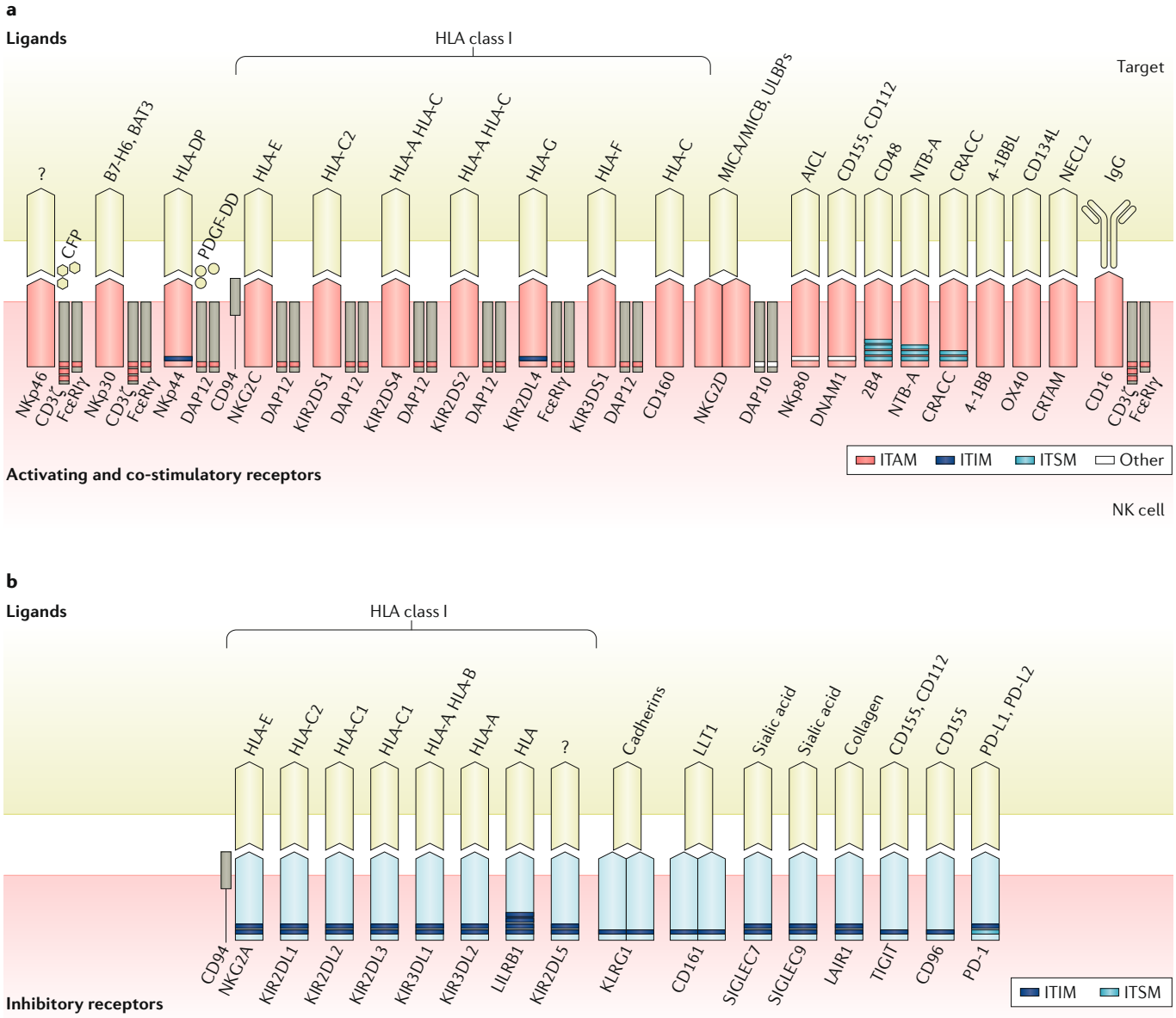
Besides downregulating MHC molecules, cancer cells may overexpress ligands for activating NK cell receptors[4–6]. For example, ligands for the activating NKG2D receptor — such as MHC class I polypeptide-related sequence A (MICA), MICB and several UL16-binding proteins (ULBPs) — are preferentially expressed in cancer cells and on cellular stress, infection or DNA damage[40,41]. Although most ligands for activating NK cell

Fig. 1 | **Activating and inhibitory NK receptors and their corresponding ligands. a** | Activating receptors transduce signals through various pathways. The natural cytotoxicity receptors (NCRs) NKp30 (also known as NCR3 or CD337) and NKp46 (also known as NCR1 or CD335) operate through the immunoreceptor tyrosine-based activation motifs (ITAMs) of FcεRIγ and CD3ζ, a mechanism also used by CD16 (also known as FCGR3A). Other receptors, such as NKp44 (also known as NCR2 or CD336), the heterodimer CD94–NKG2C and killer cell immunoglobulin-like receptors KIR2DS1, KIR2DS2, KIR2DS4 and KIR3DS1, signal via association with the ITAM-bearing DAP12 adaptor molecule, whereas NKG2D (also known as CD314) initiates signal transduction via an Src homology 2 domain-binding site in the DAP10 adaptor. NKp30 is ligated by B7-H6 (REF.[233]) and the nuclear factor HLA-B-associated transcript 3 (BAT3)[234]. Platelet-derived growth factor DD (PDGF-DD) and a subset of HLA-DP molecules can activate natural killer (NK) cells via NKp44 (REFS[43,235]), and the complement factor P (CFP; also known as properdin) can bind NKp46 (REF.[42]). The CD94–NKG2C heterodimeric receptor binds to HLA-E loaded with peptides derived from other human leukocyte antigen (HLA) class I molecules[109]. NKG2D has multiple ligands, including major histocompatibility complex class I polypeptide-related sequence A (MICA), MICB and several UL16-binding proteins (ULBPs)[40,41]. NK cell activation also depends on co-stimulation by molecules such as DNAM1 (also known as CD226), which signals through a tyrosine- and asparagine-based motif[236], 2B4 (also known as CD244), which signals through an immunoreceptor tyrosine-based switch motif (ITSM)[237], and 4-1BB (also known as CD137) and OX40 (also known as CD134), which initiate signal by recruiting tumour necrosis factor-associated factors[238]. **b** | Inhibitory receptors counterbalance signals transduced by activating receptors[4–6]. Killer cell immunoglobulin-like receptors recognize polymorphic HLA class I and use immunoreceptor tyrosine-based inhibitory motifs (ITIMs) to recruit tyrosine phosphatases and dampen signals[31]. Thus, during the interaction between NK cells and tumour cells, lower expression of HLA molecules on the latter can enhance activation. Conversely, overexpression of HLA-E in tumour cells exposed to interferon-γ secreted by activated NK and T cells can generate resistance to NK cells[112,113]. The heterodimer CD94–NKG2A engages HLA-E and transduces inhibitory signals via ITIMs[109–111,239–241], counterbalancing activating signals of CD94–NKG2C, which also binds to HLA-E but with lower affinity[242]. The balance between activation and inhibition extends to co-stimulatory molecules. Thus, CD155 and CD112, the ligands for activating DNAM1, can also bind inhibitory T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) and CD96 (REF.[243]).

Killer cell immunoglobulin-like receptors
(KIRs). Transmembrane proteins expressed by natural killer (NK) cells that interact with major histocompatibility complex/human leukocyte antigen class I molecules to modulate NK cell cytotoxicity by delivery predominantly inhibitory signals. KIR signalling during NK cell development is important for NK cell functional competency or licensing.

Licensing
A process driven by the interaction between inhibitory receptors on maturing natural killer cells and self-major histocompatibility complex molecules which increases natural killer cell responses to activating receptor signals.

Anergic
A hypofunctional state. In natural killer cells, anergy or hyporesponsiveness to activating signals might be caused by chronic stimulation during maturation through an activating receptor interacting with a self-ligand.



receptors are expressed on the cell membrane, some are soluble. For example, the complement factor P (properdin) binds NKp46, an interaction that seems to be involved in protection against bacterial infections[42]. Platelet derived growth factor DD (PDGF-DD), secreted by many tumour types, ligates the activating NKp44 receptor on NK cells, stimulating secretion of interferon-γ (IFNγ) and tumour necrosis factor (TNF)[43].

A separate mechanism for target recognition and NK cell activation is mediated by the CD16 receptor (also known as FCGR3A), which binds the constant region (Fc) of immunoglobulins. CD16 engagement by immunoglobulin-opsonized cells (cells with antibodies bound to surface membrane antigens) induces phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) domains of the high-affinity IgE receptor (FcεRIγ) and CD3ζ in NK cells, and initiates a signalling cascade that ultimately results in killing of the antibody-coated cell, a mechanism termed 'antibody-dependent cell cytotoxicity' (ADCC)[44,45]. Polymorphisms of *FCGR3A* can have marked functional consequences; receptors with valine instead of phenylalanine at position 158 have higher Fc binding and are associated with increased ADCC[44,46].

Regulatory cytokines

Ligation of individual activating receptors (with the exception of CD16) is generally insufficient to trigger cytotoxicity or cytokine secretion in naive NK cells; cytokine exposure is key to preactivating NK cells[47]. Exposure to IL-2 enhances signalling from activating receptors[47–50]. IL-15, the receptor for which shares the signalling β- and γ-subunits with the receptor for IL-2 (REF.[51]), also activates NK cells and promotes their survival and proliferation[51–54] (BOX 1). GRB2-associated binding protein 3 (GAB3) is critical for mitogen-activated protein kinase signalling triggered by IL-2 and IL-15 (REF.[55]). IL-15, secreted primarily by monocytes, macrophages and dendritic cells, forms complexes with IL-15 receptor α-chain on the surface of these cells and NK cells[56,57].

Box 1 | **Administration of IL-15**

Systemic administration of recombinant IL-15 can stimulate natural killer (NK) cell activity. In patients with metastatic malignant melanoma or renal cell cancer, bolus infusion of 0.3–3.0 μg per kg per day for 12 days caused an efflux of NK cells and $CD8^+$ T cells from the circulation within 1 h of infusion, followed by influx in the next 48 h and proliferation of NK cells up to 10-fold above baseline levels thereafter, with increase in the levels of inflammatory cytokines[53]. Dose-limiting toxic effects included hypotension, thrombocytopenia and elevations of the levels of transaminases at the higher doses. In another study, which included 14 patients with refractory solid tumours, IL-15 was given 5 days a week for 2 weeks at dosages of 0.25–3 μg per kg per day, which resulted in expansion of NK cells[54]. Grade 2 pancreatitis occurred in one patient, and cardiac chest pain with hypotension and increased troponin level occurred in another patient. Administration of recombinant IL-15 (0.3–1 μg per kg) for 12 days after NK cell infusion in patients with acute myeloid leukaemia resulted in higher rates of NK expansion than in previous trials with IL-2 but was associated with cytokine release syndrome and neurotoxicity after subcutaneous but not intravenous dosing[10]. A study assessed the effects of administering IL-15 coupled with IL-15 receptor α-chain and the crystallizable fragment of an immunoglobulin (ALT-803, a complex with higher IL-15 function) in patients with leukaemia who had relapsed after allogeneic haematopoietic stem cell transplantation[217]. No dose-limiting toxic effects or exacerbation of graft-versus-host disease was observed with subcutaneous administration, whereas NK cell activation and expansion was noted. The same agent was tested in patients with advanced-stage solid tumours, and a significant increase in NK cell numbers was observed[218].

Membrane-bound IL-15 (mbIL-15) presented on adjacent cells promoted cytotoxicity of mouse NK cells better than soluble IL-15 (REF.[57]), and IL-15 had superior bioactivity in animal models when administered as a complex with its receptor[58]. Transduction of IL-15 in human NK cells induced longer survival when IL-15 was expressed in a membrane-bound form than in a secreted form[59]. IL-15 induces expression of cytokine-inducible SH2-containing protein (CIS), which tempers NK cell activation by promoting the degradation of the tyrosine kinase JAK1 (REF.[60]). CIS deletion conferred hypersensitivity to IL-15, and augmented NK cell activity[60]. IL-21, like IL-2 and IL-15, signals via IL-2 receptor γ-chain[61]. IL-21, together with IL-15 and FMS-related tyrosine kinase 3 ligand (FLT3L), promotes the proliferation and differentiation of NK cells from human $CD34^+$ progenitor cells and can increase cytotoxicity and IFNγ production in mature NK cells[61,62].

IL-12 and IL-18 can also stimulate NK cells and are powerful inducers of IFNγ production when used in combination[63–66]. Adoptive transfer of NK cells exposed to IL-12, IL-15 and IL-18 produced increased antitumour activity and NK cell persistence in mice, whereas exposure to IL-15 or IL-2 alone had no effect[67]. Human NK cells exposed to the three cytokines for 16 h and washed and maintained in IL-15 for 1–2 weeks had greater IFNγ production after restimulation with IL-12 and IL-15 or co-culture with cell targets, suggesting induction of a 'memory-like' status[65,66]. The combined effects of different cytokines, however, can be multifaceted. For example, IL-12 stimulates expression of the inhibitory receptor NKG2A[66,68], and IL-21 can block expansion of resting NK cells induced by IL-15 (REF.[64]).

Type I interferon can also preactivate NK cells, readying them for stimulation by activating receptors. To this end, a recently uncovered mechanism starts in tumour cells with sensing of cytosolic DNA by the enzyme cyclic GMP–AMP synthase[69]. This triggers the production of the second-messenger cyclic GMP–AMP, which is transferred to non-tumour myeloid and B cells, where it binds and activates the adaptor protein signalling effector stimulator of interferon genes (STING). This interaction stimulates IFNβ production, which primes NK cells for cytotoxicity[69].

Target cell killing

NK cells in peripheral blood are recognizable morphologically as large lymphoid cells with a cytoplasm rich in granules; hence, the historic designation as 'large granular lymphocytes'[70]. When NK cells encounter a potential target cell and gets activated, a synapse with the target cell is formed, and the lytic granules, transported on microtubules, converge towards the synapse[71,72]. Additional signals from the synapse lead to polarization of the lytic granules, their docking and fusion with the plasma membrane and their release at the synapse[71,72]. The granules are lysosomal-related organelles that contain the key effectors of cytotoxicity: perforin, which inserts itself into the plasma membrane of target cells and forms pores leading to osmotic lysis, and granzymes, which transfer through the pores and activate caspases, causing apoptosis of target cells[4–6,72,73]. Remarkably, the release of a single granule can be sufficient to kill a target cell[72]. Expression on the cell surface of lysosomal-associated membrane protein 1 (LAMP1; also known as CD107a) can be used as a marker of this process, termed 'degranulation'[74]. NK cells are serial killers and, after degranulation, they can kill other target cells through a similar process[71,72]. Direct killing can also occur via expression of FAS ligand and TNF-related apoptosis-inducing ligand (TRAIL)[4–6,75].

Besides their cytotoxic capacity, NK cells can secrete multiple cytokines, chemokines and growth factors, including IFNγ, IL-13, TNF, FLT3L, CC-chemokine ligand 3 (CCL3), CCL4 and CCL5, lymphotactin (XCL1) and granulocyte–macrophage colony-stimulating factor[4–6]. Therefore, NK cells can influence the activity of other immune cells. For example, secretion of CCL5 and XCL1 attracts dendritic cells[76], FLT3L increases the number of stimulatory dendritic cells in the tumour microenvironment[77] and IFNγ promotes T helper 1 cell polarization, induces MHC class II molecules on antigen-presenting cells and activates macrophages[4–6].

NK cells in cancer

Immunosurveillance

A relation between NK cell activity and suppression of tumour occurrence has been documented in mouse models[78–82]. In humans, observations associating occurrence of malignancy and primary NK cell immunodeficiency suggest a role for NK cells in tumour immunosurveillance[83–85]. In a prospective study of more than 3,500 individuals with an 11-year follow-up, the age-adjusted relative risk of cancer was significantly higher in those individuals with low cytotoxicity of peripheral blood mononuclear cells (PBMCs) versus K562 cells, which are cell targets commonly used to measure NK cell cytotoxicity[86].

In patients with cancer, the degree of NK cell infiltration in tumour tissues was prognostic in some patient cohorts[87,88], and a reduced NK cell function has been

K562 cells
A chronic myelogenous leukaemia cell line that expresses the *BCR–ABL1* fusion gene, which lacks major histocompatibility complex/human leukocyte antigen surface expression and is commonly used as a target in natural killer (NK) cell cytotoxicity assays. Contact with K562 activates NK cells, K562 cells modified to express cytokines and stimulatory molecules are used to stimulate NK cell proliferation.

associated with worse outcome. In patients with gastrointestinal stromal tumours, higher expression of an isoform of the activating receptor NKp30 (NKp30c) — which results in a receptor with lesser stimulatory capacity — was associated with lower survival[89], whereas in patients with neuroblastoma, the ratio between NKp30a, NKp30b and NKp30c isoforms was reportedly predictive of progression-free survival in a retrospective analysis[90]. In patients with colorectal cancer, *IL15* deletion and reduction in IL-15 expression was associated with a higher risk of relapse[91].

The mechanisms by which endogenous NK cells can exert tumour immunosurveillance and influence tumour growth are not well understood. NK cell infiltration of tumours depends on their expression of heparanase[92]. Although NK cells are generally not the predominant lymphoid population in tumours, they may attract T cell infiltration and elicit inflammatory responses through cytokine and chemokine secretion[93,94]. Conceivably, NK cells might also contribute to preventing metastasis by eliminating circulating tumour cells[94,95].

Tumour resistance to NK cells

A reduction in surface expression of ligands for activating NK cell receptors is a mechanism of tumour resistance to NK cells[96]. For example, leukaemic cells with stem cell properties in acute myeloid leukaemia (AML) have low or absent expression of NKG2D ligands[97]. Reduction in surface expression of NKG2D ligands can occur through excretion in exosomes or cleavage by metalloproteinases[96,98–103]. NK cell antitumour activity in mice is increased by antibodies that block the shedding of the NKG2D ligands MICA and MICB by targeting the MICA α3 proteolytic site[104]. Prolonged stimulation by activating ligands expressed by tumour cells can induce resistance to NK cells by reducing expression of the adaptor molecules DAP10 (also known as HCST) and DAP12 (also known as TYROBP), which mediate signalling and cell activation following ligation of their associated receptors[105]. Activating ligands can also be expressed by non-tumour cells, and exposure to lactate dehydrogenase isoform 5 secreted by tumour cells can increase expression of MICB and ULBP1 on monocytes[106]. Expression of NKG2D ligands in lymph node endothelial cells has also been proposed as a desensitizing mechanism[107]. In this context, soluble ligands might counteract NK cell desensitization from membrane NKG2D ligands on tumour-associated cells, increasing rather than suppressing NK cell activity[108].

IFNγ secreted by NK and T cells stimulates expression of MHC class I molecules in tumour cells, and can suppress NK cell activity through ligation of inhibitory receptors[4–6]. For example, expression of peptide-loaded HLA-E, the ligand of inhibitory CD94–NKG2A receptor complex[109–111] (FIG. 1), is stimulated in tumour cells exposed to IFNγ[94,112]. Expression of *KLRC1* (the gene encoding NKG2A) in tumour samples correlates with that of *HLA-E*[113], and the tumour microenvironment is enriched with NK cells expressing NKG2A[114]. HLA-E is overexpressed in several tumours, and higher expression is associated with worse outcome[93,113,115,116]. These data suggest a scenario in which NK cell responses are progressively dampened by increased ligation of CD94–NKG2A. The T cell immune checkpoints programmed cell death 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA4), lymphocyte activation gene 3 protein (LAG3), and hepatitis A virus cellular receptor (HAVCR2; also known as TIM3) are expressed in some NK cells, and their ligands might play a part in dampening NK antitumour responses; blocking this interaction with checkpoint inhibitors enhances NK cell activity[117–119].

Soluble factors in the tumour milieu can suppress NK cell activation. Transforming growth factor-β (TGFβ) blocks the mechanistic target of rapamycin (mTOR) signalling pathway triggered by IL-15 in NK cells, reducing their proliferation and cytotoxicity[120]; NK cells exposed to TGFβ acquire a gene signature and phenotype characteristic of less cytotoxic innate lymphoid cells, and are unable to control tumour growth in mice[121,122]. Activin, a member of the TGFβ family, exerts a similar disabling effect on NK cells[123]. Other immunosuppressive factors produced by the tumour microenvironment that can dampen NK cell activation include prostaglandin E_2, L-kynurenine and picolinic acid derived from tryptophan catabolism by indoleamine 2,3-dioxygenase, adenosine and lactic acid[6,124–126]. Progressive overexpression of fructose 1,6-bisphosphatase in NK cells responding to tumours eventually results in impaired glycolysis that limits NK cell activity[127]. Hypoxia and low nutrient levels in the tumour microenviroment might suppress NK cell metabolism and antitumour activity[128]. Finally, accumulation of extracellular matrix and increased interstitial fluid pressure can prevent tissue penetration of immune cells[129].

NK cells as living drugs

Data from in vitro experiments and animal models unequivocally demonstrate the antitumour activity of NK cells. This body of knowledge provides a strong rationale for exploring the use of NK cells as anticancer agents. The understanding gained about the signals that regulate NK cell activation and underlying tumour resistance to NK cells offers important clues as to how NK cells can be harnessed to maximize their antitumour potential. Conversion of NK cells into living drugs, however, requires several modifications to research laboratory techniques, and must be executed within the constraints of large-scale, clinical-grade conditions that meet regulatory requirements.

Source, isolation and preactivation

NK cells for infusion can be obtained from peripheral blood, umbilical cord blood[130–132] or post-partum placenta[133] (FIG. 2). The functional differences between NK cells from these sources are still unclear, but umbilical cord sources offer the advantage of access to umbilical cord blood banks that could facilitate the selection of donors of certain HLA types and with specific NK receptor profiles. Instead of primary NK cells, some investigators have used the NK-92 cell line, which was derived from $CD56^+CD3^-$ tumour cells of a patient with non-Hodgkin lymphoma (NHL) and has cytotoxic properties similar to those of NK cells[134]. Although a continuously growing cell line that can provide unlimited tumoricidal cells simplifies the manufacturing

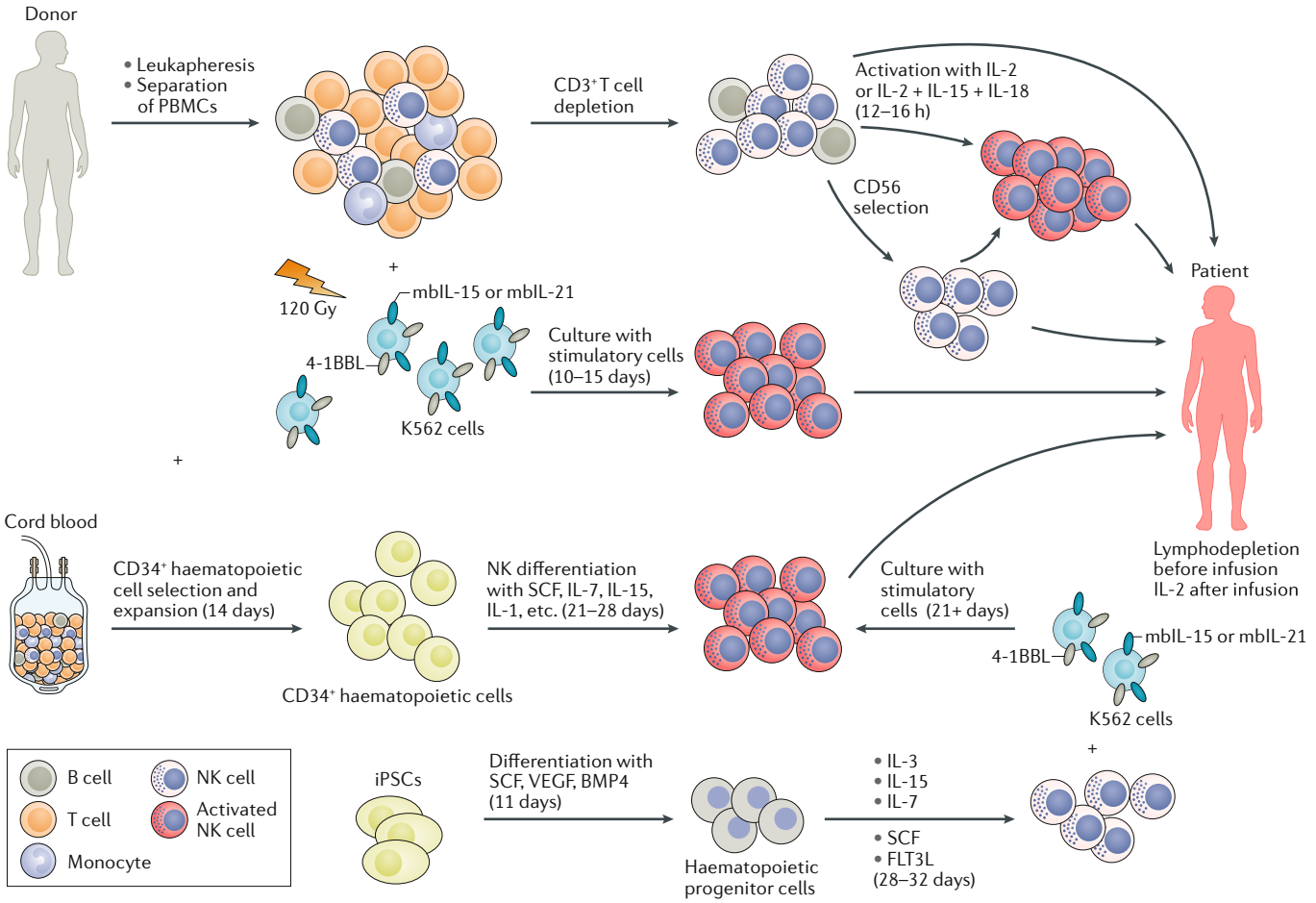


Fig. 2 | **Sources and methods for isolation, activation and propagation of allogeneic NK cells.** Peripheral blood mononucleated cells (PBMCs) can be obtained from healthy donors by leukapheresis. PBMC depletion of $CD3^+$ T cells, to prevent graft-versus-host disease after infusion, is done with anti-CD3 immunomagnetic particles, yielding a product enriched in natural killer (NK) cells but containing other cells, such as B cells and monocytes. NK cells can be further purified by $CD56^+$ cell selection with anti-CD56 immunomagnetic particles. CD3-depleted PBMCs or purified NK cells are infused as such or are activated with a 12–16-h culture with cytokines; IL-2 or a mixture of IL-12, IL-15 and IL-18 has been used in clinical studies[8,146]. NK cells can also be propagated ex vivo by a 10–15-day co-culture of PBMCs with stimulatory cells, such as the K562 cell line expressing membrane-bound IL-15 (mbIL-15) and 4-1BBL, which is irradiated before co-culture with PBMCs to prevent its expansion[39,49,135]. Residual $CD3^+$ T cells (which do not expand in these conditions) are removed after culture; cultures can also start with CD3-depleted PBMCs or purified NK cells. Umbilical cord blood NK cells can be enriched, activated and propagated like peripheral blood NK cells. Another approach includes the enrichment of $CD34^+$ haematopoietic progenitors from umbilical cord blood with anti-CD34 immunomagnetic particles. After expansion, cells are exposed to growth factors that promote their differentiation to NK cells[148]. NK cells have also been derived from induced pluripotent stem cells (iPSCs) using culture conditions that first promote haematopoietic differentiation and then NK cell differentiation, followed by propagation with a stimulatory cell line expressing mbIL-21 (REFS[149,150]). Before infusion of allogeneic NK cells, patients typically receive lymphodepleting chemotherapy, such as cyclophosphamide and fludarabine therapy, for about 1 week, which causes a transient depletion of immune cells and allows temporary engraftment of the infused NK cells. Engraftment can be supported by administration of IL-2 after infusion. BMP4, bone morphogenetic protein 4; FLT3L, FMS-related tyrosine kinase 3 ligand; SCF, stem cell factor; VEGF, vascular endothelial growth factor.

process, a major drawback is the requirement for lethal irradiation of the cells before infusion to prevent further cell proliferation and, hence, tumour transfer to patients. This drastically limits cell persistence after infusion.

For peripheral blood NK cells, which have been used in the majority of clinical trials to date, the starting material is typically a leukapheresis product; mononucleated cells can be further enriched by centrifugation on a density gradient[135]. Depletion of T cells with anti-CD3 immunomagnetic particles increases the NK cell fraction but leaves a mixture of other cells, including B lymphocytes and monocytes; B cells can be removed with anti-CD19 particles[136,137]. By combining T cell depletion with CD56 cell enrichment, highly purified NK cell populations can be isolated[136].

NK cells in peripheral blood of healthy individuals are typically in a resting state. In early studies of infusion of lymphokine-activated killer cells (LAK cells), a predecessor of NK cell infusion, these cells — which contained NK cells and T cells in various proportions[138] — with

Leukapheresis
A procedure that removes white blood cells from blood,while returning the remainder blood components to circulation. A leukapheresis product is often used as a starting material to obtain peripheral blood natural killer cells.

Lymphokine-activated killer cells
(LAK cells). Lymphocytes obtained from cancer patients, incubated with cytokines, such as IL-2, and then reinfused into patients with therapeutic intent, often in conjunction with IL-2.

enhanced cytotoxicity against tumour cells were generated by exposing PBMCs to IL-2 ex vivo[139]. NK cells are a major contributor to the antitumour activity of LAK cells[140], and exposure to IL-2 is a common method of preactivation, resulting in NK cells with considerably higher cytotoxicity than their resting equivalents[49,141]. A short (12–16-h) exposure to IL-2 at 1,000 international units (IU) per millilitre is sufficient for NK cell activation[8].

Whether preactivation with IL-15 produces more powerful NK cells than preactivation with IL-2 is unclear. Reportedly, IL-15 might maintain cytotoxicity after cytokine withdrawal for a longer period than IL-2 (REFS[142,143]). Cultures of purified NK cells with IL-15 added every 3 days for 9 days resulted in increased susceptibility to NK cell death and reduced expansion in immunodeficient mice[144]. These findings, together with other features of the resulting NK cells, suggested the occurrence of cell exhaustion, which did not occur with a single dose of IL-15 for 1 week or with a 3-day omission in the middle of the 9-day culture[144]. As mentioned earlier, combining IL-15 with IL-12 and IL-18 induces considerable biological changes and a status reminiscent of memory NK cells, including enhanced function upon restimulation, hyper-responsiveness to IL-2, greater IFNγ production and greater cytotoxicity[66,145,146]. Exposure to IL-12 (10 ng ml^{-1}), IL-15 (50 ng ml^{-1}) and IL-18 (50 ng ml^{-1}) for 12–16 h has been used to activate NK cells for infusion in patients[146]. Activation of NK cells has also been observed after exposure to an inhibitor of glycogen synthase kinase 3 (GSK3) (REF.[147]).

An alternative to isolating mature NK cells is to derive them from haematopoietic progenitor cells (FIG. 2). Spanholtz et al. enriched CD34$^+$ haematopoietic cells from umbilical cord blood, expanded them for 14 days and then differentiated them into NK cells by exposure to stem cell factor (SCF), IL-7, IL-15, IL-2 and other growth factors[148]. The resulting CD3$^-$CD56$^+$ cells expressed activating NK cell receptors and exerted cytotoxicity against K562 cells[148]. Kaufman et al. optimized methods to derive NK cells from human embryonic stem cells and iPSCs[149,150] (FIG. 2). CAR-expressing iPSCs could be used to generate CAR NK cells starting from iPSCs obtained from CD34$^+$ umbilical cord blood cells cultured with SCF, vascular endothelial growth factor (VEGF), and bone morphogenetic protein 4 (BMP4) for 11 days to induce haematopoietic differentiation, and then switching to cultures containing IL-3, IL-15, IL-7, SCF and FLT3L for 28–32 days to stimulate NK cell differentiation. NK cells were then expanded by exposure to K562 cells expressing membrane-bound IL-21 (mbIL-21)[150,151]. Of note, it took about 7 weeks of additional culture to obtain 10^9 NK cells from 10^6 human embryonic stem cell-derived NK cells[149].

Ex vivo propagation

Because NK cells represent a minority of blood lymphocytes (approximately 10%), it might be difficult to extract them in sufficient quantities to generate a high effector-to-target ratio in vivo or to perform multiple infusions. IL-2 can induce some NK cell proliferation, but this is neither consistent nor long-lasting[48,152]. In our own experiments using concentrations of IL-2 ranging from 10 to 6,000 IU ml^{-1}, we observed a maximum expansion of fourfold ($n = 29$) after 7 days of culture[49]. There was no marked improvement in NK cell proliferation on addition of IL-15, IL-12 or IL-21, alone or in combination with IL-2 (REF.[49]). Reportedly, cultures containing IL-2 (with or without IL-15) and OKT3 (an anti-CD3 antibody that can suppress T cell growth) can stimulate expansion of NK cells[131,153].

Proliferative responses in NK cells can be rapid and sustained when stimulatory cells — such as a Wilms tumour-derived cell line[154], autologous PBMCs[155,156] and Epstein–Barr virus-transformed lymphoblastoid cells[157,158] — are present in the cultures. More than three decades ago, Phillips and Lanier demonstrated that co-culture with the chronic myelogenous leukaemia-derived cell line K562 induced NK cell activation and some proliferation[159]. K562 cells transduced with mbIL-15 induce greater proliferation of NK cells than do untransduced cells[39]. Transduction of 4-1BBL, which engages the NK cell co-stimulatory molecule 4-1BB (also known as CD137), also increases proliferation[39]. The most vigorous NK cell expansion was obtained with K562 cells co-expressing both genes (K562-mbIL-15–4-1BBL cells)[22,39,49] (FIG. 2). The method has been adapted to large-scale good manufacturing practice (GMP) conditions. Expansions start with PBMCs, and T cells are depleted at the end of the cultures; alternatively, T cell depletion or CD3$^-$CD56$^+$ cell selection can be performed before culture. The protocol uses irradiated (currently 120 Gy) K562-mbIL-15–4-1BBL cells, and stem cell growth tissue culture medium (CellGenix, Freiburg, Germany) with low-dose IL-2 (10–40 IU ml^{-1} depending on the source), and cultures are carried out in G-Rex 100 chambers (Wilson Wolf, St Paul, MN, USA)[49,135,160]. GMP cultures last 10 days, producing a median NK cell expansion of 376-fold (range 90-fold to 603-fold) from leukapheresis products from healthy donors ($n = 17$) or patients with breast cancer or gastric cancer ($n = 60$), with no significant T cell expansion[39,49,135]. Therefore, this protocol allows enough NK cells to be obtained for at least four infusions at 5×10^7 per kg from one leukapheresis product[135]. Even larger numbers of NK cells can be generated by prolonging the cultures and/or adding fresh K562-mbIL-15–4-1BBL cells. However, after 8–15 weeks of continuous proliferation, senescence may ensue in NK cells derived from adult peripheral blood[22].

Other investigators have used K562 cells transduced with 4-1BBL and exogenous IL-15 (K562 cells express IL-15 receptor α-chain)[161], K562 transduced with CD64, CD86, CD19 and 4-1BBL as well as mbIL-15, mbIL-21 or both[136,151,162], or K562 transduced with OX40 ligand in cultures containing IL-2, IL-15 and IL-21 (REF.[163]). In addition to lethal irradiation, co-culture with NK cells ensures that no viable K562 cells are present in the final product[135]. Nevertheless, thorough testing must be done to ensure that no proliferating K562 cells are present after irradiation and that no viable K562 cells are detectable after NK cell expansion (K562-mbIL-15–4-1BBL cells are transduced with green fluorescent protein and are clearly detectable by flow cytometry)[135].

Non-intact cells, such as leukaemia cell lysates, mainly activate NK cells rather than induce proliferation[164]. A method to extract particles from genetically modified K562 cells has been reported[165,166]. Briefly, cells are mixed with a lysis buffer and disrupted by nitrogen cavitation, and plasma vesicles are purified by sucrose gradient centrifugation. The particles have stimulatory capacity but it is unclear whether they can promote NK cell expansions comparable to those obtained with intact cells under optimal conditions. Regardless, the method used for NK cell activation and expansion not only affects cell yield but may also influence cell function. For example, we found that the gene expression profile of NK cells expanded with K562-mbIL-15–4-1BBL cells was markedly different from the pattern in the same NK cells cultured with IL-2 alone; the former had greater expression of CD25 (also known as IL-2 receptor α-chain) and the activating receptors NKG2D and NKp46 (REF.[49]). Miller et al. reported that NK cells injected into immunodeficient mice had a different homing pattern if they were stimulated with IL-2 or expanded with genetically modified K562 cells before infusion[167].

Genetic engineering

Genetic engineering of NK cells has been performed by viral transduction or electroporation of mRNA[135,168]. When retroviral transduction is used, culture methods that induce NK cell proliferation are essential to allow DNA integration of the viral payload[39]. Although lentiviruses can transduce non-proliferating cells, gene expression is generally higher if cells are dividing. An alternative to viral transduction is electroporation of mRNA[168,169]. Potential advantages of this approach include a less time-consuming and costly preparation of mRNA compared with viral vectors and lack of concerns regarding oncogenic mutagenesis associated with viral vectors. With mRNA electroporation, however, gene expression is transient; it generally declines 2–3 days after electroporation, becoming undetectable within 5–7 days[168].

Enhancing activation and proliferation. Overexpression of activating receptors in NK cells can augment their antitumour activity by increasing sensitivity to activating ligands expressed by tumour cells (FIG. 3). NKG2D, which is physiologically expressed in association with DAP10, is a central activating NK cell receptor[40,41,170,171]. A chimeric receptor composed of NKG2D linked to CD3ζ can directly provide activation signals on ligation[172], and adding the adaptor molecule DAP10 to the vector construct promotes and stabilizes expression of NKG2D and can transduce activating signals[4]. Expression of this construct in peripheral blood NK cells further increased the already high levels of NKG2D after expansion with K562-mbIL-15–4-1BBL cells[172]. NK cells endowed with the additional NKG2D–CD3ζ–DAP10 stimulus had a substantially greater cytotoxic effect against a wide range of tumour cell lines in vitro and in xenograft models, whereas toxicity towards non-transformed cells, such as lymphocytes or mesenchymal cells, remained low[172,173]. NKG2D-modified NK cells retained cytotoxic capacity after 24 h of culture with target cells, when control NK cells had become anergic[172]. Higher expression of NKG2D should make saturation by shed NKG2D ligands less likely and, hence, less susceptible to their potentially dampening effects. A recent report indicates that NK cells expressing NKG2D–CD3ζ receptors have the unexpected property of targeting myeloid-derived suppressor cells in the tumour microenvironment, decreasing their inhibition of cytotoxic T cells and increasing the antitumour capacity of CAR T cells[174].

Another way to tilt the signalling balance in favour of activation is to decrease expression of inhibitor receptors (FIG. 3). The CD94–NKG2A complex — which transduces inhibitory signals when ligated by peptide-loaded HLA-E in tumour cells — can be targeted by using protein expression blockers. These are constructs that contain a single-chain variable fragment derived from an anti-NKG2A antibody linked to endoplasmic reticulum-retention domains, which hold newly synthesized NKG2A in the endoplasmic reticulum and prevent its transport to the cell membrane[113]. The resulting NK cells lack NKG2A surface expression and outperform control NK cells, particularly when target cells overexpressed HLA-E molecules after exposure to IFNγ or to supernatant collected from co-cultures of NK cells and tumour cells, or after transduction with HLA-E. Anti-NKG2A protein expression blockers also blocked de novo NKG2A expression generated by exposure to IL-12 (REF.[113]).

To promote expansion after infusion, NK cells can be transduced with cytokine-encoding genes (FIG. 3). Expression of mbIL-15 sustains longer survival and greater proliferation than wild-type secreted IL-15 (REF.[59]). When injected into immunodeficient mice, NK cells expressing IL-15 expanded well and infiltrated multiple tissues without exogenous IL-2; expansion could be further increased by IL-2 administration[59].

Chimeric antigen receptors. NK cells can be redirected with CARs against surface molecules expressed by tumour cells (FIG. 3). Expression of an anti-CD19 CAR by retroviral transduction in NK cells expanded by co-culture with the K562-mbIL-15–4-1BBL cell line the enhanced cytotoxic effect against CD19-positive ALL specifically and dramatically[39]. Several observations were made in that study. First, a CD8α transmembrane domain allowed highly efficient CAR expression in NK cells. Second, CARs containing either CD3ζ or DAP10 as stimulatory molecules could both activate NK cells, but CD3ζ was superior. Third, addition of the co-stimulatory molecule 4-1BB to the CAR markedly increased cytotoxicity. Fourth, CAR NK cells could efficiently kill autologous primary ALL cells, indicating that CAR expression can overcome HLA-mediated inhibitory signals[39]. During this study, it became apparent that the RD114 viral envelope protein was important for high transduction efficiency, enabling high levels of CAR expression in more than 50% (often more than 80%) of NK cells[39], which are levels similar to those achieved for T cells[175].

CAR constructs containing CD3ζ with other co-stimulatory molecules, such as CD28 or 2B4 (also known as CD244), instead of 4-1BB can also increase NK cell

cytotoxicity. CD28 was included in CARs directed against HER2 (REF.[176]), EGFR or EGFR variant III (REF.[177]), and CS1 (REF.[178]), whereas 2B4 was included in an anti-GD2 CAR[179], and both 4-1BB and CD28 were part of a CAR against CD123 (REF.[156]). A CAR against EGFR variant III containing DAP12 instead of CD3ζ and no co-stimulatory molecules has also been reported to stimulate NK cells[180]. Rezvani and colleagues expressed an anti-CD19–4-1BB–CD3ζ CAR in umbilical cord blood NK cells after stimulation with a modified K562 cell line expressing mbIL-21 and 4-1BBL; the CAR expression vector contained IL-15 as well as dimerizable caspase 9 (to allow elimination of NK cell in vivo if needed)[132]. Kaufman and colleagues expressed an anti-mesothelin CAR with the NKG2D transmembrane domain and 2B4–CD3ζ signalling in iPSC-derived NK cells[150]. Several CARs, as well as TCR variants against tumour peptides, have been expressed in NK-92 cells[181,182].

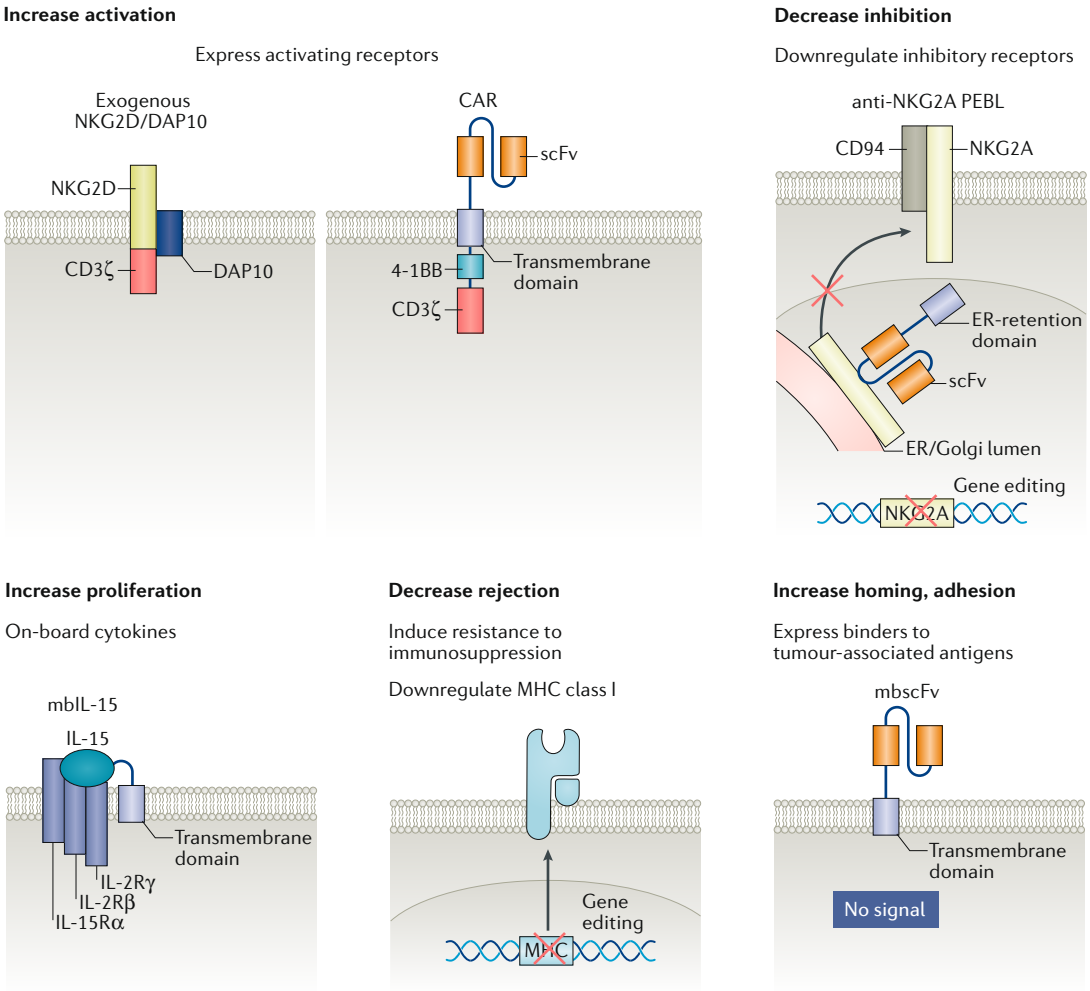


Fig. 3 | **Genetic modification approaches to increase the antitumour capacity of NK cells.** The balance of activating and inhibitory signals in natural killer (NK) cells can be tilted towards activation by transducing NK cells with activating receptors. An example is overexpression of a chimeric activating receptor consisting of NKG2D linked to CD3ζ and expressed together with DAP10, which stabilizes its surface expression; both CD3ζ and DAP10 deliver activating signals on engagement of NKG2D[172,173]. Activating signals can also be delivered by chimeric antigen receptors (CARs) containing CD3ζ and a co-stimulatory molecule (such as 4-1BB) and recognizing antigens expressed by tumour cells[39,132,168]. Conversely, activation can be promoted by decreasing expression of inhibitory receptors. This can be achieved by deleting the gene encoding the receptor of interest by gene editing methods or by using protein expression blockers (PEBLs), such as constructs that contain a single-chain variable fragment (scFv) of an antibody that targets an inhibitory receptor and that is linked to endoplasmic reticulum (ER)-retention domains. PEBLs hold the newly synthesized receptor in the ER and prevent its transport to the cell membrane[113]. Expansion of NK cells after infusion can be promoted by expressing soluble or membrane-bound cytokines[59,132]. Possible methods to avoid rejection and prolong persistence of allogeneic NK cells include induction of resistance to immunosuppressive agents, which can then be used to suppress the recipient's T lymphocytes without affecting the infused cells, and downregulation of human leukocyte antigen molecules to render the infused cells undetectable to the recipient's T lymphocytes. Finally, expression of chemokine receptors and membrane-bound scFv (mbscFv) directed against tumour-associated antigens can promote homing of NK cells to tumour sites, increase their adhesion to tumour cells and enhance cytotoxicity. IL-2Rβ, IL-2 receptor β-chain; IL-2Rγ, IL-2 receptor γ-chain; IL-15Rα, IL-15 receptor α-chain; mbIL-15, membrane-bound IL-15; MHC, major histocompatibility complex.

Table 1 | **Clinical results of non-genetically modified NK cell infusions**

Tumour type	Source	NK cell preparation	NK cell dose per kg	Lymphodepletion	IL-2 after infusion	*N*	Response	Ref.
AML, CML, MDS	Haploidentical (HSTC donor)	$CD3^-CD56^+$ selection	$(2.1–14.1)\times10^6$	None	No	5	CR in 4 patients (follow-up 8–18 months)	244
AML	Haploidentical	CD3-depleted PBMCs, IL-2 stimulation	$(8.5\pm0.5)\times10^6$	Flu/Cy	Yes	19	CR in 5 patients (including 3 of 4 patients with KIR ligand mismatch)	8
AML	Haploidentical	$CD3^-CD56^+$ selection	$(5–81)\times10^6$	Flu/Cy	Yes	10 (paediatric)	All 10 patients were in CR before infusion and remained in CR 19–39 months after infusion	9
AML	Haploidentical	$CD3^-CD56^+$ selection	$(1.1–5.0)\times10^6$	Flu/Cy	Yes	13 (5 with active disease and 2 with molecular relapse)	CR in 3 patients (1 patient with active disease and 2 patients with molecular relapse)	199
AML	Haploidentical	CD3-depleted PBMCs, IL-2 stimulation ($n=32$)	$(9.6\pm3.0)\times10^6$	Flu/Cy	Yes	42	CR in 9 patients (2 disease-free at 6 months)	200
		CD3-depleted PBMCs, CD56 selection, IL-2 stimulation ($n=10$)	$(3.4\pm0.5)\times10^6$					
AML	Haploidentical	CD3-depleted PBMCs (with or without CD56 selection), or CD3- and CD19-depleted PBMCs, IL-2 stimulation	$(26\pm15)\times10^6$	Flu/Cy, and IL-2–diphtheria toxin	Yes	15	CR in 8 patients (5 disease-free at 6 months)	200
AML	Haploidentical	CD3- and CD19-depleted PBMCs, IL-15 stimulation ($10\,ng\,ml^{-1}$)	Mean of 37×10^6	Flu/Cy	No, but IL-15 (0.3–1 µg per kg) SC or IV for 12 days after infusion	40	CR in 7 patients; CR with incomplete haematological recovery in 7 patients	10
AML	Haploidentical	$CD3^-CD56^+$ selection, IL-12, IL-15 and IL-18 stimulation	$(0.5–10.0)\times10^6$	Flu/Cy	Yes	9	CR in 4 patients; MLFS in 1 patient	146
AML, MDS, CML	Haploidentical	$CD3^-CD56^+$ selection	$(4.3\text{-}22.4)\times10^6$	Flu/Cy	Yes	8	CR in 2 patients; resolution of dysplasia in 1 patient with MDS	245
AML	Umbilical cord blood	Differentiation and expansion from $CD34^+$ cells	$(3–30)\times10^6$	Flu/Cy	No	10	CR in 10 patients before infusion; 4 patients were disease-free 16–60 months after infusion	193
AML, CML	HSCT donor	CD3 depletion, co-culture with K562-mbIL-21	$(0.01–100)\times10^6$, $\times3$ (days −2, +7 and +26 of HSCT)	HSCT conditioning	No	13	CR in 7 of 8 patients with AML and in all 5 patients with CML in the chronic phase before infusion	162
AML, MDS	Haploidentical	CD3- and CD19-depleted PBMCs, IL-2 stimulation	$(1.3–17.6)\times10^6$	Flu/Cy, TLI (2 Gy)	No	16	CR in 5 patients; PR in 1 patient; MLFS in 1 patient	195

Table 1 (cont.) | **Clinical results of non-genetically modified NK cell infusions**

Tumour type	Source	NK cell preparation	NK cell dose per kg	Lymphodepletion	IL-2 after infusion	*N*	Response	Ref.
AML	Haploidentical	$CD3^-CD56^+$ selection	$(3.6–62.6)\times10^6$	Flu/Cy	Yes	21 (paediatric)	All 21 patients were in CR before infusion. No significant increase of EFS compared with patients treated with chemotherapy alone	201
MM	Autologous or haploidentical	Co-culture with K562-mbIL-15–4-1BBL, CD3 depletion	$(22–100)\times10^6$	Bortezomib alone or with Flu/Cy and dexamethasone	Yes	7	Two patients were treatment-free for 6 months	202
B cell NHL	Haploidentical	CD3- and CD19-depleted PBMCs, IL-2 stimulation, pretreatment with rituximab	$(5.0–32.7)\times10^6$	Flu/Cy, methylprednisolone	Yes	14	CR in 2 patients; PR in 2 patients	206
Neuroblastoma	Haploidentical	$CD3^-CD56^+$ selection, pretreatment with anti-GD2	$(4.7–59.5)\times10^6$, ×3	NK cells given between courses of chemotherapy	No	11	CR in 3 patients; PR in 4 patients; SD in 4 patients	204
Neuroblastoma	Haploidentical	$CD3^-CD56^+$ selection, IL-2 stimulation, anti-GD2 after NK cell infusion	$(1–50)\times10^6$	Cy, vincristine, and topotecan	No	35	CR in 5 patients; PR in 5 patients	205
Renal cell carcinoma	Haploidentical	CD3-depleted PBMCs, IL-2 stimulation	$(2.2–15)\times10^6$	Flu	Yes	7	No response seen. Donor NK cells undetectable	8
Melanoma, renal cell carcinoma	Haploidentical	CD3-depleted PBMCs, IL-2 stimulation	$(2.2–15)\times10^6$	Cy and methylprednisolone	Yes	16	SD in 6 patients	8
Ovarian cancer, breast cancer	Haploidentical	CD3-depleted PBMCs	$(8.3–39)\times10^6$	Flu/Cy, TBI (2 Gy)	Yes	20	PR in 4 patients; SD in 12 patients	203

AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; CR, complete remission; Cy, cyclophosphamide; EFS, event-free survival; Flu, fludarabine; HSCT, haematopoietic stem cell transplantation; IV, intravenously; KIR, killer cell immunoglobulin-like receptor; mbIL-15, membrane-bound IL-15; mbIL-21, membrane-bound IL-21; MDS, myelodysplastic syndrome; MLFS, morphological leukaemia-free state; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cell; PR, partial response; SC, subcutaneously; SD, stable disease; TBI, total body irradiation; TLI, total lymphoid irradiation.

Clinical applications

Autologous versus allogeneic cells

As mentioned earlier, a predecessor of NK cell infusion was the infusion of LAK cells — autologous PBMCs cultured for 3–4 days with IL-2 (REF.[138]). LAK cells were typically administered with high-dose IL-2 (30,000–100,000 U per kg as a bolus intravenously). Rosenberg et al. reported that 8 of the 106 patients with metastatic cancer who received LAK cells had a complete response, including 4 of 36 patients with renal cell carcinoma, 2 of 26 patients with melanoma, 1 of 26 patients with colorectal cancer, and 1 of 2 patients with NHL[183]. Serious side effects, such as vascular leak syndrome and liver toxicity, were attributed to IL-2 (REF.[183]). In general, studies with unmodified autologous NK cells have yielded disappointing results. NK cells obtained from T cell-depleted peripheral blood cultured with IL-2, OKT3 and irradiated autologous peripheral blood cells for 10 days were infused into eight patients with metastatic melanoma or renal cell carcinoma following lymphodepletion, but no clinical responses were observed[155]. Likewise, infusion of a T cell-depleted leukapheresis product enriched in NK cells incubated overnight with IL-2 at 1,000 IU ml^{-1} into patients with relapsed lymphoma or metastatic breast cancer was ineffective[184].

In the absence of strong activating stimuli triggered by activating receptors or CD16 engagement with antibodies, self-HLA signals in tumour cells are likely to inhibit the antitumour activity of autologous NK cells. Allogeneic NK cells, however, may escape such inhibition. In patients undergoing haematopoietic stem cell transplantation (HSCT), allogeneic NK cells are present in the initial graft, and are also generated in vivo from the engrafted donor haematopoietic stem cells. An early study demonstrated the effectiveness of alloreactive NK cells against AML in mice[7]. This finding was corroborated by data in patients with AML undergoing HSCT that showed an association between a KIR ligand profile in donor NK cells that allowed recipient target cell killing with lower occurrence of relapse after transplantation[7]. Subsequent studies confirmed the association between donor NK cell genotypes and/or phenotypes

Vascular leak syndrome
A serious clinical condition characterized by increased vascular permeability accompanied by the escape of plasma through capillary walls. It is most commonly seen in sepsis, and it is one of the major dose-limiting toxic effects of IL-2.

with outcome after HSCT[185–190]. For example, studies segregating KIR haplotypes into a predominantly inhibitory group A and an activating group B found that overall survival in AML and NHL was significantly greater if the donor had at least one B haplotype[188,191]. Another study, including 674 patients, found an association between CMV reactivation after HSCT and higher numbers of NK cells expressing NKG2C, CD57 and low levels of CD56, a phenotype associated with NK cells responsive to CMV; patients with these features had a better outcome[189].

Allogeneic NK cells do not directly cause GVHD[7–10] if the infused product is adequately T-cell depleted. The typical safety limit for residual $CD3^+$ T cells is 5×10^4 per kg (patient weight), a threshold derived from the HSCT experience[192]. Nevertheless, one study reported unusually high rates of acute GVHD in patients who had undergone allogeneic HSCT and into whom expanded donor NK cells had been infused[161]. In that study, HSCT consisted of purified $CD34^+$ cells with 1×10^4–2×10^4 T cells per kg added back to the graft. NK cells were infused on days 7 and 35 after transplantation. Importantly, acute GVHD occurred in four of the five patients who had undergone matched-unrelated donor HSCT compared with one of the four patients with a matched-sibling donor, and donor T cell engraftment was significantly higher in patients who experienced GVHD, suggesting that GVHD was T cell mediated but might have been exacerbated by NK cells[161].

Lymphodepletion and cytokine administration

When allogeneic NK cells are infused outside the HSCT setting, rejection by the recipient's immune system limits their persistence[9,135,146,193]. Rejection can be delayed by administering lymphodepleting chemotherapy to the recipient before NK cell infusion. The lymphodepleting regimen most commonly used includes fludarabine and cyclophosphamide, two drugs that preferentially eliminate lymphoid cells[8]. Miller et al. tested two dosages for these drugs: low-dose fludarabine at 25 mg m^{-2} per day for 5 days, or that same fludarabine regimen plus cyclophosphamide at 60 mg per kg per day for 2 days (Flu/Cy)[8]. Donor-derived NK cells were detectable in blood on day 14 only in patients receiving the latter regimen, which caused deeper immunosuppression and was associated with higher levels of serum IL-15, which explained the superior engraftment[8]. It was recently reported that the number of NK cells homing to the bone marrow is proportional to the intensity of lymphodepletion[194].

Other studies observed persistence of allogenic NK cells for 14–21 days after infusion, although small numbers may be detectable in the circulation for longer[9,135,146,193,195]. In one study, the absolute levels of $CD3^+$ T cells before lymphodepleting chemotherapy were inversely related to NK cell expansion in vivo, and markers of exhaustion in recipient T cells after infusion were related to longer NK cell persistence[196]. Nevertheless, reliable predictors of persistence are lacking, and the impact of HLA matching of the donor and the recipient is unclear.

Although rejection is not an issue with autologous NK cells, lymphodepletion might 'deplete a corrupt host microenvironment'[197], including immunosuppressive myeloid-derived suppressor cells and regulatory T cells (T_{reg} cells), as well as cells that compete with the infused cells for activating cytokines, thus generating a cytokine milieu that favours their expansion[197].

To sustain survival and expansion of infused NK cells, IL-2 is often administered subcutaneously in doses ranging from 1×10^6 IU m^{-2} to 1×10^7 IU total, typically in six doses over 2 weeks[9,135,155,198,199]. In general, although low-dose IL-2 is well tolerated, there is a concern that it might stimulate immunosuppressive T_{reg} cells[198]. IL-15 and IL-15 variants (BOX 1) can also be used to support NK cells in vivo. Cooley et al. administered human recombinant IL-15 (0.3–1 μg per kg) subcutaneously or intravenously for 12 days after NK cell infusion to 40 patients with relapsed/refractory AML[10]. The rate of NK cell expansion was higher than previously observed with IL-2. Subcutaneous but not intravenous IL-15 administration was associated with cytokine release syndrome and neurotoxicity, which Cooley et al. speculated was due to direct stimulation of monocytes and T cells by IL-15, rather than an effect of NK cell activation[10].

NK cell infusions in the clinic

TABLE 1 summarizes the results of clinical trials in which NK cells were infused into patients with haematological malignancies or solid tumours. The pioneering study of Miller et al. indicated that haploidentical PBMCs enriched in NK cells by T cell depletion and cultured with IL-2 at 1,000 IU ml^{-1} for 12–16 h could induce complete remission in 5 of 19 patients with poor-prognosis AML. The response was associated with in vivo NK expansion, and no GVHD was observed[8]. In a subsequent study, this group reported responses in 17 of 57 patients[200]. NK cell expansion was detected more frequently and IL-15 serum levels were higher in the 15 patients who received Flu/Cy supplemented with IL-2–diphtheria toxin fusion protein (to deplete T_{reg} cells) than in the 42 patients who received Flu/Cy alone. The complete remission rates were 53% versus 21% and disease-free survival at 6 months was 33% versus 5%.[200] The interpretation of these data is somewhat confounded by the fact that the number of NK cells administered to patients who received the T_{reg} cell-depleting agent was higher than in the other group.

The early results of Miller et al. encouraged the use of allogeneic NK cells in patients with AML or myelodysplastic syndrome, infused either without prior activation or after activation with IL-2, or with IL-12, IL-15 and IL-18 (TABLE 1). Some studies observed responses in one third to one half of the patients. In other studies, however, the effect of NK cells was unclear[201]. Besides possible differences in cell product potency, patient characteristics and disease status are important determinants of response. In one study, for example, two patients with AML treated as soon as disease relapse was detected by molecular methods had remissions that lasted 4 and 9 months, respectively, whereas only one of the five patients treated at the time of overt disease had a response[199].

Results of trials enrolling patients with neoplasms other than AML have also been reported, including multiple myeloma[202] and ovarian and breast cancer[203], but clear antitumour activity was not observed (TABLE 1). By contrast, there were promising results in children with neuroblastoma receiving allogeneic NK cells following

Table 2 | **Selected current trials of NK cell infusion**

Ex vivo preparation	Source	Eligibility and other treatment	Phase	Patient age (years)	Trial identifiers
Isolated	Haploidentical	Haematological malignancies; after HSCT	II	0–21	NCT01807611
	Haploidentical	Neuroblastoma; after treatment with anti-GD2 mAb	II	0–18	NCT01857934
	Haploidentical	Neuroblastoma; after treatment with anti-GD2 mAb	I	Any	NCT02650648
	Haploidentical	Neuroblastoma, Ewing sarcoma, rhabdomyosarcoma, sarcoma, CNS tumours; after HSCT	II	Any	NCT02100891
	Autologous	Solid and haematological malignancies; after treatment with bortezomib	I	18–70	NCT00720785
Cytokine (IL-12, IL-15, IL-18) preactivated	Allogeneic	AML; after HSCT	I	1–30	NCT03068819
			II	≥18	NCT02782546
			I/II	≥2	NCT01898793
	Allogeneic	AML; in combination with ALT-803	I/II	≥2	NCT01898793
Pharmacologically preactivated by inhibition of glycogen synthase kinase 3	Allogeneic	AML	I	18–70	NCT03081780
	Allogeneic	Solid malignancies; in combination with trastuzumab (for $HER2^+$ cancers) or cetuximab (for $EGFR^+$ cancers)	I	18–75	NCT03319459
	Allogeneic	Ovarian, fallopian tube, primary peritoneal cancer; intraperitoneal infusion	I	18–75	NCT03213964
Expanded with stimulatory cells (K562 expressing 4-1BBL and mbIL-21)	Umbilical cord blood	NHL; after HSCT and treatment with anti-CD20 (rituximab)	II	15–70	NCT03019640
	Umbilical cord blood	MM; after treatment with anti-CD319 (elotuzumab), lenalidomide and melphalan and before autologous HSCT	II	18–75	NCT01729091
	Haploidentical	AML	I/II	≥18	NCT01787474, NCT02809092
	Allogeneic or umbilical cord blood	AML, MDS, CML; after high-dose chemotherapy and before HSCT	I/II	7–65	NCT01823198
	Allogeneic	Paediatric solid tumours	I	1–40	NCT03420963
	Autologous	Malignant posterior fossa tumours of CNS; intraventricular infusion	I	0–21	NCT02271711
Expanded with stimulatory cells as above; anti-CD19 CAR	Umbilical cord blood	NHL; before autologous HSCT	I/II	18–70	NCT03579927
	Umbilical cord blood	Relapsed refractory $CD19^+$ B lymphoid malignancies	I/II	7–80	NCT03056339
Expanded with stimulatory cells (K562 expressing mbIL-21)	Allogeneic (HLA unmatched)	Haematological/solid cancer; in combination with ALT-803	I	≥18	NCT02890758
Expanded with stimulatory cells (K562 expressing 4-1BBL and mbIL-15)	Autologous	MM; after treatment with anti-CD319 (elotuzumab) and in combination with ALT-803	II	18–75	NCT03003728
	Haploidentical	Ewing sarcoma, osteosarcoma, rhabdomyosarcoma	I/II	0–80	NCT02409576
	Haploidentical	AML, MDS, T cell ALL	I	6–80	NCT02123836
	Haploidentical	Neuroblastoma; after treatment with anti-GD2 mAb	I/II	0.5–25	NCT03242603
	Haploidentical	Neuroblastoma; after treatment with anti-GD2–IL-2 fusion protein	I	7 months to 21 years	NCT03209869
	Autologous	$EGFR^+$ NPC or HNSCC; after treatment with cetuximab	I/II	≥21	NCT02507154
	Autologous	$HER2^+$ breast or gastric cancer; after treatment with trastuzumab	I/II	21–99	NCT02030561
NK cells derived from $CD34^+$ cells	Placenta	MM; after autologous HSCT	I	18–70	NCT02955550
	Umbilical cord blood	Ovarian, fallopian tube, primary peritoneal cancer; intraperitoneal infusion	I	18–75	NCT03539406

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CAR, chimeric antigen receptor; CML, chronic myeloid leukaemia; CNS, central nervous system; HLA, human leukocyte antigen; HNSCC, head and neck squamous cell carcinoma; HSCT, haematopoietic stem cell transplantation; mAb, monoclonal antibody; mbIL-15, membrane-bound IL15; mbIL-21, membrane-bound IL-21; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NK cell, natural killer cell; NPC, nasopharyngeal carcinoma.

Minimal residual disease
Leukaemic cells undetectable by conventional morphological techniques but detectable by flow cytometry or molecular methods in peripheral blood or bone marrow. Contemporary minimal residual disease assays can detect one leukaemic cell among 10,000 or more normal cells.

administration of anti-GD2 (an antibody that recognizes a surface molecule highly expressed in neuroblastoma cells). NK cells, administered to enhance ADCC directed by the antibody, have produced partial or complete responses in about 40% of patients[204,205]. No relation between response and KIR/HLA genotype or *FCGR3A* polymorphisms was noted[205]. Haploidentical NK cells were infused to enhance rituximab-mediated ADCC in patients with refractory NHL, and four objective responses were achieved among the 14 evaluable patients, with two complete responses that lasted 3 and 9 months[206].

NK cell therapies in the future

The results of clinical trials, particularly those with activated allogeneic NK cells in patients with myeloid malignancies and neuroblastoma, are encouraging. Ongoing studies testing NK cell infusions are listed in TABLE 2. This collective experience provides a platform of knowledge onto which a next generation of NK cell therapies can be built.

Clinical trial design will be an important determinant of success. Eligibility criteria and the number of infused cells must yield an effector-to-target ratio that is likely to control or overcome tumours. In AML, for example, there may be 10^{12}–10^{13} leukaemic cells at diagnosis; after initial chemotherapy, there may still be 10^{9}–10^{10} leukaemic cells even if morphological remission is achieved[207]. In the weeks following NK cell infusion, leukaemic cells will continue to proliferate in the absence of chemotherapy. Assuming that NK cells can expand in vivo at the same rate as leukaemic cells, an infusion of, for example, 10^{7} NK cells in a patient with overt leukaemia relapse would yield an effector-to-target ratio of 1:100,000 or lower, which would clearly be inadequate to produce measurable antitumour activity. Conversely, infusion of 10^{10} NK cells into a patient who is in remission but is minimal residual disease positive could lead to an effector-to-target ratio of 1:1 or higher, which should result in a considerable reduction of leukaemia burden. Of note, severe cytokine release syndrome and neurotoxicity, which are known side effects of CAR-T cell therapies[208], were not observed in clinical trials with infusion of NK cell numbers that matched or exceeded those typically used for CAR-T cell infusion, except when IL-15 was also infused subcutaneously[10]. We propose that mbIL-15 expression in NK cells[59] can provide high-dose IL-15 stimulation in vivo without the systemic effects linked to IL-15 administration.

Most allogeneic NK cells are rejected 2–3 weeks after infusion. To increase persistence, we envisage two pathways. One is further intensification of lymphodepletion before infusion by adding, for example, total body irradiation or anti-T cell agents. However, this approach might increase toxicity and limit applicability. The second approach relies on genetic modifications that have been applied to CAR T cells, such as HLA knockdown[209], or induction of resistance to immunosuppression by, for example, deleting deoxycytidine kinase, leading to resistance to nucleoside analogues[210]. Although most studies of NK cell infusion have used haploidentical donors[8,9,201,204,205], unrelated donors, umbilical cord blood and iPSCs would considerably widen the pool of sources. Robust methods for NK cell expansion can generate cell numbers from one leukapheresis that are sufficient to treat multiple patients, such as 20 or more. In this context, it should also be possible to select NK cells with specific HLA types to improve engraftment, as well as cells with phenotypes predictive of higher antitumour activity.

NK cells are a diverse cell population[211–213], but ex vivo expansion and genetic modification enhance tumour cell killing in most NK cells recoverable from blood. Engineering NK cells with superior activation[172,173] and/or decreased inhibitory signals[113] augments their antitumour activity (FIG. 3). The CARs that have been effectively used to redirect T cells work well for NK cells too[39,132], and allogeneic CAR NK cells could be used without the additional requirement to remove TCR needed to prevent the GVHD triggered by allogeneic CAR T cells[7–10]. Nevertheless, the cellular effect of current second-generation CARs in T and NK cells differ. In T cells, CARs induce cytotoxicity and proliferation, whereas in NK cells they primarily induce cytotoxicity[39,214]. Lack of CAR-driven proliferation in NK cells could be offset by combining CARs with on-board cytokines[59,132]. An alternative strategy to CARs is genetic modification with non-signalling extracellular binders that guide homing and promote adhesion to targets, allowing the balance between activating and inhibitory receptors to decide whether cytotoxicity is triggered (Y. Zhu, A. Jain and D.C., unpublished observations) (FIG. 3). This approach would widen the range of antigens that can be targeted to include those that are preferentially, but not exclusively, expressed by tumour cells.

Future immune cell therapies might mimic more closely immune responses to microorganisms, in which different immune cell types act in a concerted fashion.

Box 2 | Activating NK cells in vivo

CD16 engagement by antibody-opsonized cells triggers natural killer (NK) cell killing and is one of the main mechanisms of action of immunotherapeutic antibodies[44]. CD16 can also be engaged with bispecific killer engagers (BiKEs), consisting of two linked single-chain variable fragments which can simultaneously bind CD16 and a tumour antigen[219]. BiKEs that target the cancer marker epithelial cell adhesion molecule (EpCAM) or CD33 enhanced NK cell killing of solid tumour cells and acute myeloid leukaemia cells, respectively[219]. Binders linking CD16 to both CD19 and CD22 (trispecific killer engagers)[219] or simultaneously delivering IL-15 have also been developed[220,221]. Engagers linking tumour antigens with CD16 and NKp46 on NK cells have recently been reported[222].

Anti-4-1BB agonistic monoclonal antibodies promote NK cell activity in mice[223]. Patients with advanced-stage solid tumours who received the anti-4-1BB monoclonal antibody urelumab showed increased levels of interferon-induced cytokines, with transaminitis at higher doses, and fatigue and nausea at lower doses[224]. In 55 patients who received anti-4-1BB utomilumab, the antibody was well tolerated, with no transaminitis; two durable responses were observed among 15 patients with Merkel cell carcinoma[225].

A monoclonal antibody, 1-7F9, which antagonizes KIR2DL1, KIR2DL2, and KIR2DL3 inhibitory receptors and increases NK cell cytotoxicity[226,227] was administered to 23 elderly patients with acute myeloid leukaemia in first complete remission[228]. Killer cell immunoglobulin-like receptor saturation was sustained for more than 2 weeks at the highest doses, with a good safety profile, and no changes in lymphocyte subsets, NK cell receptor expression or in vitro cytotoxicity[228]. In a trial that enrolled 32 patients with multiple myeloma, there was a similar safety profile, with no evidence of autoimmunity, but no objective responses were seen[229]. The inhibitory receptor NKG2A has been targeted with antagonistic antibodies in preclinical studies; clinical studies are ongoing[230–232].

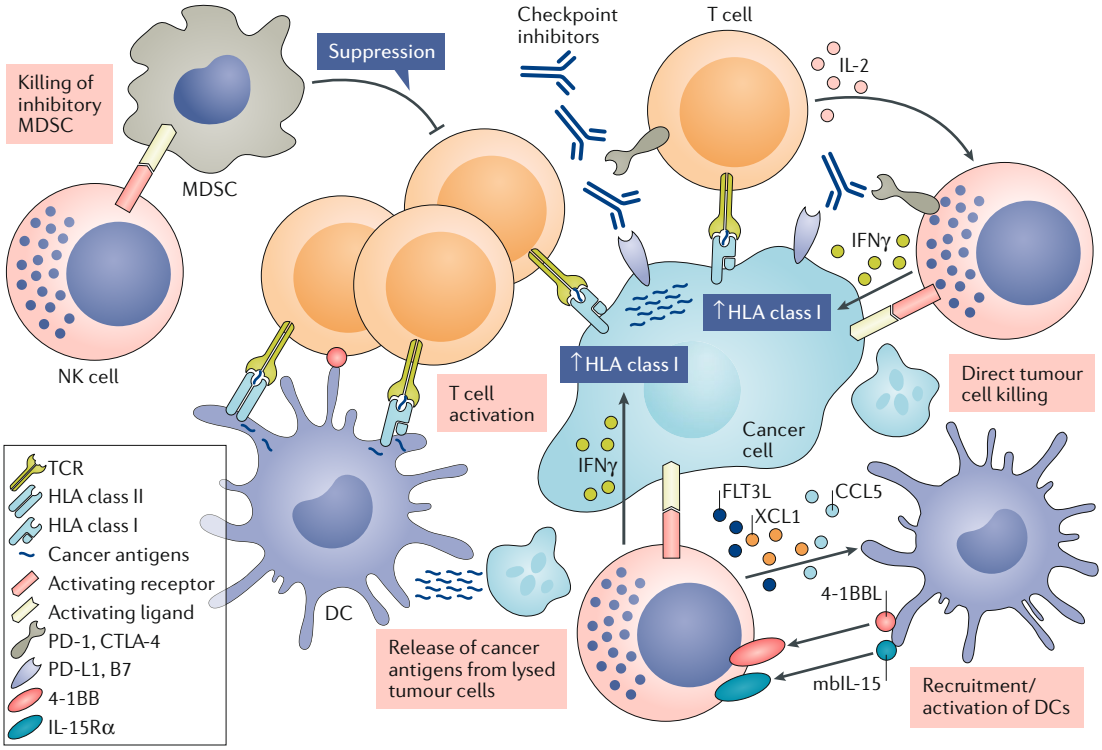


Fig. 4 | **Scenarios for interaction between NK cells and other immune cells in the tumour microenvironment.** Natural killer (NK) cells can directly kill tumour cells and complement the antitumour activity of T cells that express T cell receptors (TCRs) against tumour peptides. NK cells secrete interferon-γ (IFNγ), which increases human leukocyte antigen (HLA) class I expression in tumour cells, enhancing presentation of tumour antigens to T cells. Thus, NK cells could support the antitumour activity of T cell in the context of infusions of TCR-transduced T cells and tumour infiltrating lymphocytes and immune checkpoint inhibitor therapy. NK cells directly recruit dendritic cells (DCs) to the tumour microenvironment and stimulate their maturation via CC-chemokine ligand 5 (CCL5), XC-chemokine ligand 1 (XCL1) and FMS-related tyrosine kinase 3 ligand (FLT3L)[76,77]. DCs stimulate NK and T cells via membrane-bound IL-15 (mbIL-15) and 4-1BBL expression. Lysed tumour cells release cancer antigens, which are then presented by DCs, promoting specific T cell proliferation. Finally, NK cells can kill myeloid-derived suppressor cells (MDSCs), releasing suppression on antitumour T cells, including chimeric antigen receptors T cells[174]. CTLA4, cytotoxic T lymphocyte antigen 4; IL-15Rα, IL-15 receptor α-chain; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1.

During antibody therapy, NK cells are major effectors of ADCC, and they have been used to enhance the activity of immunotherapeutic antibodies with promising results[204–206]. Other ways to redirect them in vivo are being developed (BOX 2). T cells engineered to exert ADCC are being tested in clinical trials, further enriching the immunotherapy toolbox[215]. In patients with solid tumours, combining NK cells with infused antitumour T cells should extend the immune cell attack to tumour cells that express low levels of tumour antigens (FIG. 4). IFNγ secreted by NK cells should increase MHC class I expression in tumour cells and, thus, their vulnerability to T cells, an effect that could be magnified with checkpoint inhibitors. Increased cytotoxicity and dendritic cell stimulation should support the presentation of cancer antigens and further intensify T cell responses[76,77]. An exciting prospect, particularly in patients with haematological malignancies, is the use of CAR NK cells in combination with CAR T cells. NK cell cytotoxicity should rapidly reduce tumour load and decrease the risk of CAR T cell-related toxic effects, while the high proliferative potential and persistence of T cells should ensure elimination of minimal residual disease. Beyond immunotherapy, recent data suggest that molecularly targeted agents that induce senescence in tumour cells can elicit NK cell-mediated antitumour activity[216], further supporting the notion that NK cells are likely to be key players in future multimodal strategies to treat cancer.

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Competing interests
D.C. has received patent royalties from Juno Therapeutics (a Celgene company), Unum Therapeutics, Nkarta Therapeutics and Medisix Therapeutics; he is a co-founder of, stockholder of and consultant for Unum, Nkarta and Medisix. N.S. and D.C. are co-inventors on patent applications licensed to Nkarta or unlicensed. A.J. has no financial competing interests. None of the authors has non-financial competing interests.

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