CANCER IMMUNOTHERAPY

Therapeutic approaches to enhance natural killer cell cytotoxicity against cancer: the force awakens

Richard W. Childs and Mattias Carlsten

Abstract | Scientific insights into the human immune system have recently led to unprecedented breakthroughs in immunotherapy. In the twenty-first century, drugs and cell-based therapies developed to bolster humoral and T cell immunity represent an established and growing component of cancer therapeutics. Although natural killer (NK) cells have long been known to have advantages over T cells in terms of their capacity to induce antigen-independent host immune responses against malignancies, their therapeutic potential in the clinic has been largely unexplored. A growing number of scientific discoveries into pathways that both activate and suppress NK cell function, as well as methods to sensitize tumours to NK cell cytotoxicity, have led to the development of numerous pharmacological and genetic methods to enhance NK cell antitumour immunity. These findings, as well as advances in our ability to expand NK cells *ex vivo* and manipulate their capacity to home to the tumour, have now provided investigators with a variety of new methods and strategies to harness the full potential of NK cell-based cancer immunotherapy in the clinic.

Growing knowledge in the field of immunobiology has now established that multiple components of the immune system have important roles in protecting humans from cancer. This insight has led to systematic studies aimed at using drugs and other methods to bolster antitumour immunity in patients with cancer.

Natural killer (NK) cells are lymphocytes arising from $CD34^+$ haematopoietic progenitor cells in the bone marrow. Although NK cells are primarily found in the blood, liver, spleen, bone marrow and, to a lesser extent, lymph nodes[1], inflammation and other factors can trigger NK cell migration into almost any tissue[2]. NK cells were identified on the basis of their ability to lyse tumour cells without prior sensitization[3–6]. In contrast to B cells and T cells, NK cells do not rearrange genes to acquire antigen-specific receptors. Instead, NK cells target tumour cells via an array of germ line-encoded cell surface receptors. Based on this characteristic, NK cells have traditionally been considered to be innate immune cells. However, the observation that some NK cell subsets can be long-lived and show recall responses to certain stimuli has recently challenged this view[7,8], as these are properties characteristic of adaptive immunity.

NK cells can mediate cytotoxicity via several distinct mechanisms. The most studied pathway is degranulation, whereby NK cells release cytotoxic granulae upon interaction with target cells. This pathway is triggered by signals from activating cell surface receptors such as NK group 2 member D (NKG2D), DNAM1, 2B4 and the natural cytotoxicity receptors NKp30, NKp44 and NKp46, counterbalanced by signals from inhibitory receptors, most of which bind to major histocompatibility complex (MHC) class I molecules[9]. In the mid-1980s, Kärre and colleagues[10,11] postulated the 'missing-self' hypothesis, which describes the lack of self-MHC class I molecules on target cells as the common factor leading to NK cell cytotoxicity. Subsequent research has not only provided further insights into the intricate regulation of NK cell degranulation but also revealed that their functional capacity is tuned by interactions with self-MHC class I molecules[12,13]. In humans, killer cell immunoglobulin-like receptors (KIRs) and the CD94–NKG2A receptor are the only known receptors to mediate functional tuning (also referred to as NK cell education). However, the role for other receptors, such as leukocyte immunoglobulin-like receptor subfamily B

Hematology Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.
Correspondence to R.W.C.
e-mail: childsr@nhlbi.nih.gov
doi:10.1038/nrd4506
Published online 22 May 2015

member 1 (LIR1), in this process remains to be established. One receptor that can trigger potent degranulation without the need for simultaneous co-activation signals from other NK cell receptors is the Fc receptor CD16 which, upon interacting with antibody-coated cells, mediates antibody-dependent cellular cytotoxicity (ADCC). Other routes by which NK cells can kill target cells are the death receptor pathways: the tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)–TRAIL receptor (TRAILR) pathway and the FAS–FAS ligand (FASL) pathway (also known as the CD95–CD95 ligand pathway). Instead of triggering the release of cytotoxic granulae, death receptor pathways induce apoptosis via caspase activation inside target cells and therefore induce cytotoxicity independent of both NK cell education and signalling from receptors controlling NK cell degranulation.

More than a decade has passed since initial reports established the potential of NK cells to mediate tumour regression in patients with cancer[14,15]. These data came from studies showing that haploidentical donor NK cells can prevent leukaemia relapse following haematopoietic stem cell transplantation and induce remission after infusion of mature NK cells in patients with acute myeloid leukaemia (AML). Despite this revelation, doubts remain about the true therapeutic potential of NK cells in cancer immunotherapy. In contrast to therapy using antigen-specific T cells, enthusiasm for NK cell-based immunotherapy has been tempered by the fact that these effectors lack antigen specificity, have poor *in vivo* expansion capacity (clonal expansion) and have only modest tumour-homing capacity. Given the need to overcome inhibition by self-MHC class I molecules, the therapeutic applicability and value of NK cells may also be limited to allogeneic rather than autologous use. Moreover, NK cells in mice and non-human primates have substantial genotypic and phenotypic differences compared to humans, which presents an obstacle for using animal models to translate NK cell-based immunotherapies into clinical trials.

Several approaches have recently been developed to boost NK cell antitumour function, to support *in vivo* persistence and homeostatic proliferation, and to promote homing to the tumour microenvironment (FIG. 1). In this Review, we highlight preclinical and clinical strategies that use drugs and other approaches to bolster the therapeutic potential of NK cells against cancer (FIG. 2).

Cytokines to boost NK cells

Interleukins are involved in promoting the activation, differentiation, proliferation and survival of lymphocytes. The common γ-chain (γ_c) family of cytokines interleukin-2 (IL-2) and IL-15, as well as the pro-inflammatory cytokine IL-12, are currently being characterized in terms of their ability to stimulate NK cell antitumour immunity in humans (TABLE 1). Several *in vitro* studies have established an array of other cytokines that can activate and promote NK cell proliferation; however, because these agents remain in early preclinical development they will not be discussed here.

IL-2 was originally discovered to be a T cell growth factor more than 30 years ago. Subsequent studies showed that IL-2 promotes homeostasis, proliferation and cytotoxicity of NK cells. Recombinant IL-2 (aldesleukin/Proleukin; Prometheus) was the first cytokine used clinically to boost immune responses in patients with cancer. Although pilot studies established proof of concept of the therapeutic antitumour potential of a single exogenously administered cytokine, responses were limited and toxicity was substantial, especially when used at high doses[16,17]. Subsequently, low-dose IL-2 has been recognized to have a lower toxicity profile, and its use has been incorporated into an increasing number of investigational trials to support the *in vivo* persistence of adoptively infused immune effectors such as NK cells[14,18,19]. However, recent data suggest potential pitfalls of low-dose IL-2 use, including the mobilization of regulatory T cells (T_{Reg} cells). Indeed, even ultra-low doses of IL-2 can stimulate the expansion of host T_{Reg} cells *in vivo*, suppressing NK cell proliferation and cytotoxicity[20]. New variants of IL-2, constructed to selectively bind to the IL-2 receptor-β (IL-2Rβ) subunit expressed on NK cells rather than the IL-2Rα subunit expressed on T_{Reg} cells, are under development and may provide better *in vivo* augmentation of NK cell antitumour immunity[21].

In contrast to IL-2 that activates almost all T cells, including T_{Reg} cells, and most NK cells, IL-15 preferentially stimulates $CD8^+$ T cells and non-terminally differentiated NK cells[22,23]. IL-15 induces the association of IL-15Rα, IL-2Rβ and γ_c, which promotes NK cell development, expansion and homeostasis via long-lasting signal transducer and activator of transcription 5 (STAT5) phosphorylation and BCL-2 expression[24,25] while avoiding undesirable T_{Reg} cell mobilization[26]. The first clinical-grade version of IL-15 evaluated in patients with cancer was single-chain recombinant IL-15 (scIL-15), which was reported to have a high incidence of dose-dependent grade 3/4 toxicities[27] (TABLE 1). When used following adoptive NK cell infusion in patients with AML, scIL-15 supported both the persistence and the proliferation of NK cells[28], which appears to be a prerequisite to achieve leukaemia remission using this strategy[29]. In contrast to scIL-15, heterodimeric IL-15 (IL-15–sIL-15Rα (soluble IL-15Rα)) is more potent at stimulating NK cell proliferation and has the advantage of not requiring *trans*-presentation by IL-15Rα, which is primarily expressed on monocytes and dendritic cells[30–32]. Moreover, heterodimeric IL-15 consisting of IL-15N72D and IL-15RαSu/Fc has a much longer half-life than scIL-15, allowing for less frequent dosing[31]. Although unlikely to be of significant benefit as a single therapeutic modality, heterodimeric IL-15 may have a role in supporting NK cell antitumour immunity when used in conjunction with adoptive NK cell infusions or with drugs that induce NK cell tumour cytotoxicity. Several ongoing trials, as well as recently completed but not yet published studies, will shed more light on the value of IL-15 use in the context of cancer therapy[33].

IL-12 is a pro-inflammatory cytokine that has pleiotropic effects on the haematopoietic system. NK cells express IL-12Rβ2 (REF. 34) and show augmented function[35], upregulation of adhesion molecules (including

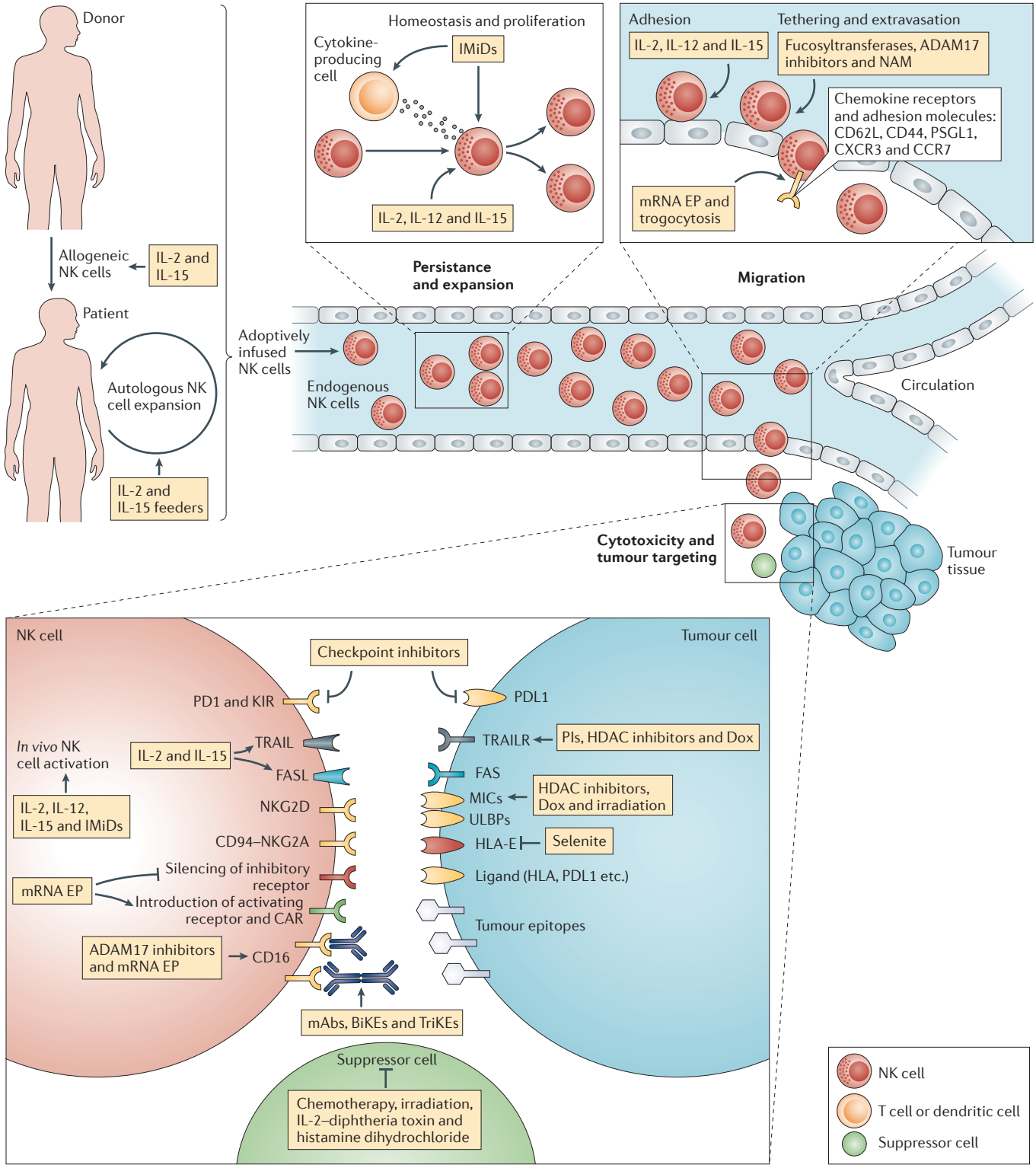

Figure 1 | **Schematic overview of drugs that bolster NK cell antitumour immunity and their interaction points.** This schematic shows how various drugs can direct natural killer (NK) cells from the circulation to migrate to the interface of NK cells with the target in the tumour microenvironment. Therapeutic approaches are depicted in yellow boxes. ADAM17, disintegrin and metalloproteinase domain-containing protein 17; BiKE, bispecific killer engager; CAR, chimeric antigen receptors CCR7, CC chemokine receptor type 7; CXCR3, CXC chemokine receptor type 3; Dox, doxorubicin; EP, electroporation; FASL, FAS ligand, HDAC, histone deacetylase; IL, interleukin; IMiD, immunomodulatory drug; KIR, killer cell immunoglobulin-like receptor; mAb, monoclonal antibody; MIC, major histocompatibility complex class I chain-related gene; NAM, nicotinamide; NKG2, NK group 2; PD1, programmed cell death protein 1; PDL1, programmed cell death ligand 1; PSGL1, P-selectin glycoprotein ligand 1; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; TRAILR, TRAIL receptor; TriKE, trispecific killer engager; ULBP, U16-binding protein.

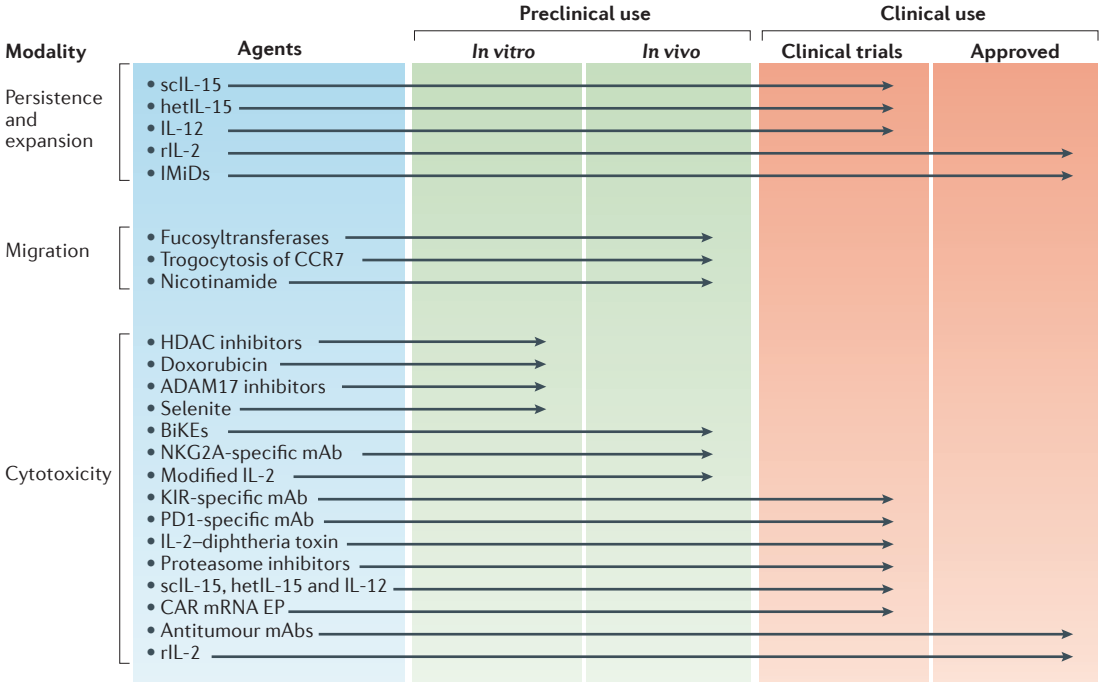

Figure 2 | **Development status of drugs designed to augment NK cell antitumour immunity.** Listed are drugs known to directly or indirectly bolster natural killer (NK) cell antitumour immunity. The indicated stage of development only refers to the development of the drug with regards to its potential to augment NK cell responses. ADAM17, disintegrin and metalloproteinase domain-containing protein 17; BiKE, bispecific killer engager; CAR, chimeric antigen receptor; CCR7, CC chemokine receptor type 7; EP, electroporation; het, heterodimeric; HDAC, histone deacetylase; IL, interleukin; IMiD, immunomodulatory drug; KIR, killer cell immunoglobulin-like receptor; mAb, monoclonal antibody; NKG2A, NK group 2 member A; PD1, programmed cell death protein 1; r, recombinant; sc, single chain.

CD62L (also known as L-selectin))[36] and enhanced migration capacity *in vivo*[37] following IL-12 stimulation. Early clinical studies of high-dose IL-12 in patients with cancer have shown that this cytokine has immunomodulatory effects on NK cells and other lymphocytes, promoting interferon-γ (IFNγ) production and margination[38–41]. Remarkably, despite limited efficacy and substantial toxicity, antitumour responses were observed in a subset of patients who had sustained blood levels of NK cell-derived IFNγ[38]. Based on preclinical mouse models, in which lower doses of IL-12 following chemotherapy-induced cytopaenias promoted multilineage haematopoietic recovery with concomitant antitumour responses[42], more recent studies in humans have focused on the use of lower doses of this agent. A recently published Phase I study of low-dose subcutaneous recombinant IL-12 in healthy subjects reported an improved toxicity profile compared with prior high-dose studies and evidence that this cytokine modulates NK cell migration[43]. However, the effects of low-dose IL-12 on NK cell function were not described. Additional studies in patients with cancer are needed to establish the possible role of IL-12 in bolstering NK cell antitumour immunity.

Drugs to augment NK cell function

Several drugs other than cytokines can directly and/or indirectly augment NK cell function *in vivo*. A number of these drugs are under clinical evaluation (TABLE 1).

Thalidomide derivatives. The immunomodulatory drugs (IMiDs) lenalidomide (Revlimid; Celgene) and pomalidomide (Pomalyst/Imnovid; Celgene) are derivatives of thalidomide. Lenalidomide, which is less toxic than thalidomide, has been widely studied in patients with cancer and now represents the standard of care for multiple myeloma and myelodysplastic syndromes. The mechanism of action of lenalidomide is not completely understood, but ample evidence shows that its intrinsic antitumour effects are mediated either directly or indirectly by stimulating antitumour immunity[44–46]. Lenalidomide indirectly augments NK cell cytotoxicity and proliferation through the release of IL-2 and IFNγ from surrounding T cells and dendritic cells via IMiD-induced downregulation of suppressor of cytokine signalling 1 (SOCS1) in these cells[45,47,48]. *In vitro*, direct exposure of NK cells to IMiDs leads to increased IFNγ production and decreases the threshold for NK cell activation[49]. Consistent with these preclinical findings, patients treated with lenalidomide have increased NK cell numbers with enhanced function, which may lead to improved clinical responses[50–53]. Emerging data also suggest that combining IMiDs with tumour-specific monoclonal antibodies (mAbs) may augment NK cell-mediated antitumour responses[44,47,48]. Although IMiDs have shown remarkable efficacy in patients with cancer, the contribution of IMiD-mediated augmentation of NK cell function to these antitumour effects has yet to be established.

Immune checkpoint inhibitors. Immune checkpoint inhibitors provide pharmaceutical blockade of central inhibitory immune receptors. Programmed cell death protein 1 (PD1) is expressed on activated T cells, B cells, monocytes and NK cells[54]. Its ligand PDL1 is expressed on a variety of tumour cells. PD1 and PDL1 have central roles in tumour recurrence and progression; signalling through this pathway suppresses lymphocytes, including NK cells[54,55]. The PD1–PDL1 pair may be particularly relevant in cancer because PD1 expression is upregulated on lymphocytes in patients with malignancies compared with healthy individuals[56,57]. *In vitro*, blockade of PD1 on NK cells from patients with multiple myeloma with the PD1-specific mAb pidilizumab augments lysis of autologous tumour cells[58]. Furthermore, when PD1-specific and PDL1-specific mAbs are combined with lenalidomide *in vitro*, NK cell killing of multiple-myeloma cells is enhanced[58]. Preclinical data also show that PD1 blockade boosts NK cell-mediated ADCC and improves NK cell tumour trafficking while simultaneously suppressing T_{Reg} cell function[44,46,48,58]. Several PD1-specific mAbs — such as pidilizumab, pembrolizumab (formerly known as lambrolizumab and marketed by Merck as Keytruda)[59,60] and nivolumab (Opdivo; Bristol-Myers Squibb)[61] — have recently gained regulatory approval or are expected to receive approval in the near future. Additional studies are needed to clarify the extent to which these mAbs bolster antitumour immunity through augmentation of endogenous NK cell function and their potential to enhance the antitumour effects of adoptively infused NK cells.

Disruption of inhibitory KIRs through mAb-mediated blockade has also been explored as a method to augment endogenous NK cell killing of tumours. The KIR-specific mAbs IPH2101 and IPH2102 (also known as lirilumab) are clinical-grade immunoglobulin G4 (IgG4) mAbs that bind to KIR2D molecules. *In vitro* studies show that IPH2101 augments NK cell-mediated lysis of KIR-ligand matched tumour cells and enhances NK cell-mediated ADCC against antibody-bound tumours[62,63]. The therapeutic potential of IPH2101 has also been demonstrated in preclinical mouse models[64,65], which have formed the basis for clinical trials evaluating IPH2101 in patients with cancer. Two Phase I clinical trials in patients with AML and in patients with multiple myeloma have demonstrated that this product is safe. The first Phase II clinical trial of IPH2101 in patients with smouldering multiple myeloma was recently published but did not establish efficacy for use in this setting[66]. It remains to be determined whether IPH2101 and IPH2102 will show efficacy in other disease settings or when used in combination with other therapies such as tumour-targeting mAbs, cytokines or IMiDs[62].

Biologics to redirect NK cell cytotoxicity

Antibodies and engineered molecules have been used to redirect NK cells to kill tumours. Tumour-specific mAbs represent a highly efficacious and distinct modality of cancer immunotherapy that has greatly improved therapeutic options for patients with a variety of malignancies. mAbs induce tumour cell death via numerous mechanisms, including growth receptor blockade, activation of complement and ADCC[67]. Below, we focus on the effects and future potential of both mAbs and engineered molecules that redirect NK cells to tumours for killing through ADCC.

mAbs. The impact of polymorphisms in the *CD16* gene on the response to mAb therapy has recently shed light on the importance of NK cells in mediating antitumour responses via ADCC. Most humans express a version of CD16 that has a relatively low affinity for IgG1 and IgG3. However, ~10% of the population has a single-nucleotide polymorphism in *CD16* that results in an amino acid substitution at position 158 (CD16-F158V)[68]. NK cells with the CD16-158V genotype have a higher affinity for IgG1 and IgG3 than those with the CD16-158F genotype and exert ADCC more efficiently. This polymorphism appears to bolster ADCC *in vivo*; two studies[69,70] reported that lymphoma patients homozygous for CD16-158V show substantially higher response rates following treatment with the CD20-specific mAb rituximab (MabThera; Hoffmann-La Roche) than those carrying the CD16-158F polymorphism (90% versus 51%[69] and 75% versus 26%[70] objective response rates at 1 year). Similar effects of *CD16* polymorphisms on efficacy have been described in patients with metastatic colon cancer treated with cetuximab (Erbitux; ImClone)[71]. Further data highlighting the importance of NK cells in mediating antitumour responses via ADCC come from a report showing improved survival of GD2-specific mAb-treated patients with neuroblastoma who lack one or more groups of KIR ligands compared with those who carry all KIR ligand groups[72].

Although the list of mAbs approved for use in patients with cancer has grown substantially over the past decade, the degree to which these mAbs mediate their antitumour responses via ADCC has been poorly characterized. Confounding this type of analysis is the fact that mAbs are routinely used in conjunction with single-agent or multi-agent chemotherapy, many of which are cytotoxic to NK cells and probably hamper their ability to mediate ADCC[73]. Combining mAb therapy with cytokines such as IL-2 or IL-15 could, theoretically, improve the efficacy of mAb therapy through their ability to activate and expand NK cells *in vivo*, to upregulate NK cell-activating receptors and to override inhibitory interactions mediated by self-MHC class I molecules that typically suppress NK cell ADCC. The recent ability to expand and infuse large numbers of *ex vivo* expanded clinical-grade NK cells now provides the opportunity to explore the full potential of mAb-mediated tumour killing by NK cell ADCC in patients with cancer.

Bispecific mAbs. Bispecific antibodies and bispecific or trispecific killer engagers (BiKEs or TriKEs) are engineered molecules that exclusively act via ADCC by crosslinking epitopes on tumour cells with the CD16 receptor on NK cells. These molecules have advantages over mAbs because they bind to a different epitope of the

CD16 molecule, resulting in stronger NK cell ADCC[74]. *In vitro*, the CD16–CD33 BiKE seems to overcome inhibitory KIR signalling, leading to robust NK cytokine production and killing of myeloid malignancies[75]. Several BiKEs and TriKEs are currently being developed and evaluated for targeting of various malignancies.

Drugs that sensitize tumours to NK cells

Strategies to render tumour cells more susceptible to NK cell killing may markedly improve the outcome of NK cell-based immunotherapies. Below, we focus on drugs that sensitize tumour cells to NK cells, some of which are currently under clinical evaluation (FIG. 2; TABLE 1).

Table 1 | **Clinical studies evaluating the ability of drugs to bolster NK cell antitumour immunity***

Drug	Effect on NK cells	Patient population	Clinical trials (number of active trials)	Comments
Cytokines				
IL-2	↑Persistence and expansion; ↑cytotoxicity	Melanoma, RCC, AML, neuroblastoma, breast cancer, ovarian carcinoma, Fallopian tube cancer and peritoneal cancer	6 (2)	Some studies combined IL-2 with antitumour mAbs
IL-15	↑Persistence and expansion; ↑cytotoxicity	Melanoma, RCC, lung cancer, SCC and multiple myeloma	4 (3)	Single-chain recombinant IL-15 and heterodimeric IL-15 used
IL-12	↑Migration; ↑cytotoxicity	Healthy volunteers	1 (1)	Lower doses used than initial studies
Cytokines after NK cell infusion				
IL-2	↑Persistence and expansion; ↑cytotoxicity	AML and myelodysplastic syndromes	58 (30)	Lower doses used than initial studies
IL-15	↑Persistence and expansion; ↑cytotoxicity	AML	3 (2)	Intended to more specifically bolster NK cell antitumour activity compared to IL-2
Checkpoint inhibitors				
PD1-specific mAbs	↑Cytotoxicity	Solid tumours and multiple myeloma	2 (2)	Used in combination with IPH2102 (lirilumab)
KIR-specific mAbs	↑Cytotoxicity	Multiple myeloma, AML, melanoma, lung cancer and peritoneal cancer	9 (4)	IPH2101 and IPH2102 (lirilumab) used
Other immunomodulatory drugs				
Lenalidomide	↑Persistence and expansion; ↑cytotoxicity	Multiple myeloma, BCL and neuroblastoma	15 (10)	–
Tumour-targeting mAbs				
CD20-specific mAbs	↑Cytotoxicity	BCL and multiple myeloma	10 (4)	Mostly rituximab but veltuzumab also used
GD2-specific mAbs	↑Cytotoxicity	Neuroblastoma	6 (5)	Several different GD2-specific mAbs are being evaluated
EGFR-specific mAbs	↑Cytotoxicity	SCC	4 (3)	Cetuximab used in all studies
ERBB2-specific mAbs	↑Cytotoxicity	Breast cancer	2 (1)	Trastuzumab used in all studies
Tumour-sensitizing agents prior to NK cell infusion				
Bortezomib	↑Cytotoxicity	CLL, RCC, lung cancer, multiple myeloma and sarcoma	1 (1)	Bortezomib administered to sensitize tumours to NK cell TRAIL
Regulatory T cell eradication prior to NK cell infusion				
IL-2–diphtheria toxin fusion protein	↑Persistence and expansion; ↑cytotoxicity	AML, non-Hodgkin lymphoma and CLL	2 (1)	In one study, IL-2-diphteria toxin fusion protein was combined with pentostatin and rituximab

AML, acute myeloid leukaemia; BCL, B cell lymphoma; CLL, chronic lymphatic leukaemia; EGFR, epidermal growth factor receptor; IL, interleukin; KIR, killer cell immunoglobulin-like receptor; mAb, monoclonal antibody; MM, multiple myeloma; NK, natural killer; PD1, programmed cell death protein 1; RCC, renal cell carcinoma; SCC, squamous cell cancer; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand.*Only trials studying the effect of drug treatment on NK cells as a primary or secondary end point are listed. Data from ClinicalTrials.gov.

Proteasome inhibitors and anthracyclines. The death ligand TRAIL, expressed on NK cells, triggers apoptosis in TRAILR-positive tumour cells by initiating cleavage of caspase 8 (REF. 76). This pathway can be augmented by exposing tumour cells to proteasome inhibitors such as bortezomib (Velcade; Millennium Pharmaceuticals) and carfilzomib (Kyprolis; Onyx Pharmaceuticals), which simultaneously upregulate TRAILRs on the tumour cell surface and increase caspase 8 activity upon TRAILR ligation. Sensitizing tumours to NK cells via this pathway has successfully been shown in animal models[77] and has therapeutic appeal because it occurs independently of signals from inhibitory NK cell receptors such as KIR and CD94–NKG2A. Importantly, in contrast to tumour cells, normal tissues express TRAIL decoy receptors (that is, TRAILR3 and TRAILR4), rendering them insensitive to TRAIL-mediated killing[78]. Proteasome inhibitors can also sensitize tumour cells to NK cells via upregulation of NKG2D receptor ligands on the tumour cell surface[79]. An ongoing Phase I clinical trial at the US National Institutes of Health (NIH) has established the safety of combining bortezomib with infusions of *ex vivo* expanded autologous NK cells. In this trial, tumour regressions were observed in patients with treatment-refractory renal cell carcinoma and chronic lymphocytic leukaemia (R.W.C., unpublished observations). Another drug that may be used in this context is the anthracyline antibiotic doxorubicin, which has broad therapeutic use for a wide range of malignancies. Similar to proteasome inhibitors, preclinical data have established that pretreatment of tumour cells with this drug enhances their susceptibility to NK cell killing via TRAIL[80]. Clinical studies combining doxorubicin with infusions of autologous or allogeneic NK cells have yet to be explored.

HDAC inhibitors. *In vitro* studies have shown that the histone deacetylase (HDAC) inhibitor valproic acid upregulates the tumour surface expression of NKG2D ligands, enhancing tumour cell susceptibility to NK cell cytotoxicity[81]. This agent can efficiently sensitize monoblastic leukaemia cells and hepatoma cells to NK cell killing, but this effect seems to be contingent upon and restricted to malignant cells having baseline expression of NKG2D ligands[82]. Romidepsin (Istodax; Celgene), another HDAC inhibitor, also upregulates NKG2D ligands and sensitizes tumour cells to NK cell killing *in vitro* [83], whereas the HDAC inhibitor depsipeptide renders tumour cells susceptible to NK cell killing through TRAIL sensitization[84].

Selenite. The inorganic compound selenite, a selenium derivative, can render mesothelioma cells susceptible to NK cells expressing CD94–NKG2A *in vitro* by reducing the tumour cell surface expression of HLA-E[88]. This compound is clinically appealing because selenium derivatives preferentially affect malignant cells while sparing normal cells[85–87]. The exact mechanism by which selenite reduces HLA-E expression on tumour cells needs further clarification, but induction of oxidative stress and subsequent endoplasmic reticulum stress has been proposed as being fundamental to this process[88].

Ex vivo manipulation of NK cells

Immunotherapeutic strategies using adoptively transferred NK cells allow the possibility to pre-activate or, by other means, manipulate NK cells prior to infusion. Several methods are being explored to modulate NK cells *ex vivo* for augmented tumour targeting following adoptive transfer. TABLE 2 presents an overview of the approaches that are being evaluated in clinical trials.

Short-term* ex vivo *NK cell activation. Adoptive transfer of short-term *ex vivo* activated allogeneic NK cells can induce clinical responses in patients with AML and in patients with multiple myeloma[14,89]. Miller *et al.*[14] pioneered this approach by using haploidentical NK cells stimulated with 1,000 IU ml^{-1} IL-2 for 8–16 hours prior to infusion. Many of these studies have used the chemotherapeutics fludarabine and cyclophosphamide with or without irradiation as a preparative regimen to avoid rejection of the infused cells; to provide immunological space for the persistence and expansion of the infused cells; and to eradicate suppressor cell populations that inhibit NK cell function. Administration of IL-2 after adoptive cell transfer is able to further promote *in vivo* expansion of infused NK cells, improving objective response rates[14].

However, because IL-2 typically leads to T_{Reg} cell expansion even when given after highly immunosuppressive conditioning, interest has recently shifted to the use of IL-15 as an alternative to induce NK cell proliferation without expanding suppressor T cell populations. Preliminary data from an ongoing study using IL-15 following NK cell infusions have shown less T_{Reg} cell mobilization while improving *in vivo* NK cell persistence and expansion, resulting in superior tumour response rates compared with studies using IL-2 (REF. 28). Modifications to these and other cytokines to more specifically and efficiently support the expansion and proliferation of endogenous and adoptively transferred NK cells are currently under development.

Ex vivo *expansion of NK cells.* Numerous methods have been developed to expand NK cells *ex vivo* from the peripheral blood, umbilical cord blood, haematopoietic progenitors, embryonic stem cells and induced pluripotent stem cells (summarized in REF. 90). *Ex vivo* expansion, in contrast to fresh or overnight cytokine stimulation, enables the use of multiple, large number infusions of highly activated NK cells. Early clinical-grade expansion methods used media containing cytokines alone, such as IL-2 and IL-15, and typically resulted in only 10–20-fold expansions after 14 days[90]. Subsequent protocols incorporating feeder cells with cytokine-containing media were able to more effectively induce NK cell proliferation, with expansions in the range of 80–10,000-fold being achieved with 14–21-day cultures[90].

NK cells can be expanded directly from peripheral blood mononuclear cells (PBMCs) with or without preceding NK cell enrichment. Expansion of NK cells from unfractionated lymphocytes usually results in high levels of T cell contamination, precluding their use in

Table 2 | **Clinical studies evaluating the efficacy of adoptively infused NK cells**

Method	Patient population	Total number of clinical trials (number of active trials)	Comments
Non-expanded NK cells			
Autologous NK cells + IL-2	Melanoma, RCC, lung cancer and nasopharyngeal cancer	3 (1)	–
Autologous NK cells + IL-15	Neuroblastoma, sarcoma, Wilms tumour and rhabdomyosarcoma	1 (1)	Intended to more specifically bolster NK cell antitumour activity than IL-2
Allogeneic NK cells + IL-2	AML, multiple myeloma, myelodysplastic syndromes, lymphoma, ovarian carcinoma, melanoma, neuroblastoma, Ewing sarcoma, breast cancer and Fallopian tube cancer	55 (29)	Most data published on adoptive NK cell therapy are from these studies
Allogeneic NK cells + IL-15	AML and myelodysplastic syndromes	2 (1)	Intended to more specifically bolster NK cell antitumour activity than IL-2
Expanded NK cells			
Autologous NK cells	CLL, RCC, lung cancer, multiple myeloma, sarcoma, colon cancer, melanoma, neuroblastoma, prostate cancer, ALL and pancreatic cancer	7 (6)	Various expansion methods used, including EBV-LCL and membrane-bound cytokine or 4-1BBL feeder cells; some studies use IL-2 post NK cell infusion
Allogeneic NK cells	AML, myelodysplastic syndromes, T cell lymphoma and multiple myeloma	11 (8)	Various expansion methods used, including EBV-LCL and membrane-bound cytokine or 4-1BBL feeder cells; some studies use IL-2 post NK cell infusion
Genetically manipulated NK cells			
CD19 CAR mRNA (expanded NK cells)	BCL	2 (2)	Designed to redirect tumour targeting. Haploidentical NK cells expanded with K562 membrane-bound IL-15 or 4-1BBL feeder cells; in Phase II clinical trials
NK cell lines			
NK-92	AML, multiple myeloma and lymphoma	2 (2)	Off-the-shelf NK cells; in dose-escalating Phase I clinical trials

4-1BBL, 4-1BB ligand; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BCL, B cell lymphoma; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukaemia; EBV-LCL, Epstein–Barr virus-transformed lymphoblastoid cell line; IL, interleukin; LC, lung cancer; MDS, myelodysplastic syndromes; NK, natural killer; RCC, renal cell carcinoma. Data from ClinicalTrials.gov.

allogeneic settings given concerns that contaminating T cells could lead to graft-versus-host disease[90]. Various irradiated cell populations — including PBMCs, T cells, Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines and K562 cells — have been used as feeders for NK cell expansion cultures. Genetically modified K562 cells that express membrane-bound 4-1BBL (also known as TNFSF9) and membrane-bound cytokines such IL-15 or IL-21 are highly efficient at preferentially expanding large numbers of NK cells directly from unmodified PBMCs[91–97]. However, to avoid T cell contamination so that the expanded NK cell product can be used in the allogeneic setting, CD3 depletion of PBMCs used for NK cell expansion cultures is usually required.

At the NIH, we have established a clinical-grade method for NK cell expansion using an irradiated EBV-transformed lymphoblastoid feeder cell line and IL-2-supplemented X-VIVO 20 media[98]. By positively selecting $CD56^+$ NK cells from a T cell-depleted apheresis product, this method generates up to 10,000-fold expansions of highly activated pure (>95%) NK cells within 14–21 days (R.W.C., unpublished observations). Preliminary data from our Phase I clinical trial show that multiple rounds of up to 2.5×10^8 autologous NK cells per kilogram can be safely infused into patients with cancer, with some patients demonstrating regression of metastatic tumours[90] (R.W.C., unpublished observations). However, murine and non-human primate models, as well as *in vivo* correlative data in humans, have raised concerns regarding the ability of these adoptively transferred NK cell populations to home to the bone marrow, where the vast majority of haematological malignancies are located[99]. As a consequence, we and others have now begun to modify culture conditions with the goal of altering the phenotype of expanded NK cells to increase the likelihood of their homing to the bone marrow. Frei *et al.*[100]

recently reported a clinical-grade method to expand NK cells from CD3-depleted apheresis products in feeder-free minimum essential medium-α (MEM-α) containing IL-2, IL-15 and the vitamin B3 derivative nicotinamide, a potent inhibitor of NAD^+-dependent enzymes involved in controlling redox reactions, cell metabolism and cell motility. Using G-Rex flasks, up to 80-fold expansions of >95% pure NK cells were achieved within 14 days of culture. Remarkably, infusions of these NK cells into irradiated non-obese diabetic–severe combined immunodeficiency mice showed improved persistence and preferential homing to bone marrow compared with NK cells expanded under the same conditions without nicotinamide[101]. Subsequent analysis of the nicotinamide-expanded cells revealed that they possess substantially higher surface expression of L-selectin, a molecule pivotal for trafficking and homeostatic proliferation of lymphocytes. Ongoing preclinical and clinical studies should soon provide insights into optimal methods to expand NK cells to maximize their chances of *in vivo* proliferation, survival and homing to critical organs where cancers are located.

Genetic manipulation of NK cells. The development of efficient methods to genetically manipulate NK cells has long been perceived as a necessity to optimize their *in vivo* persistence, homing to disease sites and tumour cytotoxicity following adoptive transfer. However, in contrast to T cells, the genetic manipulation of primary NK cells has been challenging because viral transduction is highly inefficient and results in significant cell death. Recently, electroporation of mRNA has been discovered to be an alternative and effective strategy to genetically modify NK cells. This approach offers a method to genetically modify NK cells using clinically compliant conditions without the need for viral vectors, which requires high-level biosafety laboratories and incurs considerable cost and delays in time to bring to the clinic. As demonstrated recently, NK cells electroporated with mRNA encoding a CD19-specific chimeric antigen receptor (CAR) showed distinct augmentation in their cytotoxicity against B cell malignancies *in vitro* and in preclinical animal models[102,103], which has led to a trial exploring their use in humans. In addition to mRNA transfection of CARs, this approach could be used to modify cellular elements of infused NK cells that critically affect their cytotoxic function and homing properties. The availability of good manufacturing practice-compliant methods to reprogramme NK cells using mRNA transfection offers a new, rapid and cost-efficient method to explore a wide range of genetic approaches to enhance NK cell immunotherapy in the clinic[104]. A complementary approach to mRNA electroporation of primary NK cells is viral transduction of NK cell lines that, in contrast to primary cells, can be genetically manipulated to express CARs and other molecules using adenoviral and lentiviral vectors[105]. The advantage of this approach is that stable transgene expression is achieved; however, infusions of an allogeneic NK cell line require conditioning of the patient to avoid rapid rejection of the infused cells by the host immune system.

Pharmacological and cellular approaches to improve NK cell homing to the tumour. The ability of adoptively infused NK cells to mediate clinically meaningful antitumour effects may, in large part, be determined by their ability to traffic to the tumour microenvironment. Recruitment of circulating leukocytes into the bone marrow is thought to be dependent on endothelial cell E-selectin binding to its cognate ligands, which are expressed on lymphocytes and haematopoietic progenitor cells. Importantly, only E-selectin ligands bearing sialyl Lewis X with a terminal fucose (referred to as glycosylated or fucosylated) have functional activity to bind to E-selectin. Recently, animal models have established that pretreatment of human cord blood stem cells with fucosyltransferases can improve their homing to the bone marrow[106]. The human recombinant enzyme TZ101 (recombinant α1-3 fucosyltransferase VI; Targazyme Inc.) can be used *ex vivo* to efficiently fucosylate E-selectin ligands on haematopoietic stem cells and is currently being studied in a clinical trial for its capacity to improve engraftment following umbilical cord blood transplantation.

One of the hurdles of using cytokine-activated and *ex vivo* expanded NK cells for immunotherapy is that they express mostly non-glycosylated ligands for E-selectin, potentially limiting their ability to home to the bone marrow following adoptive transfer. Ligands for E-selectin expressed on the surface of expanded NK cells can be rapidly and efficiently glycosylated *ex vivo* using TZ101, which significantly enhances their ability to bind to recombinant E-selectin. Infusion of glycosylated NK cells into mice and non-human primates is able to increase the number of NK cells infiltrating the bone marrow[107]. These data suggest that forced fucosylation of NK cells could be used as a novel approach to improve the antitumour effects of adoptive NK cell infusions in patients with haematological malignancies.

An alternative and highly novel strategy to improve NK cell migration to target tissues is to alter their phenotype by having them incorporate homing receptors expressed on feeder cell populations into their cell membrane. It was recently demonstrated that co-culturing *ex vivo* expanded NK cells with K562 cells that were genetically modified to express the homing receptor CC chemokine receptor type 7 (CCR7) led to short-term transfer of CCR7-containing membrane fractions to the NK cell surface via trogocytosis[108]. Importantly, when infused into mice, these CCR7-expressing NK cells show improved homing to the lymph nodes expressing the CCR7 ligands CCL19 and CCL21 (REF. 108). *Ex vivo* culture approaches that engineer NK cells to express desired surface molecules via trogocytosis could represent a more practical strategy to modify the phenotype and function of NK cells than viral transduction strategies, which are less efficient and more expensive.

Where do we go from here?

To date, NK cells have been largely unappreciated in terms of their contribution and ability to mediate effective immune responses against cancer. Nevertheless, increasing evidence has established that these cells can

induce tumour regression, particularly following treatment with drugs such as cytokines, IMiDs and mAbs or when infused over HLA barriers (allogeneic NK cells). Although tumour responses are occasionally observed following treatment with cytokines (that is, IL-2, IL-15 and IL-12), these and other drugs administered to boost NK cell antitumour immunity do so in a nonspecific manner and are unlikely to have a significant therapeutic impact when used as single agents. By contrast, mAbs and mAb-like molecules can specifically redirect NK cell cytotoxicity against tumours, potentially overcoming the immune tolerance that occurs in advanced stages of cancer. Preclinical evidence suggests that trials using multiple simultaneous strategies to bolster NK cell immunity — including methods to enhance NK cell activation, proliferation and tumour-homing, as well as methods to sensitize the tumour to NK cell killing — may be most effective in the clinic. For instance, NK cell-mediated ADCC could be markedly enhanced by combining mAbs or mAb-like molecules with cytokines that expand NK cells *in vivo* and/or upregulate NK cell surface expression of CD16 (that is, IL-12)[109], or with drugs such as disintegrin and metalloproteinase domain-containing protein 17 (ADAM17) inhibitors that prevent both CD16 shedding and shedding of NKG2D ligands on tumour cells[75,110,111]. However, early clinical evaluation of strategies that combine new agents and approaches are often limited by the lack of established clinical trial models and/or by regulatory obstacles. Furthermore, the development of methods to expand large numbers of clinical-grade NK cells *ex vivo* and the ability to genetically modify these cells to express or overexpress activating receptors (that is, CARs, NKG2D or DNAM1) and/or silence inhibitory receptors (that is, KIRs, NKG2A or PD1) now provide an opportunity to study the full potential of NK cell immunotherapy in the clinic.

Until recently, the homing capacity of NK cells, particularly for cells used in the context of adoptive NK cell therapy, has been largely unexplored. For adoptively infused NK cells to mediate antitumour effects, it is intuitive that they must have the capacity to home to the tumour. Therapeutic strategies designed to induce expression of adhesion molecules and homing receptors such as L-selectin, CD62E, CCR7 and CXCR4 could improve NK cell extravasation and subsequent delivery to the lymph nodes and bone marrow, where the majority of haematological cancers are located. Culturing NK cells in media containing nicotinamide, fucosylating NK cells following *ex vivo* expansion or modifying the genetic expression and phenotype of NK cells through mRNA electroporation are some of the more promising methods being explored to improve tumour homing of adoptively infused NK cells.

Homeostatic proliferation and *in vivo* persistence are additional factors that are critical for the success of adoptive NK cell transfer. In this regard, the use of exogenous IL-2, IL-15 and other cytokines plays an important part in sustaining the homeostatic proliferation of NK cells. Furthermore, preconditioning the host prior to adoptive NK cell transfer can enhance endogenous IL-15 production, which has an independent and important role in promoting NK cell proliferation *in vivo*[14]. Although the fusion protein of IL-2 and diphtheria toxin denileukin diftitox (Ontak; Eisai) is able to efficiently promote leukaemia clearance by suppressing T_{Reg} cells[112], new and promising cytokines that selectively promote NK cell proliferation without expanding T_{Reg} cells are currently in the developmental pipeline. The identification of long-lived memory-like NK cells in mice and now humans provides evidence that exogenous cytokine support may be unnecessary for some forms of NK cell immunotherapy. Human NK cells that co-express NKG2C and a self-HLA-binding inhibitory receptor are able to expand following cytomegalovirus (CMV) reactivation[7,113], such cells can be transferred from donors to patients following allogeneic transplantation and expand in the recipient upon CMV reactivation[8]. Remarkably, early CMV reactivation following allogeneic stem cell transplantation of patients with AML has been associated with reduced risk of leukaemia relapse, indicating that these cells may also have antileukaemia properties[114,115]. In conclusion, one may infer from these data that adoptive transfer of selected NK cell subsets with memory-like properties may preclude the obligate use of drugs to support their persistence and activity *in vivo*.

Concluding remarks

Durable and complete regression of treatment-refractory B cell malignancies following CAR T cell therapy has led to an unprecedented realization of the potential of the human immune system to eradicate cancer. Although more than 40 years have passed since the discovery that NK cells represent one of the most tumour-cytotoxic components of the human immune system, we are still awaiting their role as therapeutic antitumour agents to be realized in the clinic. Discrepancies between preclinical predictions and measured clinical effects, the absence of informative animal models and the perception that breakthroughs in the use of adaptive immune effectors to treat cancer preclude value to the use of antigen-nonspecific innate immune effectors have, in part, tempered enthusiasm and slowed developments in the field of NK cell immunotherapy.

Insights into a growing number of recently characterized factors that regulate NK cell function now provide the platform for the development of novel strategies that boost NK cell antitumour immunity. Key components to the success of future trials include the incorporation of modalities that harness NK cell cytotoxicity while promoting *in vivo* survival, homeostatic proliferation and trafficking to the tumour. Newly developed drugs that trigger NK cell tumour killing via ADCC or sensitization of the target and drugs that promote NK cell tumour homing, as well as the development of novel expansion methods and methods to genetically modify NK cells, all represent promising strategies with the potential to establish NK cell immunotherapy as a therapeutic option for patients with cancer. Given the advances summarized in this Review, we foresee an exciting future in the field of NK cell-based cancer immunotherapy and predict that NK cells soon will be an appreciated and natural component in the fight against cancer.

1. Moretta, L., Ciccone, E., Mingari, M. C., Biassoni, R. & Moretta, A. Human natural killer cells: origin, clonality, specificity, and receptors. *Adv. Immunol.* **55**, 341–380 (1994).
2. Gregoire, C. *et al.* The trafficking of natural killer cells. *Immunol. Rev.* **220**, 169–182 (2007).
3. Herberman, R. B., Nunn, M. E., Holden, H. T. & Lavrin, D. H. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer* **16**, 230–239 (1975).
4. Herberman, R. B., Nunn, M. E. & Lavrin, D. H. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer* **16**, 216–229 (1975).
5. Kiessling, R., Klein, E., Pross, H. & Wigzell, H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol.* **5**, 117–121 (1975).
6. Kiessling, R., Klein, E. & Wigzell, H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur. J. Immunol.* **5**, 112–117 (1975).
7. Foley, B. *et al.* Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C$^+$ natural killer cells with potent function. *Blood* **119**, 2665–2674 (2012).
8. Foley, B. *et al.* Human cytomegalovirus (CMV)-induced memory-like NKG2C$^+$ NK cells are transplantable and expand *in vivo* in response to recipient CMV antigen. *J. Immunol.* **189**, 5082–5088 (2012).
9. Bryceson, Y. T. & Long, E. O. Line of attack: NK cell specificity and integration of signals. *Curr. Opin. Immunol.* **20**, 344–352 (2008).
10. Kärre, K. in *Mechanisms of Cytotoxicity by NK Cells* (eds Callewaert, D. & Herberman, R. B.) 81–91 (Academis Press., 1985).
11. Ljunggren, H. G. & Karre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* **11**, 237–244 (1990).
12. Anfossi, N. *et al.* Human NK cell education by inhibitory receptors for MHC class I. *Immunity* **25**, 331–342 (2006).
13. Kim, S. *et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* **436**, 709–713 (2005).
14. Miller, J. S. *et al.* Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood* **105**, 3051–3057 (2005).
This is the first study to show that infusion of short-term IL-2-activated allogeneic haploidentical NK cells to patients with refractory leukaemia can induce remission.
15. Ruggeri, L. *et al.* Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* **295**, 2097–2100 (2002).
This seminal paper describes the role for NK cells in preventing leukaemia relapse following haploidentical haematopoietic stem cell transplantation in patients with AML and patients with acute lymphoblastic leukaemia.
16. Rosenberg, S. A. *et al.* Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N. Engl. J. Med.* **313**, 1485–1492 (1985).
17. West, W. H. *et al.* Constant-infusion recombinant interleukin-2 in adoptive immunotherapy of advanced cancer. *N. Engl. J. Med.* **316**, 898–905 (1987).
18. Curti, A. *et al.* Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. *Blood* **118**, 3273–3279 (2011).
19. Geller, M. A. *et al.* A Phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. *Cytotherapy* **13**, 98–107 (2011).
20. Ito, S. *et al.* Ultra-low dose interleukin-2 promotes immune-modulating function of regulatory T cells and natural killer cells in healthy volunteers. *Mol. Ther.* **22**, 1388–1395 (2014).
21. Levin, A. M. *et al.* Exploiting a natural conformational switch to engineer an interleukin-2 'superkine'. *Nature* **484**, 529–533 (2012).
22. Leclercq, G., Debacker, V., de Smedt, M. & Plum, J. Differential effects of interleukin-15 and interleukin-2 on differentiation of bipotential T/natural killer progenitor cells. *J. Exp. Med.* **184**, 325–336 (1996).
23. Pillet, A. H., Bugault, F., Theze, J., Chakrabarti, L. A. & Rose, T. A programmed switch from IL-15- to IL-2-dependent activation in human NK cells. *J. Immunol.* **182**, 6267–6277 (2009).
24. Pillet, A. H., Theze, J. & Rose, T. Interleukin (IL)-2 and IL-15 have different effects on human natural killer lymphocytes. *Hum. Immunol.* **72**, 1013–1017 (2011).
25. Carson, W. E. *et al.* A potential role for interleukin-15 in the regulation of human natural killer cell survival. *J. Clin. Invest.* **99**, 937–943 (1997).
26. Berger, C. *et al.* Safety and immunologic effects of IL-15 administration in nonhuman primates. *Blood* **114**, 2417–2426 (2009).
27. Conlon, K. C. *et al.* Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J. Clin. Oncol.* **33**, 74–82 (2015).
28. Cooley, S. *et al.* Recombinant human IL-15 promotes *in vivo* expansion of adoptively transferred NK cells in a first-in-human Phase I dose escalation study in patients with AML. *Blood* **615**, 894 (2012).
29. Miller, J. S. Therapeutic applications: natural killer cells in the clinic. *Hematol. Am. Soc. Hematol. Educ. Program* **2013**, 247–253 (2013).
30. Dubois, S., Patel, H. J., Zhang, M., Waldmann, T. A. & Muller, J. R. Preassociation of IL-15 with IL-15R α-IgG1-Fc enhances its activity on proliferation of NK and CD8$^+$/CD44^{high} T cells and its antitumor action. *J. Immunol.* **180**, 2099–2106 (2008).
31. Han, K. P. *et al.* IL-15:IL-15 receptor α superagonist complex: high-level co-expression in recombinant mammalian cells, purification and characterization. *Cytokine* **56**, 804–810 (2011).
32. Wu, Z. & Xu, Y. IL-15R α-IgG1-Fc enhances IL-2 and IL-15 anti-tumor action through NK and CD8$^+$ T cells proliferation and activation. *J. Mol. Cell. Biol.* **2**, 217–222 (2010).
33. Steel, J. C., Waldmann, T. A. & Morris, J. C. Interleukin-15 biology and its therapeutic implications in cancer. *Trends Pharmacol. Sci.* **33**, 35–41 (2012).
34. Pistoia, V., Cocco, C. & Airoldi, I. Interleukin-12 receptor β2: from cytokine receptor to gatekeeper gene in human B-cell malignancies. *J. Clin. Oncol.* **27**, 4809–4816 (2009).
35. Kobayashi, M. *et al.* Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* **170**, 827–845 (1989).
36. Lehmann, D. *et al.* IL-12 directs further maturation of *ex vivo* differentiated NK cells with improved therapeutic potential. *PLoS ONE* **9**, e87131 (2014).
37. Allavena, P. *et al.* Interleukin-12 is chemotactic for natural killer cells and stimulates their interaction with vascular endothelium. *Blood* **84**, 2261–2268 (1994).
38. Gollob, J. A. *et al.* Phase I trial of twice-weekly intravenous interleukin 12 in patients with metastatic renal cell cancer or malignant melanoma: ability to maintain IFN-γ induction is associated with clinical response. *Clin. Cancer Res.* **6**, 1678–1692 (2000).
39. Gollob, J. A. *et al.* Phase I trial of concurrent twice-weekly recombinant human interleukin-12 plus low-dose IL-2 in patients with melanoma or renal cell carcinoma. *J. Clin. Oncol.* **21**, 2564–2573 (2003).
40. Trudeau, C. *et al.* A single administration of recombinant human interleukin-12 is associated with increased expression levels of interferon-γ and signal transducer and activator of transcription in healthy subjects. *J. Clin. Pharmacol.* **45**, 649–658 (2005).
41. Robertson, M. J. *et al.* Interleukin 12 immunotherapy after autologous stem cell transplantation for hematological malignancies. *Clin. Cancer Res.* **8**, 3383–3393 (2002).
42. Basile, L. A., Gallaher, T. K., Shibata, D., Miller, J. D. & Douer, D. Multilineage hematopoietic recovery with concomitant antitumor effects using low dose Interleukin-12 in myelosuppressed tumor-bearing mice. *J. Transl. Med.* **6**, 26 (2008).
43. Gokhale, M. S. *et al.* Single low-dose rHuIL-12 safely triggers multilineage hematopoietic and immune-mediated effects. *Exp. Hematol. Oncol.* **3**, 11 (2014).
44. Zhu, D., Corral, L. G., Fleming, Y. W. & Stein, B. Immunomodulatory drugs Revlimid (lenalidomide) and CC-4047 induce apoptosis of both hematological and solid tumor cells through NK cell activation. *Cancer Immunol. Immunother.* **57**, 1849–1859 (2008).
45. Gorgun, G. *et al.* Immunomodulatory effects of lenalidomide and pomalidomide on interaction of tumor and bone marrow accessory cells in multiple myeloma. *Blood* **116**, 3227–3237 (2010).
46. Davies, F. E. *et al.* Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. *Blood* **98**, 210–216 (2001).
47. Reddy, N. *et al.* Immunomodulatory drugs stimulate natural killer-cell function, alter cytokine production by dendritic cells, and inhibit angiogenesis enhancing the anti-tumour activity of rituximab *in vivo*. *Br. J. Haematol.* **140**, 36–45 (2008).
48. Hayashi, T. *et al.* Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. *Br. J. Haematol.* **128**, 192–203 (2005).
49. Lagrue, K., Chopra, R. & Davis, D. M. The immunomodulatory drug Lenalidomide alters the threshold for NK cell activation and augments NK cell effector functions. *Immunology* **140** (Suppl. 1), 71 (2013).
50. Berg, S. L. *et al.* Safety, pharmacokinetics, and immunomodulatory effects of lenalidomide in children and adolescents with relapsed/refractory solid tumors or myelodysplastic syndrome: a Children's Oncology Group Phase I Consortium report. *J. Clin. Oncol.* **29**, 316–323 (2011).
51. Chanan-Khan, A. A. *et al.* Biological effects and clinical significance of lenalidomide-induced tumour flare reaction in patients with chronic lymphocytic leukaemia: *in vivo* evidence of immune activation and antitumour response. *Br. J. Haematol.* **155**, 457–467 (2011).
52. Eve, H. E. *et al.* Single-agent lenalidomide in relapsed/refractory mantle cell lymphoma: results from a UK phase II study suggest activity and possible gender differences. *Br. J. Haematol.* **159**, 154–163 (2012).
53. Wolschke, C. *et al.* Postallograft lenalidomide induces strong NK cell-mediated antimyeloma activity and risk for T cell-mediated GvHD: results from a Phase I/II dose-finding study. *Exp. Hematol.* **41**, 134–142.e3 (2013).
54. Keir, M. E., Butte, M. J., Freeman, G. J. & Sharpe, A. H. PD-1 and its ligands in tolerance and immunity. *Annu. Rev. Immunol.* **26**, 677–704 (2008).
55. Dong, H. *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* **8**, 793–800 (2002).
56. MacFarlane, A. W. 4th *et al.* PD-1 expression on peripheral blood cells increases with stage in renal cell carcinoma patients and is rapidly reduced after surgical tumor resection. *Cancer Immunol. Res.* **2**, 320–331 (2014).
57. Wiesmayr, S. *et al.* Decreased NKp46 and NKG2D and elevated PD-1 are associated with altered NK-cell function in pediatric transplant patients with PTLD. *Eur. J. Immunol.* **42**, 541–550 (2012).
58. Benson, D. M. Jr *et al.* The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* **116**, 2286–2294 (2010).
This study demonstrates that antibody blockade of the PD1–PDL1 signalling axis can significantly bolster NK cell tumour reactivity.
59. Hamid, O. *et al.* Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N. Engl. J. Med.* **369**, 134–144 (2013).
60. Robert, C. *et al.* Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a Phase 1 trial. *Lancet* **384**, 1109–1117 (2014).
61. Weber, J. S. *et al.* Safety, efficacy, and biomarkers of nivolumab with vaccine in ipilimumab-refractory or -naive melanoma. *J. Clin. Oncol.* **31**, 4311–4318 (2013).
62. Benson, D. M. Jr *et al.* IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. *Blood* **118**, 6387–6391 (2011).
63. Romagne, F. *et al.* Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. *Blood* **114**, 2667–2677 (2009).
64. Sola, C. *et al.* Genetic and antibody-mediated reprogramming of natural killer cell missing-self recognition *in vivo*. *Proc. Natl Acad. Sci. USA* **106**, 12879–12884 (2009).
65. Vahlne, G. *et al. In vivo* tumor cell rejection induced by NK cell inhibitory receptor blockade: maintained tolerance to normal cells even in the presence of IL-2. *Eur. J. Immunol.* **40**, 813–823 (2010).
66. Korde, N. *et al.* A phase II trial of pan-KIR2D blockade with IPH2101 in smoldering multiple myeloma. *Haematologica* **99**, e81–e83 (2014).

67. Maloney, D. G. Anti-CD20 antibody therapy for B-cell lymphomas. *N. Engl. J. Med.* **366**, 2008–2016 (2012).
68. Wu, J. *et al.* A novel polymorphism of FcγRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. *J. Clin. Invest.* **100**, 1059–1070 (1997).
69. Cartron, G. *et al.* Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene. *Blood* **99**, 754–758 (2002).
70. Weng, W. K. & Levy, R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J. Clin. Oncol.* **21**, 3940–3947 (2003).
References 69 and 70 provide the first data on the importance of NK cells in mediating tumour regression following mAb therapy, showing a survival advantage and improved response rates among rituximab-treated CD16-158V homozygous follicular lymphoma patients compared to non-CD16-158V homozygous patients.
71. Pander, J. *et al.* Correlation of *FCGR3A* and *EGFR* germline polymorphisms with the efficacy of cetuximab in *KRAS* wild-type metastatic colorectal cancer. *Eur. J. Cancer* **46**, 1829–1834 (2010).
72. Tarek, N. *et al.* Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. *J. Clin. Invest.* **122**, 3260–3270 (2012).
73. Kim, D. H. *et al. FCGR3A* gene polymorphisms may correlate with response to frontline R-CHOP therapy for diffuse large B-cell lymphoma. *Blood* **108**, 2720–2725 (2006).
74. Gleason, M. K. *et al.* Bispecific and trispecific killer cell engagers directly activate human NK cells through CD16 signaling and induce cytotoxicity and cytokine production. *Mol. Cancer Ther.* **11**, 2674–2684 (2012).
75. Wiernik, A. *et al.* Targeting natural killer cells to acute myeloid leukemia *in vitro* with a CD16 × 33 bispecific killer cell engager and ADAM17 inhibition. *Clin. Cancer Res.* **19**, 3844–3855 (2013).
This study shows that the use of CD16–CD33 BiKEs can overcome self-inhibitory signals and effectively elicit NK cell-mediated killing of CD33$^+$ AML cells, with this effect being boosted by preventing CD16 shedding via inhibition of ADAM17.
76. Thorburn, A. Death receptor-induced cell killing. *Cell. Signal.* **16**, 139–144 (2004).
77. Lundqvist, A., Yokoyama, H., Smith, A., Berg, M. & Childs, R. Bortezomib treatment and regulatory T-cell depletion enhance the antitumor effects of adoptively infused NK cells. *Blood* **113**, 6120–6127 (2009).
This study shows that the proteasome inhibitor bortezomib can be used to sensitize tumour cells *in vivo* to NK cell killing via the TRAIL–DR5 death receptor pathway.
78. Merino, D. *et al.* Differential inhibition of TRAIL-mediated DR5–DISC formation by decoy receptors 1 and 2. *Mol. Cell. Biol.* **26**, 7046–7055 (2006).
79. Vales-Gomez, M., Chisholm, S. E., Cassady-Cain, R. L., Roda-Navarro, P. & Reyburn, H. T. Selective induction of expression of a ligand for the NKG2D receptor by proteasome inhibitors. *Cancer Res.* **68**, 1546–1554 (2008).
80. Wennerberg, E. *et al.* Doxorubicin sensitizes human tumor cells to NK cell- and T-cell-mediated killing by augmented TRAIL receptor signaling. *Int. J. Cancer* **133**, 1643–1652 (2013).
81. Diermayr, S. *et al.* NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. *Blood* **111**, 1428–1436 (2008).
82. Armeanu, S. *et al.* Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. *Cancer Res.* **65**, 6321–6329 (2005).
83. Skov, S. *et al.* Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3-dependent expression of MHC class I-related chain A and B. *Cancer Res.* **65**, 11136–11145 (2005).
84. Lundqvist, A. *et al.* Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. *Cancer Res.* **66**, 7317–7325 (2006).
85. Bandura, L., Drukala, J., Wolnicka-Glubisz, A., Bjornstedt, M. & Korohoda, W. Differential effects of selenite and selenate on human melanocytes, keratinocytes, and melanoma cells. *Biochem. Cell Biol.* **83**, 196–211 (2005).
86. Husbeck, B., Nonn, L., Peehl, D. M. & Knox, S. J. Tumor-selective killing by selenite in patient-matched pairs of normal and malignant prostate cells. *Prostate* **66**, 218–225 (2006).
87. Kandas, N. O., Randolph, C. & Bosland, M. C. Differential effects of selenium on benign and malignant prostate epithelial cells: stimulation of LNCaP cell growth by noncytotoxic, low selenite concentrations. *Nutr. Cancer* **61**, 251–264 (2009).
88. Enqvist, M. *et al.* Selenite induces posttranscriptional blockade of HLA-E expression and sensitizes tumor cells to CD94/NKG2A-positive NK cells. *J. Immunol.* **187**, 3546–3554 (2011).
89. Shi, J. *et al.* Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. *Br. J. Haematol.* **143**, 641–653 (2008).
90. Childs, R. W. & Berg, M. Bringing natural killer cells to the clinic: *ex vivo* manipulation. *Hematol. Am. Soc. Hematol. Educ. Program* **2013**, 234–246 (2013).
91. Fujisaki, H., Kakuda, H., Imai, C., Mullighan, C. G. & Campana, D. Replicative potential of human natural killer cells. *Br. J. Haematol.* **145**, 606–613 (2009).
92. Garg, T. K. *et al.* Highly activated and expanded natural killer cells for multiple myeloma immunotherapy. *Haematologica* **97**, 1348–1356 (2012).
93. Imai, C., Iwamoto, S. & Campana, D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* **106**, 376–383 (2005).
94. Lapteva, N. *et al.* Large-scale *ex vivo* expansion and characterization of natural killer cells for clinical applications. *Cytotherapy* **14**, 1131–1143 (2012).
95. Liu, Y. *et al.* Growth and activation of natural killer cells *ex vivo* from children with neuroblastoma for adoptive cell therapy. *Clin. Cancer Res.* **19**, 2132–2143 (2013).
96. Zhang, H. *et al.* Activating signals dominate inhibitory signals in CD137L/IL-15 activated natural killer cells. *J. Immunother.* **34**, 187–195 (2011).
97. Denman, C. J. *et al.* Membrane-bound IL-21 promotes sustained *ex vivo* proliferation of human natural killer cells. *PLoS ONE* **7**, e30264 (2012).
98. Berg, M. *et al.* Clinical-grade *ex vivo*-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. *Cytotherapy* **11**, 341–355 (2009).
99. Miller, J. S. *et al.* Expansion and homing of adoptively transferred human natural killer cells in immunodeficient mice varies with product preparation and *in vivo* cytokine administration: implications for clinical therapy. *Biol. Blood Marrow Transplant* **20**, 1252–1257 (2014).
100. Frei, G. *et al.* Nicotinamide, a form of vitamin B3, promotes expansion of natural killer cells that display increased *in vivo* survival and cytotoxic activity. *Blood* **703**, 4035 (2011).
101. Frei, G. *et al.* Improved homing to bone marrow, spleen and lung of adoptively infused NK cells expanded *ex vivo* with the small molecule nicotinamide using feeder-free conditions. *Blood* **122**, 897 (2013).
102. Li, L. *et al.* Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. *Cancer Gene Ther.* **17**, 147–154 (2010).
103. Shimasaki, N. *et al.* A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. *Cytotherapy* **14**, 830–840 (2012).
104. Carlsten, M. *et al.* Clinical-grade mRNA electroporation of NK cells: a novel and highly efficient method to genetically reprogram human NK cells for cancer immunotherapy. *Blood* **801**, 2153 (2014).
105. Chu, J. *et al.* CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance *in vitro* and *in vivo* antitumor activity against human multiple myeloma. *Leukemia* **28**, 917–927 (2014).
106. Xia, L., McDaniel, J. M., Yago, T., Doeden, A. & McEver, R. P. Surface fucosylation of human cord blood cells augments binding to P-selectin and E-selectin and enhances engraftment in bone marrow. *Blood* **104**, 3091–3096 (2004).
107. Onishi, T. *et al.* Forced fucosylation with ASC-101 enhances the binding of *ex vivo* expanded human NK cells to E-selectin: a novel method to improve the homing of adoptively transferred NK cells to the bone marrow in patients with hematological malignancies. *Blood* **122**, 4499 (2013).
108. Somanchi, S. S., Somanchi, A., Cooper, L. J. & Lee, D. A. Engineering lymph node homing of *ex vivo*-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. *Blood* **119**, 5164–5172 (2012).
This is one of the first studies to describe how phenotypic modification of NK cells can be used to redirect their homing to target tissues *in vivo* following adoptive infusion.
109. Loza, M. J. & Perussia, B. The IL-12 signature: NK cell terminal CD56^{+high} stage and effector functions. *J. Immunol.* **172**, 88–96 (2004).
110. Romee, R. *et al.* NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood* **121**, 3599–3608 (2013).
111. Boutet, P. *et al.* Cutting edge: the metalloproteinase ADAM17/TNF-α-converting enzyme regulates proteolytic shedding of the MHC class I-related chain B protein. *J. Immunol.* **182**, 49–53 (2009).
112. Bachanova, V. *et al.* Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. *Blood* **123**, 3855–3863 (2014).
This study provides evidence that the depletion of T_{Reg} cells can be used to improve NK cell *in vivo* expansion and is associated with higher frequencies of complete remission in patients with AML.
113. Lopez-Verges, S. *et al.* Expansion of a unique CD57$^+$NKG2C^{hi} natural killer cell subset during acute human cytomegalovirus infection. *Proc. Natl Acad. Sci. USA* **108**, 14725–14732 (2011).
114. Elmaagacli, A. H. *et al.* Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood* **118**, 1402–1412 (2011).
115. Green, M. L. *et al.* CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood* **122**, 1316–1324 (2013).

Acknowledgements
The authors acknowledge all the members of the US National Heart, Lung and Blood Institute's Laboratory of Transplantation Immunotherapy and the Dean R. O'Neill Memorial Fellowship for their many contributions and support for the original research described in this Review.

Competing interests statement
The authors declare no competing interests.

FURTHER INFORMATION
ClinicalTrials.gov: https://clinicaltrials.gov

ALL LINKS ARE ACTIVE IN THE ONLINE PDF