Please cite this article in press as: Rezvani et al., Engineering Natural Killer Cells for Cancer Immunotherapy, Molecular Therapy (2017), http://dx.doi.org/10.1016/j.ymthe.2017.06.012

Molecular Therapy
Review

Engineering Natural Killer Cells for Cancer Immunotherapy

Katayoun Rezvani,[1] Rayne Rouce,[2] Enli Liu,[1] and Elizabeth Shpall[1]

[1]Department of Stem Cell Transplantation and Cellular Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA; [2]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston Methodist Hospital and Texas Children's Hospital, Houston, TX 77030, USA

The past several years have seen tremendous advances in the engineering of immune effector cells as therapy for cancer. While chimeric antigen receptors (CARs) have been used extensively to redirect the specificity of autologous T cells against hematological malignancies with striking clinical results, studies of CAR-modified natural killer (NK) cells have been largely preclinical. In this review, we focus on recent advances in NK cell engineering, particularly on preclinical evidence suggesting that NK cells may be as effective as T cells in recognizing and killing targets after genetic modification. We will discuss strategies to introduce CARs into both primary NK cells and NK cell lines in an effort to provide antigen specificity, the challenges of manufacturing engineered NK cells, and evidence supporting the effectiveness of this approach from preclinical and early-phase clinical studies using CAR-engineered NK cells. CAR-NK cells hold great promise as a novel cellular immunotherapy against refractory malignancies. Notably, NK cells can provide an "off-the-shelf" product, eliminating the need for a personalized and patient-specific product that plagues current CAR-T cell therapies. The ability to more potently direct NK cell-mediated cytotoxicity against refractory tumors through the expression of CAR is likely to contribute to the recent paradigm shift in cancer treatment.

The past several years have seen tremendous advances in the engineering of immune effector cells as therapy for cancer. Chimeric antigen receptors (CARs) have been used extensively to redirect the specificity of autologous T cells against lymphoid leukemia and lymphoma with striking clinical results. The most success has been reported in acute lymphoblastic leukemia (ALL), with several groups reporting complete remission (CR) rates as high as 90% after administration of a single dose of CD19-CAR-T cells following lymphodepleting chemotherapy, although the responses have been short lived in some cases. The favorable responses in early-phase trials of CD19-CAR-T cells (even in heavily pretreated children and adults)[1–3] have resulted in ongoing commercialization efforts in an attempt to make this therapy more widely available.[4,5]

However, CAR-modified T cells have a number of practical limitations. The generation of an autologous CAR-T cell product for each individual patient is logistically cumbersome and too restrictive for general clinical use. The manufacturing of CAR-T cells often takes a number of weeks, making it impractical for the treatment of a patient with rapidly advancing disease. Furthermore, it is not always possible to collect enough lymphocytes from heavily pretreated (and often lymphopenic) patients to allow for the successful generation of clinically relevant doses of CAR-T cells. An allogeneic "off-the-shelf" product could overcome these logistic challenges; however, allogeneic T cells (even if human leukocyte antigen [HLA] matched) carry a significant risk of graft-versus-host disease (GVHD) mediated through their native α-β T cell receptor (TCR).

Natural killer (NK) cells are highly cytotoxic immune effectors, killing their targets in a non-specific manner.[6,7] NK cells lack the potential to cause GVHD[8–11] and thus open opportunities to produce an off-the-shelf allogeneic product that could be readily available for immediate clinical use. Moreover, as engineered NK cells should also retain their full array of native receptors, they have the potential to exert anti-cancer activity through mechanisms other than that dictated by the specificity of the CAR, which in principle could reduce the risk of relapse or resistance mediated by loss of CAR-targeted antigen, as reported for CAR-T cell therapy.[3,12] Thus, the inherent qualities of NK cells make them promising candidates for immunotherapy. Augmenting NK cell antitumor responses by introducing antigen specificity through genetic modification is a subject of intense investigation in the field of cancer immuno-oncology.

In this review, we focus on recent advances in NK cell engineering, particularly on preclinical evidence suggesting that NK cells may be as equally effective as T cells in recognizing and killing targets after genetic modification. We will discuss strategies to introduce CARs into both primary NK cells and NK cell lines in an effort to provide antigen specificity, the challenges of manufacturing engineered NK cells, and evidence supporting the effectiveness of this approach from preclinical and early-phase clinical studies using CAR-engineered NK cells.

NK Biology and Adoptive Immunotherapy

NK cells are innate immune effectors with the ability to exert rapid cytotoxicity against cancer and virus-infected cells without prior

http://dx.doi.org/10.1016/j.ymthe.2017.06.012.

Correspondence: Katayoun Rezvani, Department of Stem Cell Transplantation and Cellular Therapy, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA.
E-mail: krezvani@mdanderson.org

Please cite this article in press as: Rezvani et al., Engineering Natural Killer Cells for Cancer Immunotherapy, Molecular Therapy (2017), http://dx.doi.org/10.1016/j.ymthe.2017.06.012

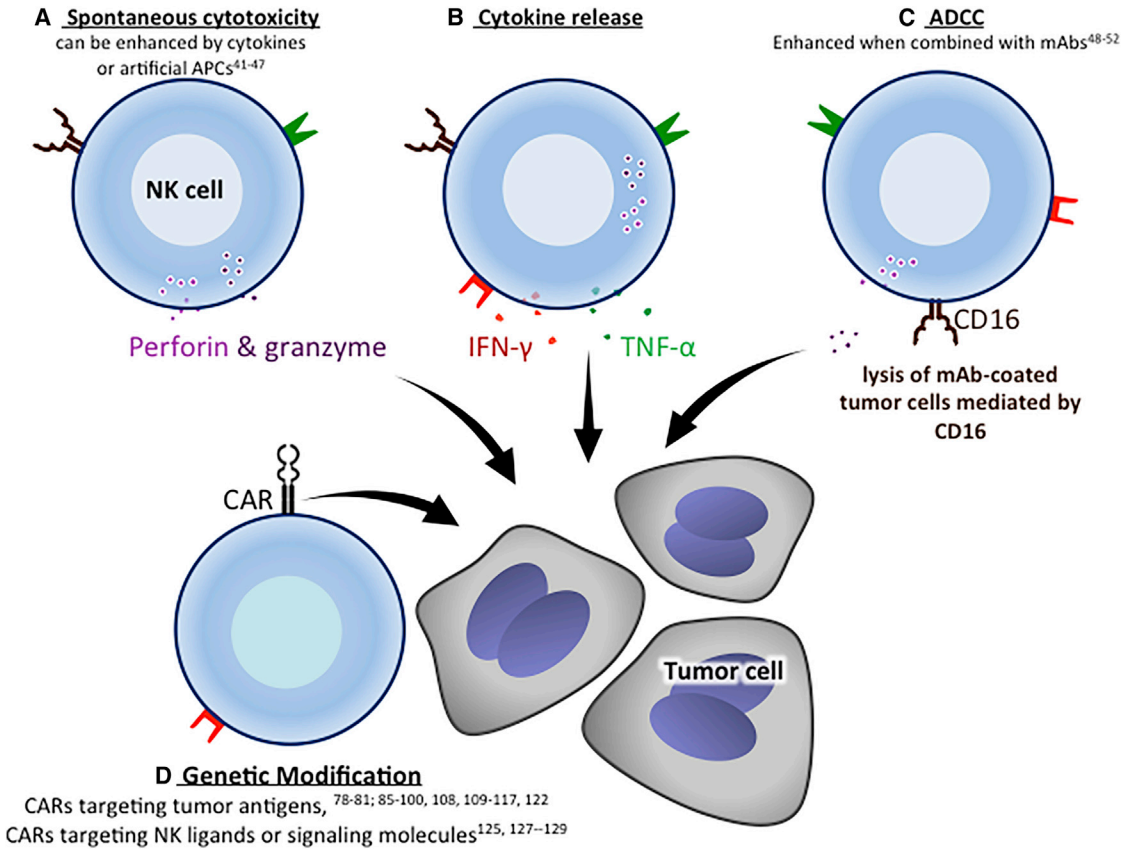

Figure 1. Mechanisms of NK-Mediated Cytotoxicity

(A) Spontaneous cytotoxicity. (B) Cytokine release. (C) ADCC. (D) Genetic modification. ADCC, antibody-dependent cell-mediated cytotoxicity; mAb, monoclonal antibody.

sensitization, hence the designation "natural" killers. NK-mediated cytotoxicity occurs in a HLA-unrestricted fashion, a desirable quality for cancer immunotherapy, although NK cells acquire cytotoxic function after encountering and recognizing self-HLA molecules during a process termed "licensing" or NK cell education.[13–15] NK cells are characterized by a lack of CD3/TCR molecules and expression of CD16 and CD56 surface antigens. While the majority of NK cells are in the blood, liver, spleen, and bone marrow, they are also found to a lesser extent in lymph nodes.[15–18]

NK cell functions, including degranulation, cytokine release, and cytotoxicity, are governed by a balance between signals received from inhibitory receptors (notably, the killer Ig-like receptors [KIRs] and the heterodimeric C-type lectin receptor [NKG2A]) and activating receptors (in particular, the natural cytotoxicity receptors [NCRs] NKp46, NKp30, NKp44, and the C-type lectin-like activating immunoreceptor NKG2D[7]) that recognize ligands on their cellular targets. These receptors therefore require mechanisms to prevent unintentional activation against normal tissues, referred to as "tolerance to self." Engagement of inhibitory KIRs (iKIRs) by HLA class I molecules leads to transmission of an inhibitory signal that blocks NK cell triggering during effector responses. Through a concept known as the "missing self" hypothesis, when mature NK cells encounter transformed cells lacking HLA class I, which occurs upon viral or malignant transformation, the inhibitory receptors on the surface of the NK cell are not engaged. In this context, the NK cell does not receive inhibitory signals.[19,20] In parallel, cellular stress and DNA damage, both of which occur in cells during viral or malignant transformation, result in upregulation of "stress ligands" that can be recognized by activating NK receptors, resulting in a positive signal for NK cells to kill the target.[20–22] NK cells can directly kill tumor cells through release of cytoplasmic granules containing perforin and granzyme,[22–24] and expression of tumor necrosis factor (TNF) family members such as FasL or TNF-related apoptosis-inducing ligand (TRAIL), which induce tumor cell apoptosis by interacting with their respective receptors.[22–25] Additionally, antibody-dependent cellular cytotoxicity (ADCC), mediated by the Fc receptor CD16, can trigger NK cell degranulation against antibody-coated target cells.[7] $CD56^{dim}$ NK cells express high levels of CD16, while $CD56^{bright}$ NK cells are CD16 dim or negative[16] (Figure 1).

Adoptive Transfer of NK Cells to Target Tumors

Their rapid killing and broad cytotoxicity make NK cells uniquely primed for use in adoptive therapy. In vitro, NK cells exert

Please cite this article in press as: Rezvani et al., Engineering Natural Killer Cells for Cancer Immunotherapy, Molecular Therapy (2017), http://dx.doi.org/10.1016/j.ymthe.2017.06.012

cytotoxicity against a number of hematologic malignancies, including acute myeloid leukemia (AML),[26–28] ALL,[29–33] multiple myeloma (MM),[34–37] as well as many solid tumors including neuroblastoma, ovarian, colon, renal cell, and gastric carcinomas.[33,38–42] However, many tumors develop evasion strategies to circumvent killing by autologous NK cells.[27,31] For example, some leukemia and lymphoma cells maintain high surface expression of HLA molecules, making them invisible to NK attack,[31,32] or may lack ligands that signal through activating NK cell receptors.[31] Thus, many groups have explored strategies to enhance the activity of NK cells, including the use of cytokines and artificial antigen-presenting cells (APCs) with enhanced costimulatory molecules as feeder cells[43–46] for their in vivo expansion. Following incubation with cytokines, particularly interleukin (IL)-2 or IL-15, NK cells acquire the capacity to lyse a broad array of fresh and cultured tumor targets not normally sensitive to NK lysis.[43–48] Another strategy is combination therapy with monoclonal antibodies to boost ADCC.[49–53]

Functional NK cells for immunotherapy can be derived from several different sources, as demonstrated by a number of groups.[6–11,54–61] Although adoptive transfer of expanded, activated autologous NK cells has been safely tolerated in clinical trials, efficacy has been limited.[6,41,47] This is likely related to the inhibition of autologous NK cells by self-HLA molecules. NK cells from an allogeneic source provide a promising alternative for immunotherapy. One of the most promising features of alloreactive NK cells is their ability to contribute to graft-versus-leukemia/tumor (GvL/GvT) effect without causing GVHD, as evidenced by preclinical[30,36,62,63] and clinical studies using adoptively transferred haploidentical NK cells in the pre- and post-transplant settings.[6,9–11,64,65] Adoptively transferred allogeneic NK cells are shown to be safe in patients with hematologic and solid tumors, although their clinical activity has been modest at best.[9–11,43,54] A number of groups, including the authors, are now exploring different strategies to genetically reprogram NK cells to further improve their capacity to kill tumors.

Advantages and Challenges of CAR-NK Cell Therapy

CARs were developed to equip immune effector cells with the ability to recognize antigens on the surface of tumors and kill their targets in an HLA-unrestricted fashion.[66,67] CARs are composed of an extracellular antigen-binding domain, typically the single-chain variable fragment (scFv) derived from a monoclonal antibody, and an intracellular signaling domain, usually the CD3ζ chain of the TCR.[68] While CARs have been used extensively to redirect the specificity of T cells for clinical use, studies of CAR-modified NK cells have been largely preclinical to date. However, the dramatic clinical responses and prolonged remissions observed after the infusion of CD19 CAR-T cells in patients with lymphoid malignancies, particularly ALL,[1–5] have rekindled the enthusiasm to further study and optimize CAR-modified NK cells for clinical use.

There are several advantages to using CAR-NK cells compared to T cells. For example, adoptively transferred autologous and allogeneic NK cells have limited in vivo persistence. The lack of clonal expansion of NK cells and immune-mediated rejection of allogeneic NK cells within days to weeks makes life-threatening toxicities such as cytokine release syndrome (CRS) less likely. Furthermore, although a number of patients have received allogeneic CAR-T cells without an increased incidence of GVHD,[69–73] the majority of CAR-T cell studies to date have used autologous T cells. This is because allogeneic T cells (even if HLA matched) may induce GVHD mediated through their native α-β TCR. NK cells, on the other hand, do not cause GVHD, as evidence by preclinical[62,63,74] and clinical studies of haploidentical and cord blood (CB) NK cell infusions in numerous patients with both hematologic and solid malignancies.[6,9–11,34,47] Thus, NK cells offer opportunities to produce an off-the-shelf allogeneic product that could be readily available for immediate clinical use. Additionally, unlike CAR-T cells, CAR-NK cells will retain their intrinsic capacity to recognize and target tumor cells through their native receptors, making disease escape through downregulation of the CAR target antigen less likely than it is with CAR-T cells.[3,12] One could potentially exploit this property by selecting donors either for NK-CAR production based on KIR-ligand mismatch with the recipient or based on haplotype B KIR gene content, as both have been shown to be beneficial in the setting of allogeneic stem cell transplantation.[27,75–78]

Initial reluctance to utilize NK cells for CAR-modified therapy was largely based on the uncertainty regarding their ability to migrate to and penetrate tumor tissues, and their presumed overall inferiority to CAR-T cells.[79] Part of this reluctance is related to the limited in vivo persistence of NK cells, which, while desirable from a safety standpoint, may be limiting from an efficacy standpoint. Additionally, until relatively recently, the genetic manipulation of NK cells has proven challenging.[80,81] Viral transduction, successful for T cells, has often been associated with low or variable levels of transgene expression and unfavorable effects on cell viability in NK cells.[80,81] However, recent optimization in viral transduction and electroporation technologies[47,80,81] has revived the enthusiasm for studies to evaluate the genetic modification of NK cells. Perhaps the biggest challenge to the development of suitable CAR therapies, regardless of the source of effector cells, is the identification of appropriate target antigens. An ideal target antigen is one that is ubiquitously expressed by tumor cells, with little to no expression on normal tissue, thus limiting on-target off-tumor effects.

Primary human NK cells derived from peripheral blood (PB) or CB, or hematopoietic stem cell progenitors (HSCPs), as well as NK cell lines have been successfully engineered to express CARs against a number of targets,[82] many of which have also been targeted using CAR-T cells. CAR-transduced NK cells mediate efficient in vitro and in vivo killing of tumor targets,[80,81] although no clinical data on the use of CAR-NK cell therapy have been reported to date.

Studies with CAR-Modified Primary NK Cells

Functional NK cells for adoptive therapy can be derived from several different sources.[80] While autologous NK cells can be reproducibly generated in vitro, they have limited activity against the patient's

Please cite this article in press as: Rezvani et al., Engineering Natural Killer Cells for Cancer Immunotherapy, Molecular Therapy (2017), http://dx.doi.org/10.1016/j.ymthe.2017.06.012

own tumor cells,[27,31,83,84] which may not be easily overcome by engineering the cells to express a CAR.[82] Allogeneic NK cells can be generated from the PB of healthy donors or expanded from CB.

Irrespective of the source, there are several features of expanded, activated CB, or PB-derived NK cells that make them useful effectors for gene modification. Expanded, activated NK cells generally express a vast range of activating receptors, including CD16, NKG2D, and the NCRs (NKp44 and NKp46), despite donor-to-donor variability. These activated NK cells are equipped with KIRs and are "licensed to kill."[47,58,80] Their in vivo expansion and persistence capacity, although inferior to transferred T cells, is clearly associated with antitumor activity in trials involving hematologic malignancies such as AML.[10,11,80] Additionally, ex vivo expanded primary human NK cells produce a different spectrum of cytokines than T cells, including interferon (IFN)-γ, IL-3, and granulocyte macrophage colony-stimulating factor (GM-CSF),[85,86] which may be associated with a lower risk of CRS. As mentioned above, the overwhelming majority of experience with CAR-NK cells remains in the preclinical arena, with CAR-modified primary human NK cells redirected against a number of hematologic and solid tumor antigens, including CD19, CD20, GD2, and HER-2.[87–102]

Many of the initial preclinical studies of CAR-NK cells focused on targeting B cell malignancies with anti-CD19 and CD20-CARs.[88–90] Although infusion of CD19-CAR-T cells following lymphodepletion has resulted in dramatic responses in patients with relapsed or refractory $CD19^+$ malignancies,[1–5] the results for patients with refractory Burkitt lymphoma (BL) have been underwhelming. Chu et al.[91] therefore set out to target CD20 on BL with a second-generation CD20-targeted NK-CAR molecule, generated using mRNA nucleofection. This anti-CD20-4-1BB-CD3ζ CAR was used to genetically modify PB NK cells from healthy donors that were activated in the presence of a K-562-based feeder cell line expressing membrane-bound IL-15 and 4-1BB ligand (K562-mbIL15-41BBL). A mere 16 hr following CAR mRNA nucleofection, 50%–95% of expanded PB NK cells expressed the CAR molecules, and exhibited markedly enhanced in vitro cytolytic activity against rituximab-sensitive and resistant BL cells compared to rituximab alone, while extending survival of Raji-xenografted mice.[91] While non-viral expression techniques such as nucleofection or electroporation can produce robust CAR-mediated killing, the short-lived nature of these CAR molecules would likely dictate the need for repeated infusions in the clinical setting.

Our group has recently developed a novel approach to the generation of CAR-$CD19^+$ NK cells that we believe addresses all of the limitations described above. We genetically modified CB-derived NK cells with a retroviral vector (iC9/CAR.19/IL15)[82] that (1) incorporates the gene for CAR CD19 to redirect specificity to CD19; (2) ectopically produces IL-15, a cytokine crucial for NK cell survival and proliferation[103]; and (3) expresses a suicide gene, inducible caspase-9 (iC9), that can be pharmacologically activated to eliminate transduced cells as needed.[104] These genetic modifications enabled the engineered NK cells to persist in sufficient numbers with impressive functional competence to effectively kill B cell leukemia or lymphoma cells in a xenograft mouse model.[82]

Nonetheless, data from primary NK cell reports must take into account the dramatic variability in inherent NK function among donors, different transduction methods used to modify the cells (both viral and non-viral), and the differing expansion strategies. While the majority of preclinical reports in primary NK cells utilize retrovirus- or lentivirus-based vectors, a wide range of transduction efficiencies have been reported, ranging from 1% to upward of 90%.[88,89,105] Lentiviral transduction possesses some unique benefits compared to retrovirus. For one, it does not require actively dividing cells, thus making transduction of primary, non-activated cells a possibility (although there is rarely a circumstance where a non-expanded primary NK cell would be utilized). While lenti- and retroviral vectors are designed to ensure persistent transgene expression by integrating into the host genome, alternative non-viral transduction methods are also being explored. These methods include electroporation, which introduces CAR-encoding mRNA through pores in the cell membrane, resulting in immediate expression of the CAR molecule. However, considering mRNA electroporation or a single lentiviral transduction results in markedly lower efficiencies in PB or umbilical CB-derived NK cells (<10% and <30%, respectively, in one report), retroviral transduction may be more suitable when modifying primary or CB NK cells. One way around this issue is the possibility of expressing the CAR in induced pluripotent stem cells (iPSCs) and their subsequent differentiation into mature NK cells,[56,106] as discussed in the section on alternative sources of NK cells below. Table 1 summarizes the results of preclinical studies using primary CAR-NK cells published to date.

Studies with CAR-Modified NK Cell Lines

Despite the encouraging data supporting the use of expanded, activated primary CAR-NK cells, the majority of published reports have utilized NK cell lines to express CAR molecules. Although several NK cell lines exist (NKG, YT, NK-YS, HANK-1, YTS cells, and NKL cells),[80,107] the most widely studied by far is NK-92, a human NK-like cell line originally established from a patient with non-Hodgkin's Lymphoma (NHL).[60,61] NK-92 cells lack almost all inhibitory KIRs except KIR2DL4.[60,61] The lack of KIRs on NK-92 cells may, at least in part, account for their marked in vitro activity against a broad spectrum of tumor targets.[8] Promising in vitro results have led to early-phase administration of NK-92 cells to over 40 human subjects with advanced cancers.[61,108] However, despite the safety of repeated infusions of NK-92 cells, efficacy remains limited; therefore, a number of groups are exploring the use of CAR modification to enhance the antitumor activity of these cells.[82,88–102,109]

There are a number of theoretical advantages to genetically modifying NK-92 cell lines over primary NK cells. NK-92 is an established and well-characterized homogeneous cell line that has been successfully reproduced and expanded using good manufacturing practice (GMP)-compliant cryopreserved master cell banks.[110] It is a continuously expanding line and "limitless" in number, allowing the

Please cite this article in press as: Rezvani et al., Engineering Natural Killer Cells for Cancer Immunotherapy, Molecular Therapy (2017), http://dx.doi.org/10.1016/j.ymthe.2017.06.012

Table 1. Preclinical Studies of Primary NK CARs

Disease (Antigen Targeted)	Source	scFv Clone	Construct; Signaling Domain	Expansion Method	Costimulatory Domain	Genetic Modification Method	In Vitro Cytotoxicity	In Vivo Cytotoxicity	Reference
B cell malignancies (CD19)	human PBMCs	FMC63	CD19 CD3ζ CD19-DAP10ζ CD19-4-1BBζ CD19 truncated	K562.41BB.IL15	4-1BB	lentiviral transduction	Y	N	[88]
B cell malignancies (CD19)	human PBMCs (expanded NK, (unstimulated NK)	MSCV	CD19-4-1BBζ	K562.41BB.IL15	4-1BB	mRNA transfection	Y	N	[89]
B cell malignancies (CD19)	human PBMCs (expanded NK, fresh NK)	FMC63	CD19-4-1BBζ	K562.41BB.IL15	4-1BB	mRNA electroporation; retroviral transduction	Y	Y	[90]
B cell malignancies (CD20)	human PBMCs	NR	CD20-4-1BBζ	K562.41BB.IL15	4-1BB	mRNA nucleofection	Y	Y	[91]
B cell malignancies (CD19); NBL (GD2)	human PBMCs (healthy and patient derived)	14G2A	CD19 CD3ζ CD19-2B4 CD19-2B4ζ 14.G2a-2B4ζ	K562.41BB.IL15	2B4	retroviral transduction	Y	N	[87]
B cell malignancies (CD19)	UCB	FMC63	iC9/CD19.CD3ζ/ IL-15	K562.41BB.IL15	none	retroviral transduction	Y	Y	[82]
B cell malignancies (CD19)	human PBMCs	FMC63	CD19.CD28.4-1BBζ	IL-2	CD284-1BB	retroviral (α, γ) and lentiviral transduction	Y	N	[105]
Osteosarcoma; prostate, colon, HCC, and breast cancer (NKG2D)	human PBMCs	NR	NKG2D-DAP10-CD3ζ	K562.41BB.IL15	DAP10	retroviral transduction	Y	Y	[126]
Ovarian and breast cancer (HER-2)	human PBMCs	C6.5	HER-2	B-LCL, CD80/86 costimulated	CD28ζ	retroviral transduction	Y	Y	[92]
Breast cancer metastasis (EGFR)	human PBMCs	528	EGFR.CD28.CD3ζ + oHSV	IL-2	CD28ζ	lentiviral transduction	Y	Y	[114]
Ewing sarcoma (GD2)	human PBMCs	14G2A	14.G2A-2B4ζ 14.G2A-4-1BB 14.G2A-2B4ζ.4-1BB	NR	2B44-1BB	retroviral transduction	Y	N	[42]

EGFR, epidermal growth factor receptor; HCC, hepatocellular carcinoma; N, no; NBL, neuroblastoma; NR, not reported; oHSV, oncolytic herpes simplex virus; PBMC, peripheral blood mononuclear cell; scFV, single-chain fragment variable; UCB, umbilical cord blood; Y, yes.

Please cite this article in press as: Rezvani et al., Engineering Natural Killer Cells for Cancer Immunotherapy, Molecular Therapy (2017), http://dx.doi.org/10.1016/j.ymthe.2017.06.012

generation of sufficient numbers of NK cells for CAR therapy.[95] In recent years, several groups have engineered NK-92 cells to express various CARs targeting both hematologic and solid malignancies, including CD19 and CD20 for B cell leukemia/lymphoma,[93,94,96] CD38 and CS-1 for multiple myeloma,[97,109] and HER-2 for epithelial cancers.[100,101] CAR-modified NK-92 cells can also be administered via intratumoral injection, allowing them to traffic to tumor sites and exert their effect via a vaccine-like mechanism.[111] Moreover, while transduction efficiencies vary widely with primary NK cells, there is more consistent CAR expression when transducing NK-92 cells, largely owing to the uniformity of the cell line. The transduction efficiencies of NK-92 cells average around 50%, even when using non-viral methods such as electroporation or nucleofection.[94,95,112]

While a number of studies have established the feasibility of large-scale expansion and safety of administering NK-92 cells as allogeneic cellular immunotherapy,[93–102,109,113–118] NK-92 cells have inherent drawbacks that must be taken into account. The most clinically significant drawbacks include their potential tumorigenicity (since NK-92 cells are derived from a patient with NHL), multiple cytogenetic abnormalities, and latent infection with Epstein-Barr virus (EBV).[60,119] Thus, for safety purposes, NK-92 cells are irradiated prior to clinical use.[119] While most groups agree that 1,000 cGy of radiation is sufficient to prevent engraftment of NK-92 cells with minimal effect on their in vitro cytotoxicity, irradiation prior to infusion is likely to negatively impact their in vivo proliferation, persistence, and long-term antitumor efficacy.[100] The efficacy of CAR-T cells is accepted to be dependent on persistence,[4,67] and similarly, the in vivo efficacy of adoptively infused NK cells has been shown to depend on their in vivo expansion and persistence.[45] Moreover, we have shown the importance of in vivo persistence of CAR-expressing NK cells for effective and durable antitumor immunity in an in vivo mouse model of lymphoma.[82] While repeated infusions of irradiated NK-92 cells are feasible and may be used as a means to circumvent their limited persistence, it is likely that such an approach will result in induction of antibodies and cellular immunity against the allogeneic cell line and more rapid rejection with each infusion. Additionally, NK-92 cells lack expression of several typical activating receptors (e.g., NKp44 and NKp46[110,120]), and they do not express endogenous Fc receptors and thus are not capable of mediating ADCC. To counteract the latter shortcoming, an NK-92 cell derivative expressing the high-affinity variant of FcγRIII has been developed.[121,122] This high-affinity variant can also prove useful in primary NK cells, as only 10% of the population naturally carry this polymorphism. Table 2 summarizes the results of preclinical studies with CAR-modified NK cell lines.

Alternative Sources of NK Cells

Another source of NK cells suitable for CAR expression are NK cells derived from human pluripotent stem cells (HPSCs). Both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) can be maintained indefinitely to provide an almost limitless supply of NK cells.[56,57] Lowe et al.[123] recently described a protocol for the differentiation of NK cells from $CD34^+$ human HPSCs isolated from cryopreserved CB, which were then modified to express CD19-CAR molecules. The same group also described a feeder-free protocol for the generation of gene-modified NK cells from HPSCs using insulin-like growth factor 1, which could potentially be used as a platform to express other CAR molecules.[123]

CARs Targeting Activating Receptors or Other NK Cell Signaling Molecules

All CAR-NK constructs discussed thus far incorporate the intracellular signaling chain CD3ζ, conferring specific cytotoxicity to surface-expressed tumor-associated antigens.[67] Another attractive strategy is the development of CAR-NK cells that target ligands for activating NK receptors such as NKG2D. NKG2D ligands, including major histocompatibility complex (MHC) class I chain-related A (MICA), MICB, and several UL-16-binding proteins (ULBPs), are upregulated on the surface of many tumor cells and virally infected cells.[124,125] Thus, an NKG2D CAR has the potential to recognize approximately 90% of human tumor types.[126] An additional benefit of such an approach is that an NKG2D CAR not only recognizes tumor cells but also the NKG2D ligands expressed on immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs).[126,127] However, NKG2D ligands are also induced under a variety of physiological circumstances such as inflammation, which raises concerns about "on-target/off-tumor" toxicity. Even though NK cells normally express NKG2D, expression of an NKG2D CAR has been shown to greatly enhance their activity beyond the endogenous NKG2D receptor alone.[126] NKG2D lacks a signaling motif in its cytoplasmic domain and upon its ligation, signal transduction occurs via the phosphorylation of DNAX-activating protein 10 (DAP10), which in turn recruits downstream signaling effector molecules and ultimately results in cytotoxicity.[128,129] To test whether supraphysiologic activating signals can enhance NK-mediated cytotoxicity, Chang et al.[126] co-expressed DAP10 with the NKG2D/CD3ζ CAR and tested the activity of NK cells transduced with this CAR against multiple cell lines derived from a number of different malignancies, with the best responses demonstrated against ALL, osteosarcoma, prostate carcinoma, and rhabdomyosarcoma. One drawback to this strategy is the downregulation or loss of activating ligands on the surface of some primary hematologic malignancies,[31] which could affect NKG2D-mediated cytotoxicity. Interestingly, the authors found no significant correlation between the overall level of NKG2D ligand expression and NKG2D-DAP10-CD3ζ receptor-mediated cytotoxicity.

In an effort to establish a CAR that provides an alternative route to activate NK cells, Topfer et al.[130] incorporated DNAX-activation protein 12 (DAP12) as an intracellular signaling domain, as well as the prostate stem cell antigen (PSCA) scFv (derived from the hybridoma 7F5), in both primary NK cells and the NK cell line YTS. DAP12 is expressed in NK cells and associates with a number of activating receptors, including the C-type lectin receptor NKG2C, the natural cytotoxicity receptor NKp44, and the activating KIRs KIR3DS1 and KIR2DS1/2/5.[130] The anti-PSCA-DAP12 CAR expressed in both primary NK cells and the YTS-NK cell line was able to lyse otherwise

Please cite this article in press as: Rezvani et al., Engineering Natural Killer Cells for Cancer Immunotherapy, Molecular Therapy (2017), http://dx.doi.org/10.1016/j.ymthe.2017.06.012

Table 2. Preclinical studies of NK Cell Line CARs

Disease (Antigen Targeted)	Source	scFv Clone	Construct; Signaling Domain	Costimulatory Domain	Genetic Modification Method	In Vitro Cytotoxicity	In Vivo Cytotoxicity	Reference
B cell malignancies (CD19)	NK-92	FMC63	CD19 CD3ζ	none	retroviral transduction	Y	N	93
CLL (CD19)	NK-92	FMC63	CD19 CD3ζ	none	mRNA electroporation	Y	N	94
B cell malignancies (CD19 and CD20)	NK-92	FMC63	CD19 CD3ζ	none	lentiviral transduction	Y	Y	95
B cell malignancies (CD20)	NK-92	Leu-16	CD19 CD3ζ	none	retroviral transduction	Y	Y	96
B cell malignancies (CD19)	NK-92	FMC63	CD19 CD3ζ CD28 CD3ζ CD137 CD3ζ	CD28/4-1BB	lentiviral transduction	Y	Y	113
MM (CD138)	NK-92MI	4B3	CD138 CD3ζ	none	lentiviral transduction	Y	Y	97
MM (CS1)	NK-92	NR	CD19 CD3ζ CD28ζ	CD28	lentiviral transduction	Y	Y	109
NBL (GD2)	NK-92	Ch14;18	GD2 CD3ζ	none	retroviral transduction	Y	N	98
Breast/ovarian cancer (ErbB2/HER-2)	NK-92	FRP5	FRP5 CD3ζ	none	retroviral transduction	Y	Y	99
Breast cancer/RCC (HER-2)	NK-92	FRP5	HER-2. CD3ζ HER-2. CD28.CD3ζ HER-2. CD137.CD3ζ	CD28/4-1BB	lentiviral transduction	Y	Y	100
Breast cancer (EpCAM)	NK-92	MOC31	EpCAM. CD28. CD3ζ -IL-15	CD28	lentiviral transduction	Y	N	101
Breast cancer metastasis (EGFR)	NK-92	528	EGFR CD28.CD3ζ + oHSV	CD28ζ	lentiviral transduction	Y	Y	114
Melanoma (GPA7)	NK-92	NR	-GPA7-CD3ζ	None	mRNA electroporation	Y	Y	102
GBM (EGFR, EGFRvIII)	NK-92NKL	528	CD28.CD3ζ	CD28ζ	lentiviral transduction	Y	Y	115
GBM (EGFR, EGFRvIII, common epitope)	NK-92	R1 MR1-1 225	EGFR.CD28.CD3ζ EGFRvIII CD28.CD3ζ common epitope-.CD28.CD3ζ	CD28ζ	lentiviral transduction	Y	Y	116
$(EBV)^+$ T cells, EBNA3C	NK-92MI	EBNA clone 315 (HLA A2)	4-1BB.CD3ζ	4-1BB	retroviral transduction	Y	N	117
T cell malignancies (CD5)	NK-92	TIB104	CD5-CD28.4-1BB.CD3ζ	CD28ζ 4-1BBζ	lentiviral transduction	Y	Y	118

B-ALL, B cell acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; EBV, Epstein-Barr virus; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; GBM, glioblastoma multiforme; MM, multiple myeloma; NBL, neuroblastoma; NR, not reported; oHSV, oncolytic herpes simplex virus; RCC, renal cell carcinoma; scFv, single-chain fragment variable.

resistant $PSCA^+$ HLA-B/C- and HLA-C-matched tumor cells, both in in vitro and in mouse models. Interestingly, the YTS-NK CAR incorporating the DAP-12 signaling domain improved the specific cytotoxicity compared with NK cells expressing an anti-PSCA-CD3ζ-based CAR. To our knowledge, this work is the first to demonstrate the ability of a single immunoreceptor tyrosine-based activation motif (ITAM)-containing DAP12-CAR to initiate downstream signaling as efficiently as a CD3ζ-based CAR, which contains three ITAMs. Importantly, this DAP12-signaling CAR did not require additional costimulatory signaling molecules for in vitro activation and cytotoxicity, which the authors hypothesized are not necessary for DAP12-mediated NK cell activation.

Aside from CAR modification, another promising immunotherapeutic strategy involves engineering NK cells to express cytokines known to be important for boosting NK cell cytotoxicity. This approach may also prolong NK cell persistence and would obviate the need for in vivo cytokine supplementation, which is toxic.

Please cite this article in press as: Rezvani et al., Engineering Natural Killer Cells for Cancer Immunotherapy, Molecular Therapy (2017), http://dx.doi.org/10.1016/j.ymthe.2017.06.012

However, an obvious concern is the risk of inducing CRS and other cytokine-induced systemic toxicity. There is also a more than theoretical risk of inducing malignant transformation in the transduced cells following persistent autocrine stimulation, which has been observed for IL-2-engineered T cells.[131] One group sought to avoid toxicities by temporarily introducing genes coding for IL-2 or IL-15 using short-lived (but stable) expression models such as mRNA electroporation of NK cells.[79,97]

Special Considerations for Clinical Translation of CAR-NK Cell Therapy

Despite the promising preclinical results with CAR-NK cells, the well-established safety profile of unmodified NK cells, and the recent clinical successes with CAR-T cells, as of December 2016, only two clinical trials of CAR-NK cell therapy have actively enrolled patients (NCT00995137 from St. Jude Children's Research Hospital and NCT01974479 from The National University Health System, Singapore). Both trials are targeting refractory $CD19^+$ ALL with an identical second-generation anti-CD19 CAR that incorporates the 4-1BB costimulatory domain (anti-CD19-BB-ζ), transduced into haploidentical donor-derived NK cells.[90] While the St. Jude trial was only open to pediatric patients and is closed to enrollment, the Singapore trial is actively enrolling both children and adults. In this dose-escalation trial, pediatric and adult patients receive a single intravenous (i.v.) infusion of anti-CD19-BB-ζ NK cells at doses of 0.5×10^7 to 1×10^8 $CD56^+$ cells/kg. The clinical results of these studies are awaited.

Within the last few months, several other trials involving CAR-NK cell therapy have been registered on ClinicalTrials.gov. PersonGen BioTherapeutics, based in China, obtained regulatory approval for several trials to administer sequential doses of third-generation (relevant scFv attached to TCRζ, CD28, and 4-1BB signaling domains) CAR-transduced NK-92 cells (on days 0, 3, and 5). The group is targeting refractory $CD7^+$ leukemia/lymphomas in adults (NCT02742727), $CD33^+$ myeloid malignancies in children and adults (NCT02944162), and refractory $CD19^+$ malignancies in children and adults planned to undergo hematopoietic stem cell transplantation (HSCT) (NCT02892695). The group is also targeting $MUC1^+$ relapsed and refractory solid tumors (NCT02839954). Our group also plans a clinical study to test the safety and efficacy of escalating doses of off-the-shelf CB-derived NK cells, engineered to express iC9.CAR19.CD28-ζ-2A-IL-15 in patients with relapsed or refractory B-lymphoid malignancies in early 2017.

A number of scientific questions and regulatory hurdles remain to be addressed before CAR-NK therapy can be extended to larger cohorts of patients. Although powerful methods for isolation, expansion, and transduction of NK cells have been described, the preparation of the cells remains burdensome. Compared to lentiviral constructs, retroviral constructs confer higher transduction efficiencies but also carry a higher potential risk of insertional mutagenesis.[132,133] This risk, which has remained largely theoretical (aside from in select populations with X-linked severe combined immunodeficiency [SCID], Wiskott-Aldrich, or chronic granulomatous disease[132–136]), comes with distinct regulatory hurdles prior to use in humans. Electroporation, besides being technically simpler and less stringently regulated, avoids the use of viral vectors and the associated risks of oncogene activation and insertional mutagenesis.[112] However, while some groups report transfection efficiencies of up to 80%–90% in NK-92 cells with clinical-grade electroporators,[90] the majority of published studies with ex vivo expanded primary NK cells have been largely disappointing, with transfection efficiencies as low as 10%.[94] Moreover, CAR molecule expression is short lived, typically lasting < 7 days,[137] which is likely to negatively impact the long-term efficacy of the CAR-NK cells. While few reports have overtly explored the effects of transduction on the viability, phenotype, and functionality of the NK cells, existing data suggest a minimal effect on these parameters.[95]

An additional concept that has yet to be explored is whether lymphodepletion will be necessary to prevent rejection of the infused allogeneic CAR-NK cells. Aside from inducing lymphopenia, lymphodepleting chemotherapy depletes other immunosuppressive cells within the tumor microenvironment such as Tregs and MDSCs, which may negatively impact NK cell cytotoxicity or their in vivo expansion.[79] Lastly, given the recent safety concerns associated with infusion of CAR-modified T cells, careful consideration of whether a suicide system (e.g., based on caspase-9 or thymidine kinase) deserves incorporation into the construct must be taken into account.[67,104,138]

Further unanswered questions include whether repeated infusions could trigger immunogenicity, especially since the majority of scFvs are murine-based, and may elicit human anti-mouse antibodies (HAMAs) or cellular-mediated rejection/sensitization. While the development of HAMA immunogenicity has been studied extensively following CAR-T cell infusions, it has not been a significant problem in CAR-T cell studies, likely because most patients to date have received a single infusion of autologous cells. Fewer reports have discussed cell-mediated responses, although the use of allogeneic NK cells in this context may make this problem less theoretical and more practical.

Further studies exploring the optimal vector, construct, and transduction method are necessary to identify the "perfect NK CAR" for immunotherapy.

What Does the Future Hold?

The next several years will provide clinical validation of genetically engineered NK cells and are likely to shape the future of immunotherapy. Combinatorial techniques will be tested to improve the efficacy of tumor-specific NK cells, whether through harnessing the innate power of the NK cell, inhibiting or knock out of immune checkpoints,[139] or targeting of the tumor microenvironment. Additional gene editing techniques currently being explored in the setting of adoptive T cell therapy, such as CRISPR/Cas9 and transcription activator-like effector nuclease (TALEN), are also likely to be tested

in the setting of NK cells, with some groups demonstrating preclinical feasibility of these techniques (Navarro, 2016, Society for Natural Immunity, conference). Such strategies come with significant regulatory hurdles of their own, and to date, only one clinical trial utilizing this technology is actively recruiting patients,[140–142] although trials targeting NY-ESO1 and PD-1 are in development.

Despite the questions that remain, CAR-NK cells hold great promise as a novel cellular immunotherapy against refractory malignancies. Notably, NK cells can provide an off-the-shelf product that can be used as an allogeneic product to treat patients, eliminating the need for a personalized and patient-specific product that plagues current CAR-T cell therapies. The ability to more potently direct NK cell-mediated cytotoxicity against refractory tumors through the expression of CAR is likely to contribute to the recent paradigm shift in cancer treatment.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH(R01 CA211044-01) and the Cancer Prevention Research Institute of Texas (RSG-15-218-01-LIB).

REFERENCES

1. Maude, S.L., Frey, N., Shaw, P.A., Aplenc, R., Barrett, D.M., Bunin, N.J., Chew, A., Gonzalez, V.E., Zheng, Z., Lacey, S.F., et al. (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. *371*, 1507–1517.
2. Davila, M.L., Riviere, I., Wang, X., Bartido, S., Park, J., Curran, K., Chung, S.S., Stefanski, J., Borquez-Ojeda, O., Olszewska, M., et al. (2014). Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. Sci. Transl. Med. *6*, 224ra25.
3. Lee, D.W., Kochenderfer, J.N., Stetler-Stevenson, M., Cui, Y.K., Delbrook, C., Feldman, S.A., Fry, T.J., Orentas, R., Sabatino, M., Shah, N.N., et al. (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet *385*, 517–528.
4. June, C.H., Riddell, S.R., and Schumacher, T.N. (2015). Adoptive cellular therapy: a race to the finish line. Sci. Transl. Med. *7*, 280ps7.
5. Sadelain, M. (2015). CAR therapy: the CD19 paradigm. J. Clin. Invest. *125*, 3392–3400.
6. Locatelli, F., Moretta, F., Brescia, L., and Merli, P. (2014). Natural killer cells in the treatment of high-risk leukemia. Semin. Immunol. *26*, 173–179.
7. Farag, S.S., and Caligiuri, M.A. (2006). Human natural killer cell development and biology. Blood Rev. *20*, 123–137.
8. Moretta, L., Locatelli, F., Pende, D., Marcenaro, E., Mingari, M.C., and Moretta, A. (2011). Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. Blood *117*, 764–771.
9. Yoon, S.R., Lee, Y.S., Yang, S.H., Ahn, K.H., Lee, J.H., Lee, J.H., Kim, D.Y., Kang, Y.A., Jeon, M., Seol, M., et al. (2010). Generation of donor natural killer cells from CD34(+) progenitor cells and subsequent infusion after HLA-mismatched allogeneic hematopoietic cell transplantation: a feasibility study. Bone Marrow Transplant. *45*, 1038–1046.
10. Miller, J.S., Soignier, Y., Panoskaltsis-Mortari, A., McNearney, S.A., Yun, G.H., Fautsch, S.K., McKenna, D., Le, C., Defor, T.E., Burns, L.J., et al. (2005). Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood *105*, 3051–3057.
11. Rubnitz, J.E., Inaba, H., Ribeiro, R.C., Pounds, S., Rooney, B., Bell, T., Pui, C.H., and Leung, W. (2010). NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. J. Clin. Oncol. *28*, 955–959.
12. Sotillo, E., Barrett, D.M., Black, K.L., Bagashev, A., Oldridge, D., Wu, G., Sussman, R., Lanauze, C., Ruella, M., Gazzara, M.R., et al. (2015). Convergence of acquired mutations and alternative splicing of CD19 enables resistance to CART-19 immunotherapy. Cancer Discov. *5*, 1282–1295.
13. Kim, S., Poursine-Laurent, J., Truscott, S.M., Lybarger, L., Song, Y.J., Yang, L., French, A.R., Sunwoo, J.B., Lemieux, S., Hansen, T.H., and Yokoyama, W.M. (2005). Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature *436*, 709–713.
14. Davies, J.O., Stringaris, K., Barrett, A.J., and Rezvani, K. (2014). Opportunities and limitations of NK cells as adoptive therapy for malignant disease. Cytotherapy *16*, 1453–1466.
15. Wagner, J.A., Berrien-Elliott, M.M., Rosario, M., Leong, J.W., Jewell, B.A., Schappe, T., Abdel-Latif, S., and Fehniger, T.A. (2016). Cytokine-induced memory-like differentiation enhances unlicensed natural killer cell antileukemia and FcγRIIIa-triggered responses. Biol. Blood Marrow Transplant *23*, 398–404.
16. Cooper, M.A., Fehniger, T.A., Turner, S.C., Chen, K.S., Ghaheri, B.A., Ghayur, T., Carson, W.E., and Caligiuri, M.A. (2001). Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood *97*, 3146–3151.
17. Campbell, J.J., Qin, S., Unutmaz, D., Soler, D., Murphy, K.E., Hodge, M.R., Wu, L., and Butcher, E.C. (2001). Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J. Immunol. *166*, 6477–6482.
18. De Maria, A., Bozzano, F., Cantoni, C., and Moretta, L. (2011). Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation. Proc. Natl. Acad. Sci. USA *108*, 728–732.
19. Kärre, K. (2002). Immunology. A perfect mismatch. Science *295*, 2029–2031.
20. Campbell, K.S., and Hasegawa, J. (2013). Natural killer cell biology: an update and future directions. J. Allergy Clin. Immunol. *132*, 536–544.
21. Caligiuri, M.A. (2008). Human natural killer cells. Blood *112*, 461–469.
22. Bradley, M., Zeytun, A., Rafi-Janajreh, A., Nagarkatti, P.S., and Nagarkatti, M. (1998). Role of spontaneous and interleukin-2-induced natural killer cell activity in the cytotoxicity and rejection of Fas1 and Fas- tumor cells. Blood *92*, 4248–4255.
23. Screpanti, V., Wallin, R.P., Ljunggren, H.G., and Grandien, A. (2001). A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. J. Immunol. *167*, 2068–2073.
24. Kayagaki, N., Yamaguchi, N., Nakayama, M., Takeda, K., Akiba, H., Tsutsui, H., Okamura, H., Nakanishi, K., Okumura, K., and Yagita, H. (1999). Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. J. Immunol. *163*, 1906–1913.
25. Joncker, N.T., Shifrin, N., Delebecque, F., and Raulet, D.H. (2010). Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. J. Exp. Med. *207*, 2065–2072.
26. Lotzová, E., Savary, C.A., and Herberman, R.B. (1987). Inhibition of clonogenic growth of fresh leukemia cells by unstimulated and IL-2 stimulated NK cells of normal donors. Leuk. Res. *11*, 1059–1066.
27. Stringaris, K., Sekine, T., Khoder, A., Alsuliman, A., Razzaghi, B., Sargeant, R., Pavlu, J., Brisley, G., de Lavallade, H., Sarvaria, A., et al. (2013). Leukemia-induced phenotypic and functional defects in natural killer cells predict failure to achieve remission in acute myeloid leukemia. Haematologica *99*, 836–847.
28. Fauriat, C., Just-Landi, S., Mallet, F., Arnoulet, C., Sainty, D., Olive, D., and Costello, R.T. (2007). Deficient expression of NCR in NK cells from acute myeloid leukemia: evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. Blood *109*, 323–330.
29. Satwani, P., Bavishi, S., Saha, A., Zhao, F., Ayello, J., van de Ven, C., Chu, Y., and Cairo, M.S. (2014). Upregulation of NKG2D ligands in acute lymphoblastic leukemia and non-Hodgkin lymphoma cells by romidepsin and enhanced in vitro and in vivo natural killer cell cytotoxicity. Cytotherapy *16*, 1431–1440.
30. Jin, F., Lin, H., Gao, S., Hu, Z., Zuo, S., Sun, L., Jin, C., Li, W., and Yang, Y. (2016). The anti-tumor role of NK cells in vivo pre-activated and restimulated by interleukins in acute lymphoblastic leukemia. Oncotarget *7*, 79187.

31. Rouce, R.H., Shaim, H., Sekine, T., Weber, G., Ballard, B., Ku, S., Barese, C., Murali, V., Wu, M.F., Liu, H., et al. (2016). The TGF-β/SMAD pathway is an important mechanism for NK cell immune evasion in childhood B-acute lymphoblastic leukemia. Leukemia *30*, 800–811.

32. Pfeiffer, M., Schumm, M., Feuchtinger, T., Dietz, K., Handgretinger, R., and Lang, P. (2007). Intensity of HLA class I expression and KIR-mismatch determine NK-cell mediated lysis of leukaemic blasts from children with acute lymphatic leukaemia. Br. J. Haematol. *138*, 97–100.

33. Bachanova, V., and Miller, J.S. (2014). NK cells in therapy of cancer. Crit. Rev. Oncog. *19*, 133–141.

34. Shah, N., Martin-Antonio, B., Yang, H., Ku, S., Lee, D.A., Cooper, L.J., Decker, W.K., Li, S., Robinson, S.N., Sekine, T., et al. (2013). Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity. PLoS ONE *8*, e76781.

35. El-Sherbiny, Y.M., Meade, J.L., Holmes, T.D., McGonagle, D., Mackie, S.L., Morgan, A.W., Cook, G., Feyler, S., Richards, S.J., Davies, F.E., et al. (2007). Requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. Cancer Res. *67*, 8444–8449.

36. Swift, B.E., Williams, B.A., Kosaka, Y., Wang, X.H., Medin, J.A., Viswanathan, S., Martinez-Lopez, J., and Keating, A. (2012). Natural killer cell lines preferentially kill clonogenic multiple myeloma cells and decrease myeloma engraftment in a bioluminescent xenograft mouse model. Haematologica *97*, 1020–1028.

37. Davies, F.E., Raje, N., Hideshima, T., Lentzsch, S., Young, G., Tai, Y.T., Lin, B., Podar, K., Gupta, D., Chauhan, D., et al. (2001). Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood *98*, 210–216.

38. Liu, Y., Wu, H.W., Sheard, M.A., Sposto, R., Somanchi, S.S., Cooper, L.J., Lee, D.A., and Seeger, R.C. (2013). Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy. Clin. Cancer Res. *19*, 2132–2143.

39. Rusakiewicz, S., Semeraro, M., Sarabi, M., Desbois, M., Locher, C., Mendez, R., Vimond, N., Concha, A., Garrido, F., Isambert, N., et al. (2013). Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. Cancer Res. *73*, 3499–3510.

40. Mamessier, E., Sylvain, A., Thibult, M.L., Houvenaeghel, G., Jacquemier, J., Castellano, R., Gonçalves, A., André, P., Romagné, F., Thibault, G., et al. (2011). Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. J. Clin. Invest. *121*, 3609–3622.

41. Gras Navarro, A., Björklund, A.T., and Chekenya, M. (2015). Therapeutic potential and challenges of natural killer cells in treatment of solid tumors. Front. Immunol *6*, 202.

42. Kailayangiri, S., Altvater, B., Spurny, C., Jamitzky, S., Schelhaas, S., Jacobs, A.H., Wiek, C., Roellecke, K., Hanenberg, H., Hartmann, W., et al. (2016). Targeting Ewing sarcoma with activated and GD2-specific chimeric antigen receptor-engineered human NK cells induces upregulation of immune-inhibitory HLA-G. OncoImmunology *6*, e1250050.

43. Koepsell, S.A., Miller, J.S., and McKenna, D.H., Jr. (2013). Natural killer cells: a review of manufacturing and clinical utility. Transfusion *53*, 404–410.

44. Leong, J.W., Chase, J.M., Romee, R., Schneider, S.E., Sullivan, R.P., Cooper, M.A., and Fehniger, T.A. (2014). Pre-activation with IL-12, IL-15, and IL-18 induces CD25 and a functional high affinity IL-2 receptor on human cytokine-induced memory-like NK cells. Biol. Blood Marrow Transplant *20*, 463–473.

45. Miller, J.S., Rooney, C.M., Curtsinger, J., McElmurry, R., McCullar, V., Verneris, M.R., Lapteva, N., McKenna, D., Wagner, J.E., Blazar, B.R., and Tolar, J. (2014). 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.

46. Berg, M., Lundqvist, A., McCoy, P., Jr., Samsel, L., Fan, Y., Tawab, A., and Childs, R. (2009). Berg. 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.

47. Rezvani, K., and Rouce, R.H. (2015). The application of natural killer cell immunotherapy for the treatment of cancer. Front. Immunol. *6*, 578.

48. Ni, J., Miller, M., Stojanovic, A., Garbi, N., and Cerwenka, A. (2012). Sustained effector function of IL-12/15/18-preactivated NK cells against established tumors. J. Exp. Med. *209*, 2351–2365.

49. Romain, G., Senyukov, V., Rey-Villamizar, N., Merouane, A., Kelton, W., Liadi, I., Mahendra, A., Charab, W., Georgiou, G., Roysam, B., et al. (2014). Antibody Fc engineering improves frequency and promotes kinetic boosting of serial killing mediated by NK cells. Blood *124*, 3241–3249.

50. Kanazawa, T., Hiramatsu, Y., Iwata, S., Siddiquey, M., Sato, Y., Suzuki, M., Ito, Y., Goshima, F., Murata, T., and Kimura, H. (2014). Anti-CCR4 monoclonal antibody mogamulizumab for the treatment of EBV-associated T- and NK-cell lymphoproliferative diseases. Clin. Cancer Res. *20*, 5075–5084.

51. Lin, W., Voskens, C.J., Zhang, X., Schindler, D.G., Wood, A., Burch, E., Wei, Y., Chen, L., Tian, G., Tamada, K., et al. (2008). Fc-dependent expression of CD137 on human NK cells: insights into "agonistic" effects of anti-CD137 monoclonal antibodies. Blood *112*, 699–707.

52. Benson, D.M., Jr., Bakan, C.E., Zhang, S., Collins, S.M., Liang, J., Srivastava, S., Hofmeister, C.C., Efebera, Y., Andre, P., Romagne, F., et al. (2011). IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. Blood *118*, 6387–6391.

53. Terszowski, G., Klein, C., and Stern, M. (2014). KIR/HLA interactions negatively affect rituximab- but not GA101 (obinutuzumab)-induced antibody-dependent cellular cytotoxicity. J. Immunol *192*, 5618–5624.

54. Curti, A., Ruggeri, L., D'Addio, A., Bontadini, A., Dan, E., Motta, M.R., Trabanelli, S., Giudice, V., Urbani, E., Martinelli, G., et al. (2011). 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.

55. Rubnitz, J.E., Inaba, H., Kang, G., Gan, K., Hartford, C., Triplett, B.M., Dallas, M., Shook, D., Gruber, T., Pui, C.H., and Leung, W. (2015). Natural killer cell therapy in children with relapsed leukemia. Pediatr. Blood Cancer *62*, 1468–1472.

56. Woll, P.S., Grzywacz, B., Tian, X., Marcus, R.K., Knorr, D.A., Verneris, M.R., and Kaufman, D.S. (2009). Human embryonic stem cells differentiate into a homogeneous population of natural killer cells with potent in vivo antitumor activity. Blood *113*, 6094–6101.

57. Chouaib, S., Pittari, G., Nanbakhsh, A., El Ayoubi, H., Amsellem, S., Bourhis, J.H., and Spanholtz, J. (2014). Improving the outcome of leukemia by natural killer cell-based immunotherapeutic strategies. Front. Immunol. *5*, 95.

58. Shaim, H., and Yvon, E. (2015). Cord blood: a promising source of allogeneic natural killer cells for immunotherapy. Cytotherapy *17*, 1–2.

59. Denman, C.J., Senyukov, V.V., Somanchi, S.S., Phatarpekar, P.V., Kopp, L.M., Johnson, J.L., Singh, H., Hurton, L., Maiti, S.N., Huls, M.H., et al. (2012). Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS ONE *7*, e30264.

60. Klingemann, H.G., Wong, E., and Maki, G. (1996). A cytotoxic NK-cell line (NK-92) for ex vivo purging of leukemia from blood. Biol. Blood Marrow Transplant. *2*, 68–75.

61. Tonn, T., Schwabe, D., Klingemann, H.G., Becker, S., Esser, R., Koehl, U., Suttorp, M., Seifried, E., Ottmann, O.G., and Bug, G. (2013). Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy *15*, 1563–1570.

62. Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W.D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., et al. (2002). Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science *295*, 2097–2100.

63. Olson, J.A., Leveson-Gower, D.B., Gill, S., Baker, J., Beilhack, A., and Negrin, R.S. (2010). NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. Blood *115*, 4293–4230.

64. Passweg, J.R., Tichelli, A., Meyer-Monard, S., Heim, D., Stern, M., Kühne, T., Favre, G., and Gratwohl, A. (2004). Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. Leukemia *18*, 1835–1838.

65. Koehl, U., Kalberer, C., Spanholtz, J., Lee, D.A., Miller, J.S., Cooley, S., Lowdell, M., Uharek, L., Klingemann, H., Curti, A., et al. (2015). Advances in clinical NK cell

studies: donor selection, manufacturing and quality control. OncoImmunology *5*, e1115178.

66. Eshhar, Z., Waks, T., Gross, G., and Schindler, D.G. (1993). Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc. Natl. Acad. Sci. USA *90*, 720–724.
67. Rouce, R.H., Sharma, S., Huynh, M., and Heslop, H.E. (2017). Recent advances in T-cell immunotherapy for haematological malignancies. Br. J. Haematol *176*, 688–704.
68. Sadelain, M. (2016). Chimeric antigen receptors: driving immunology towards synthetic biology. Curr. Opin. Immunol. *41*, 68–76.
69. Kebriaei, P., Singh, H., Huls, M.H., Figliola, M.J., Bassett, R., Olivares, S., Jena, B., Dawson, M.J., Kumaresan, P.R., Su, S., et al. (2016). Phase I trials using sleeping beauty to generate CD19-specific CAR T cells. J. Clin. Invest *126*, 3363–3376.
70. Cruz, C.R., Micklethwaite, K.P., Savoldo, B., Ramos, C.A., Lam, S., Ku, S., Diouf, O., Liu, E., Barrett, A.J., Ito, S., et al. (2013). Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. Blood *122*, 2965–2973.
71. Ghosh, A., Smith, M., James, S.E., Davila, M.L., Velardi, E., Argyropoulos, K.V., Gunset, G., Perna, F., Kreines, F.M., Levy, E.R., et al. (2017). Donor CD19 CAR T cells exert potent graft-versus-lymphoma activity with diminished graft-versus-host activity. Nat. Med. *23*, 242–249.
72. Ren, J., Liu, X., Fang, C., Jiang, S., June, C.H., and Zhao, Y. (2017). Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. Clin. Cancer Res. *23*, 2255–2266.
73. Brudno, J.N., Somerville, R.P., Shi, V., Rose, J.J., Halverson, D.C., Fowler, D.H., Gea-Banacloche, J.C., Pavletic, S.Z., Hickstein, D.D., Lu, T.L., et al. (2016). Allogeneic T cells that express an anti-CD19 chimeric antigen receptor induce remissions of B-cell malignancies that progress after allogeneic hematopoietic stem-cell transplantation without causing graft-versus-host disease. J. Clin. Oncol *34*, 1112–1121.
74. Geller, M.A., and Miller, J.S. (2011). Use of allogeneic NK cells for cancer immunotherapy. Immunotherapy *3*, 1445–1459.
75. Cooley, S., Trachtenberg, E., Bergemann, T.L., Saeteurn, K., Klein, J., Le, C.T., Marsh, S.G., Guethlein, L.A., Parham, P., Miller, J.S., and Weisdorf, D.J. (2009). Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. Blood *113*, 726–732.
76. Sekine, T., Marin, D., Cao, K., Li, L., Mehta, P., Shaim, H., Sobieski, C., Jones, R., Oran, B., Hosing, C., et al. (2016). Specific combinations of donor and recipient KIR-HLA genotypes predict for large differences in outcome after cord blood transplantation. Blood *128*, 297–312.
77. Bao, X., Wang, M., Zhou, H., Zhang, H., Wu, X., Yuan, X., Li, Y., Wu, D., and He, J. (2016). Donor killer immunoglobulin-like receptor profile Bx1 imparts a negative effect and centromeric B-specific gene motifs render a positive effect on standard-risk acute myeloid leukemia/myelodysplastic syndrome patient survival after unrelated donor hematopoietic stem cell transplantation. Biol. Blood Marrow Transplant. *22*, 232–239.
78. Oevermann, L., Michaelis, S.U., Mezger, M., Lang, P., Toporski, J., Bertaina, A., Zecca, M., Moretta, L., Locatelli, F., and Handgretinger, R. (2014). KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. Blood *124*, 2744–2747.
79. Carlsten, M., and Childs, R.W. (2015). Genetic manipulation of NK cells for cancer immunotherapy: techniques and clinical implications. Front. Immunol. *6*, 266.
80. Glienke, W., Esser, R., Priesner, C., Suerth, J.D., Schambach, A., Wels, W.S., Grez, M., Kloess, S., Arseniev, L., and Koehl, U. (2015). Advantages and applications of CAR-expressing natural killer cells. Front. Pharmacol. *6*, 21.
81. Tettamanti, S., Marin, V., Pizzitola, I., Magnani, C.F., Giordano Attianese, G.M., Cribioli, E., Maltese, F., Galimberti, S., Lopez, A.F., Biondi, A., et al. (2013). Targeting of acute myeloid leukaemia by cytokine-induced killer cells redirected with a novel CD123-specific chimeric antigen receptor. Br. J. Haematol. *161*, 389–401.
82. Liu, E., Tong, Y., Dotti, G., Savoldo, B., Muftuoglu, M., Kondo, K., Mukherjee, M., Orange, J.S., Sobieski, C., Alsuliman, A., et al. (2016). Cord blood derived natural killer cells engineered with a chimeric antigen receptor targeting CD19 and expressing IL-15 have long term persistence and exert potent anti-leukemia activity. Blood *126*, 3091.
83. Kloess, S., Huenecke, S., Piechulek, D., Esser, R., Koch, J., Brehm, C., Soerensen, J., Gardlowski, T., Brinkmann, A., Bader, P., et al. (2010). IL-2-activated haploidentical NK cells restore NKG2D-mediated NK-cell cytotoxicity in neuroblastoma patients by scavenging of plasma MICA. Eur. J. Immunol. *40*, 3255–3267.
84. Raffaghello, L., Prigione, I., Airoldi, I., Camoriano, M., Levreri, I., Gambini, C., Pende, D., Steinle, A., Ferrone, S., and Pistoia, V. (2004). Downregulation and/or release of NKG2D ligands as immune evasion strategy of human neuroblastoma. Neoplasia *6*, 558–568.
85. Huenecke, S., Zimmermann, S.Y., Kloess, S., Esser, R., Brinkmann, A., Tramsen, L., Koenig, M., Erben, S., Seidl, C., Tonn, T., et al. (2010). IL-2-driven regulation of NK cell receptors with regard to the distribution of CD16+ and CD16- subpopulations and in vivo influence after haploidentical NK cell infusion. J. Immunother. *33*, 200–210.
86. Klingemann, H.G. (2013). Cellular therapy of cancer with natural killer cells-where do we stand. Cytotherapy *15*, 1185–1194.
87. Altvater, B., Landmeier, S., Pscherer, S., Temme, J., Schweer, K., Kailayangiri, S., Campana, D., Juergens, H., Pule, M., and Rossig, C. (2009). 2B4 (CD244) signaling by recombinant antigen-specific chimeric receptors costimulates natural killer cell activation to leukemia and neuroblastoma cells. Clin. Cancer Res. *15*, 4857–4866.
88. Imai, C., Iwamoto, S., and Campana, D. (2005). Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood *106*, 376–383.
89. Li, L., Liu, L.N., Feller, S., Allen, C., Shivakumar, R., Fratantoni, J., Wolfraim, L.A., Fujisaki, H., Campana, D., Chopas, N., et al. (2010). Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. Cancer Gene Ther. *17*, 147–154.
90. Shimasaki, N., Fujisaki, H., Cho, D., Masselli, M., Lockey, T., Eldridge, P., Leung, W., and Campana, D. (2012). A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. Cytotherapy *14*, 830–840.
91. Chu, Y., Hochberg, J., Yahr, A., Ayello, J., van de Ven, C., Barth, M., Czuczman, M., and Cairo, M.S. (2015). Targeting CD20+ aggressive B-cell non-Hodgkin lymphoma by anti-CD20 CAR mRNA-modified expanded natural killer cells in vitro and in NSG mice. Cancer Immunol. Res. *3*, 333–344.
92. Kruschinski, A., Moosmann, A., Poschke, I., Norell, H., Chmielewski, M., Seliger, B., Kiessling, R., Blankenstein, T., Abken, H., and Charo, J. (2008). Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. Proc. Natl. Acad. Sci. USA *105*, 17481–17486.
93. Romanski, A., Uherek, C., Bug, G., Seifried, E., Klingemann, H., Wels, W.S., Ottmann, O.G., and Tonn, T. (2016). CD19-CAR engineered NK-92 cells are sufficient to overcome NK cell resistance in B-cell malignancies. J. Cell. Mol. Med. *20*, 1287–1294.
94. Boissel, L., Betancur, M., Wels, W.S., Tuncer, H., and Klingemann, H. (2009). Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leuk. Res. *33*, 1255–1259.
95. Boissel, L., Betancur-Boissel, M., Lu, W., Krause, D.S., Van Etten, R.A., Wels, W.S., and Klingemann, H. (2013). Retargeting NK-92 cells by means of CD19- and CD20-specific chimeric antigen receptors compares favorably with antibody-dependent cellular cytotoxicity. OncoImmunology *2*, e26527.
96. Müller, T., Uherek, C., Maki, G., Chow, K.U., Schimpf, A., Klingemann, H.G., Tonn, T., and Wels, W.S. (2008). Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells. Cancer Immunol. Immunother. *57*, 411–423.
97. Jiang, H., Zhang, W., Shang, P., Zhang, H., Fu, W., Ye, F., Zeng, T., Huang, H., Zhang, X., Sun, W., et al. (2014). Transfection of chimeric anti-CD138 gene enhances natural killer cell activation and killing of multiple myeloma cells. Mol. Oncol. *8*, 297–310.
98. Esser, R., Müller, T., Stefes, D., Kloess, S., Seidel, D., Gillies, S.D., Aperlo-Iffland, C., Huston, J.S., Uherek, C., Schönfeld, K., et al. (2012). NK cells engineered to express a GD2 -specific antigen receptor display built-in ADCC-like activity against tumour cells of neuroectodermal origin. J. Cell. Mol. Med. *16*, 569–581.

99. Uherek, C., Tonn, T., Uherek, B., Becker, S., Schnierle, B., Klingemann, H.G., and Wels, W. (2002). Retargeting of natural killer-cell cytolytic activity to ErbB2-expressing cancer cells results in efficient and selective tumor cell destruction. Blood *100*, 1265–1273.

100. Schönfeld, K., Sahm, C., Zhang, C., Naundorf, S., Brendel, C., Odendahl, M., Nowakowska, P., Bönig, H., Köhl, U., Kloess, S., et al. (2015). Selective inhibition of tumor growth by clonal NK cells expressing an ErbB2/HER2-specific chimeric antigen receptor. Mol. Ther. *23*, 330–338.

101. Sahm, C., Schönfeld, K., and Wels, W.S. (2012). Expression of IL-15 in NK cells results in rapid enrichment and selective cytotoxicity of gene-modified effectors that carry a tumor-specific antigen receptor. Cancer Immunol. Immunother *61*, 1451–1461.

102. Zhang, G., Liu, R., Zhu, X., Wang, L., Ma, J., Han, H., Wang, X., Zhang, G., He, W., Wang, W., et al. (2013). Retargeting NK-92 for anti-melanoma activity by a TCR-like single-domain antibody. Immunol. Cell Biol. *91*, 615–624.

103. Tagaya, Y., Bamford, R.N., DeFilippis, A.P., and Waldmann, T.A. (1996). IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels. Immunity *4*, 329–336.

104. Di Stasi, A., Tey, S.K., Dotti, G., Fujita, Y., Kennedy-Nasser, A., Martinez, C., Straathof, K., Liu, E., Durett, A.G., Grilley, B., et al. (2011). Inducible apoptosis as a safety switch for adoptive cell therapy. N. Engl. J. Med. *365*, 1673–1683.

105. Suerth, J.D., Morgan, M.A., Kloess, S., Heckl, D., Neudörfl, C., Falk, C.S., Koehl, U., and Schambach, A. (2016). Efficient generation of gene-modified human natural killer cells via alpharetroviral vectors. J. Mol. Med. (Berl.) *94*, 83–93.

106. Hermanson, D.L., and Kaufman, D.S. (2015). Utilizing chimeric antigen receptors to direct natural killer cell activity. Front. Immunol. *6*, 195.

107. Yagita, M., Huang, C.L., Umehara, H., Matsuo, Y., Tabata, R., Miyake, M., Konaka, Y., and Takatsuki, K. (2000). A novel natural killer cell line (KHYG-1) from a patient with aggressive natural killer cell leukemia carrying a p53 point mutation. Leukemia *14*, 922–930.

108. Arai, S., Meagher, R., Swearingen, M., Myint, H., Rich, E., Martinson, J., and Klingemann, H. (2008). Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. Cytotherapy *10*, 625–632.

109. Chu, J., Deng, Y., Benson, D.M., He, S., Hughes, T., Zhang, J., Peng, Y., Mao, H., Yi, L., Ghoshal, K., et al. (2014). 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.

110. Suck, G., Odendahl, M., Nowakowska, P., Seidl, C., Wels, W.S., Klingemann, H.G., and Tonn, T. (2016). NK-92: an 'off-the-shelf therapeutic' for adoptive natural killer cell-based cancer immunotherapy. Cancer Immunol. Immunother *65*, 485–492.

111. Boissel, L., Klingemann, H., Khan, J., and Soon-Shiong, P. (2016). Intra-tumor injection of CAR-engineered NK cells induces tumor regression and protection against tumor re-challenge. Blood *128*, 466.

112. Klingemann, H. (2014). Are natural killer cells superior CAR drivers? OncoImmunology *3*, e28147.

113. Oelsner, S., Friede, M.E., Zhang, C., Wagner, J., Badura, S., Bader, P., Ullrich, E., Ottmann, O.G., Klingemann, H., Tonn, T., and Wels, W.S. (2017). Continuously expanding CAR NK-92 cells display selective cytotoxicity against B-cell leukemia and lymphoma. Cytotherapy *19*, 235–249.

114. Chen, X., Han, J., Chu, J., Zhang, L., Zhang, J., Chen, C., Chen, L., Wang, Y., Wang, H., Yi, L., et al. (2016). A combinational therapy of EGFR-CAR NK cells and oncolytic herpes simplex virus 1 for breast cancer brain metastases. Oncotarget *7*, 27764–27777.

115. Han, J., Chu, J., Keung Chan, W., Zhang, J., Wang, Y., Cohen, J.B., Victor, A., Meisen, W.H., Kim, S.H., Grandi, P., et al. (2015). CAR-engineered NK cells targeting wild-type EGFR and EGFRvIII enhance killing of glioblastoma and patient-derived glioblastoma stem cells. Sci. Rep. *5*, 11483.

116. Genßler, S., Burger, M.C., Zhang, C., Oelsner, S., Mildenberger, I., Wagner, M., Steinbach, J.P., and Wels, W.S. (2015). Dual targeting of glioblastoma with chimeric antigen receptor-engineered natural killer cells overcomes heterogeneity of target antigen expression and enhances antitumor activity and survival. OncoImmunology *5*, e1119354.

117. Tassev, D.V., Cheng, M., and Cheung, N.K. (2012). Retargeting 92 cells using an HLA-A2-restricted, EBNA3C-specific chimeric antigen receptor. Cancer Gene Ther *19*, 84–100.

118. Chen, K.H., Wada, M., Pinz, K.G., Liu, H., Lin, K.W., Jares, A., Firor, A.E., Shuai, X., Salman, H., Golightly, M., et al. (2017). Preclinical targeting of aggressive T cell malignancies using anti-CD5 chimeric antigen receptor. Leukemia, Published online February 10, 2017. http://dx.doi.org/10.1038/leu.2017.8.

119. Uphoff, C.C., Denkmann, S.A., Steube, K.G., and Drexler, H.G. (2010). Detection of EBV, HBV, HCV, HIV-1, HTLV-I and -II, and SMRV in human and other primate cell lines. J. Biomed. Biotechnol. *2010*, 904767.

120. Maki, G., Klingemann, H.G., Martinson, J.A., and Tam, Y.K. (2001). Factors regulating the cytotoxic activity of the human natural killer cell line, NK-92. J. Hematother. Stem Cell Res. *10*, 369–383.

121. Weitzman, J., Betancur, M., Boissel, L., Rabinowitz, A.P., Klein, A., and Klingemann, H. (2009). Variable contribution of monoclonal antibodies to ADCC in patients with chronic lymphocytic leukemia. Leuk. Lymphoma *50*, 1361–1368.

122. Jochems, C., Hodge, J.W., Fantini, M., Fujii, R., Morillon, Y.M., 2nd, Greiner, J.W., Padget, M.R., Tritsch, S.R., Tsang, K.Y., Campbell, K.S., et al. (2016). An NK cell line (haNK) expressing high levels of granzyme and engineered to express the high affinity CD16 allele. Oncotarget *7*, 86359–86373.

123. Lowe, E., Truscott, L.C., and De Oliveira, S.N. (2016). In vitro generation of human NK cells expressing chimeric antigen receptor through differentiation of gene-modified hematopoietic stem cells. Methods Mol. Biol. *1441*, 241–251.

124. Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., and Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science *285*, 727–729.

125. Gasser, S., Orsulic, S., Brown, E.J., and Raulet, D.H. (2005). The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature *436*, 1186–1190.

126. Chang, Y.H., Connolly, J., Shimasaki, N., Mimura, K., Kono, K., and Campana, D. (2013). A chimeric receptor with NKG2D specificity enhances natural killer cell activation and killing of tumor cells. Cancer Res. *73*, 1777–1786.

127. Lindau, D., Gielen, P., Kroesen, M., Wesseling, P., and Adema, G.J. (2013). The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. Immunology *138*, 105–115.

128. Wu, J., Song, Y., Bakker, A.B., Bauer, S., Spies, T., Lanier, L.L., and Phillips, J.H. (1999). An activating immunoreceptor complex formed by NKG2D and DAP10. Science *285*, 730–732.

129. Upshaw, J.L., Arneson, L.N., Schoon, R.A., Dick, C.J., Billadeau, D.D., and Leibson, P.J. (2006). NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. Nat. Immunol. *7*, 524–532.

130. Töpfer, K., Cartellieri, M., Michen, S., Wiedemuth, R., Müller, N., Lindemann, D., Bachmann, M., Füssel, M., Schackert, G., and Temme, A. (2015). DAP12-based activating chimeric antigen receptor for NK cell tumor immunotherapy. J. Immunol. *194*, 3201–3212.

131. Hassuneh, M.R., Nagarkatti, P.S., and Nagarkatti, M. (1997). Evidence for the participation of interleukin-2 (IL-2) and IL-4 in the regulation of autonomous growth and tumorigenesis of transformed cells of lymphoid origin. Blood *89*, 610–620.

132. Baum, C., and Fehse, B. (2003). Mutagenesis by retroviral transgene insertion: risk assessment and potential alternatives. Curr. Opin. Mol. Ther. *5*, 458–462.

133. Wu, C., and Dunbar, C.E. (2011). Stem cell gene therapy: the risks of insertional mutagenesis and approaches to minimize genotoxicity. Front. Med. *5*, 356–371.

134. Stein, S., Ott, M.G., Schultze-Strasser, S., Jauch, A., Burwinkel, B., Kinner, A., Schmidt, M., Krämer, A., Schwäble, J., Glimm, H., et al. (2010). Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. Nat. Med. *16*, 198–204.

135. Braun, C.J., Boztug, K., Paruzynski, A., Witzel, M., Schwarzer, A., Rothe, M., Modlich, U., Beier, R., Göhring, G., Steinemann, D., et al. (2014). Gene therapy

for Wiskott-Aldrich syndrome–long-term efficacy and genotoxicity. Sci. Transl. Med. *6*, 227ra33.

136. Hacein-Bey-Abina, S., Garrigue, A., Wang, G.P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., et al. (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J. Clin. Invest. *118*, 3132–3142.

137. Zhao, Y., Moon, E., Carpenito, C., Paulos, C.M., Liu, X., Brennan, A.L., Chew, A., Carroll, R.G., Scholler, J., Levine, B.L., et al. (2010). Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. Cancer Res. *70*, 9053–9061.

138. Zhou, X., Dotti, G., Krance, R.A., Martinez, C.A., Naik, S., Kamble, R.T., Durett, A.G., Dakhova, O., Savoldo, B., Di Stasi, A., et al. (2015). Inducible caspase-9 suicide gene controls adverse effects from alloreplete T cells after haploidentical stem cell transplantation. Blood *125*, 4103–4113.

139. Kwon, H.J., Kim, N., and Kim, H.S. (2017). Molecular checkpoints controlling natural killer cell activation and their modulation for cancer immunotherapy. Exp. Mol. Med. *49*, e311.

140. Poirot, L., Philip, B., Schiffer-Mannioui, C., Le Clerre, D., Chion-Sotinel, I., Derniame, S., Potrel, P., Bas, C., Lemaire, L., Galetto, R., et al. (2015). Multiplex genome-edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies. Cancer Res. *75*, 3853–3864.

141. Qasim, W., Zhan, H., Samarasinghe, S., Adams, S., Amrolia, P., Stafford, S., Butler, K., Rivat, C., Wright, G., Somana, K., et al. (2017). Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci. Transl. Med. *9*, eaaj2013.

142. Qasim, W., Amrolia, P.J., Samarasinghe, S., Ghorashian, S., Zhan, H., Stafford, S., Butler, K., Ahsan, G., Gilmour, K., Adams, S., et al. (2015). First clinical application of Talen engineered universal CAR19 T cells in B-ALL. Blood *126*, 2046.