Outlook for New CAR-Based Therapies with a Focus on CAR NK Cells: What Lies Beyond CAR-Engineered T Cells in the Race against Cancer

May Daher and Katayoun Rezvani

ABSTRACT

Chimeric antigen receptor (CAR) engineering of T cells has revolutionized the field of cellular therapy for the treatment of cancer. Despite this success, autologous CAR-T cells have recognized limitations that have led to the investigation of other immune effector cells as candidates for CAR modification. Recently, natural killer (NK) cells have emerged as safe and effective platforms for CAR engineering. In this article, we review the advantages, challenges, and preclinical and clinical research advances in CAR NK cell engineering for cancer immunotherapy. We also briefly consider the feasibility and potential benefits of applying other immune effector cells as vehicles for CAR expression.

Significance: CAR engineering can redirect the specificity of immune effector cells, converting them to a much more potent weapon to combat cancer cells. Expanding this strategy to immune effectors beyond conventional T lymphocytes could overcome some of the limitations of CAR T cells, paving the way for safer and more effective off-the-shelf cellular therapy products.

INTRODUCTION

The field of cellular therapy for cancer is growing at an unprecedented pace, especially since the advent of chimeric antigen receptor (CAR)–engineered T cells. This strategy has proven effective against B-cell malignancies and is showing promising activity in clinical trials for other hematologic cancers, with the potential for inducing beneficial responses in solid tumors (1). Some of the obstacles faced in the clinical application of CAR T-cell therapy are cytokine release syndrome (CRS) and immune effector cell–associated neurotoxicity syndrome (ICANS), both of which increase the length of patient hospitalization and the cost of therapy (2). Other limitations of CAR T-cell therapy are the logistic hurdles of generating an autologous product (3) and the fact that further genetic modification is necessary to produce a safe allogeneic T-cell product, given the risk of graft versus host disease (GVHD) mediated by the T-cell receptor (4). The suboptimal results of CAR T-cell therapy for solid tumors have been attributed mainly to the unique challenges presented by the immunosuppressive microenvironment and the physical barriers imposed by the tumor stroma (5). Given these shortcomings of conventional CAR-transduced αβ-T cells, there has been a surge of interest in other candidate immune cell subsets in both the innate and adaptive compartments, as well as improved strategies of CAR engineering. Natural killer (NK) cells have gained much recent attention as a promising alternative platform for CAR engineering owing to their unique biological attributes, their specialized cytotoxicity against tumors, their safety profile and their potential use as an off-the-shelf cellular therapy (3).

In this article, we review the development and performance of CAR-modified NK cells in cancer immunotherapy, discussing their advantages, including both mechanisms of antitumor activity and safety features, as well as the roadblocks and challenges that must be overcome before CAR NK cells can become a central player in the struggle against cancer. We also discuss the progress being made in exploring other immune effector cells as platforms for CAR-based therapies.

CAR NK CELLS IN CANCER THERAPY

NK cells, which represent 5% to 10% of peripheral blood lymphocytes, are an essential component of the innate immune
system and play a critical role in our first-line defense against pathogens and cancer cells (6). As their name implies, NK cells are specialized killers with a natural ability to eliminate abnormal cells that have been compromised by viral infections or malignant transformation. Unlike T cells, NK cells lack expression of the T-cell receptor (TCR) and CD3 and can be identified by surface expression of CD56 and CD16 (7). Depending on their level of CD56 and CD16 expression, NK cells can be further subdivided into two major subsets: CD16+CD56dim, which represents a more mature and cytotoxic subset mostly present in the peripheral blood, and CD16 CD56high, a less mature and more immunoregulatory subset found in tissues (8).

**Intrinsic Killing Capacity**

NK cells are highly specialized cytotoxic cells that rely on the integration of multiple signals from activating receptors, inhibitory receptors, cytokine receptors, and chemokine receptors to recognize and eliminate their targets (9). Once committed to a cytotoxic state, NK cells (unlike T cells) do not require any prior antigen priming before they attack their targets, and therefore can quickly and effectively kill their target cells through a variety of mechanisms (8). NK cells can release cytotoxic granules containing perforin and granzyme, leading to target cell lysis (10). In addition, by releasing members of the tumor necrosis factor (TNF) family of molecules, NK cells upregulate death ligands on their surface, including FAS ligand and TRAIL, which can bind to death receptors on tumor cells, thus activating the caspase pathway to induce apoptosis of target cells (11). Furthermore, upon engagement, NK cells produce IFNγ, which activates the adaptive immune response through its pleiotropic effects on other immune effector cells, such as macrophages and dendritic cells (9). Finally, NK cells have the ability to kill cancer cells through antibody-dependent cellular cytotoxicity (ADCC) mediated by CD16, which binds to the Fc portion of IgG1 antibodies opsonized on the surface of tumor cells (12).

**Engineered Killing Capacity**

Engineering immune cells to express a CAR redirects their specificity and focuses their killing capacity on a particular antigen. The basic structure of a CAR molecule includes an antigen recognition domain comprised of a single-chain variable fragment (scFv), an extracellular hinge domain, a transmembrane domain, and an intracellular signaling domain. Incremental improvements in CAR design can be appreciated from differences in the multiple generations of these constructs. While first-generation CARs consist of the basic structure with one signaling region (13), second-generation CARs contain an additional costimulatory domain such as CD28 or 4-1BB (14, 15), and third-generation CARs possess multiple costimulatory domains (16, 17). Several costimulatory domains have been investigated, including members of the immunoglobulin superfamily (CD28 and ICOS), members of the TNF receptor superfamily (4-1BB, CD27, OX40, and CD40), and others such as CD40L and TLRs (18). Early preclinical studies exploring CAR NK cells used CAR constructs optimized for T-cell signaling and function. Although some signaling/costimulatory domains used in CAR design such as CD3ζ and 4-1BB are shared between NK cells and T cells, the role of other costimulatory molecules such as CD28 in NK cells is less well understood (19). This led many investigators to study costimulatory domains with greater specificity for NK-cell signaling, such as DAP10, DAP12, or 2B4 (refs. 20, 21; Fig. 1). DAP10 and DAP12 are adaptor molecules that contain immunoreceptor tyrosine-based activation motifs (ITAM) and transmit activating signals to NK cells; DAP10 is critical for transmitting signals for the activating receptor NKG2D, while DAP12 mediates signaling through NKG2C, Nkp44, and activating killer immunoglobulin receptors (KIR). 2B4, another activating receptor, belongs to the signaling lymphocytic activation molecule (SLAM) family of proteins, and upon binding to its natural ligand CD48, recruits adaptor molecules such as SLAM-associated protein (SAP) through its immunoreceptor tyrosine-based switch motif (ITSM) to mediate signal transduction (22). NK cells transduced with an anti-CD19 CAR containing either a DAP10 or CD3ζ signaling domain were shown to successfully trigger NK-cell cytotoxicity (23), but the best response was achieved when both domains were included in the CAR construct (24). Likewise, CAR NK cells targeting prostate stem cell antigen (PSCA) displayed enhanced cytotoxicity when DAP12 was incorporated into the CAR construct, compared with results when CD3ζ was used alone (25). Other studies reported superior in vitro and in vivo activity of NK cells expressing 2B4 containing CAR constructs targeting mesothelin (26) or CD5 (27) in the relevant tumor models.

More recently, fourth-generation CARs, also known as “armored CARs” or TRUCKs (T cells Redirected for Universal Cytokine Killing), have been investigated (28). These constructs incorporate a transgenic “payload” designed to improve the proliferation, persistence, and antitumor activity of CAR-engineered NK cells. Results published to date support the superior activity of these latest revisions to CAR design, with the promise of broadening the role of CAR-engineered NK cells within the available repertoire of cancer immunotherapies (29, 30).

**SAFETY FEATURES OF CAR NK CELLS**

**Intrinsic Features**

NK-cell killing is specific for transformed or otherwise abnormal cells in the body. To recognize and spare healthy cells, NK cells rely primarily on inhibitory receptors [such as killer cell immunoglobulin-like receptors (KIR) and NKG2A] that bind to MHC class I or class I-like molecules that are constitutively present on normal cells and act as a safety switch to inhibit NK-cell cytotoxicity (9, 31). As opposed to T cells, NK cells do not induce GVHD, opening the way for their broad application in the allogeneic settings and for the generation of off-the-shelf cellular therapy products (3). More recently, NK cells were also demonstrated to have an important edge over T cells as platforms for CAR engineering, as they lacked any evidence of serious toxicities, including CRS or ICANS (29).

**Engineered Features**

Suicide genes have been investigated as safety switches mainly in long-lived genetically modified effectors, such as CAR T cells. NK cells, in contrast, are typically short-lived and safe, raising questions as to the need for a suicide switch in adoptive NK-cell therapy (32). Use of inducible caspase 9...
CAR NK Cells and Other Alternative CAR-Based Platforms

REVIEW

(iCas9), activated by a chemical inducer of dimerization (CID; ref. 33), has proven effective at eliminating CAR NK cells both in vitro and in vivo in preclinical studies (30, 34). In the first-in-human clinical trial of CAR NK cells for the treatment of relapsed/refractory B-cell lymphoid malignancies, our group tested cord blood–derived NK cells transduced with a retroviral vector encoding a single-chain variable fragment (scFv) against CD19, IL15 to enhance proliferation and in vivo persistence, and iCas9 as a cell suicide switch. Given further advances in construct design, nonviral methods of cell engineering and gene editing technologies, it is likely that future generations of CAR T and CAR NK cells will induce fewer toxicities, eliminating the need for suicide genes or other types of safety switches; nonetheless, it is possible that fourth-generation CARs could cause unanticipated toxicity due to excessive cytokine production, supporting incorporation of a suicide gene in certain cases, depending on the construct and the cytokine transgene.

**CHALLENGES FACING CAR NK-CELL THERAPY**

**Limited Persistence**

One of the major limitations of adoptive NK-cell therapy is the lack of in vivo persistence of the infused cells in the absence of cytokine support. Although this feature might be desirable from a safety standpoint, it may well limit the efficacy of the NK-cell immunotherapy. The administration of exogenous cytokines has been shown to enhance the proliferation and persistence of adoptively infused NK cells; however, it can also induce undesirable toxicities (35), as well as the expansion of other immune subsets that might be immunosuppressive, such as regulatory T cells (Tregs; ref. 36). Hence, several groups have reported encouraging results by engineering NK cells with transgenes encoding for cytokines that are either expressed on the membrane or secreted constitutively. In one study, NK-92 cell lines or primary NK cells transduced with retroviral vectors expressing IL2 or IL15 showed enhanced proliferation and persistence in tumor-bearing mice (37). Our group has shown that incorporating the IL15 transgene into the CAR construct enhances NK-cell proliferation and in vivo persistence with improved antitumor activity, without increasing systemic levels of IL15 or toxicity in patients with high-risk lymphoid malignancies (29, 30). Other armored CAR NK cells engineered with cytokine transgenes are under development, but published reports are not yet available. Another strategy to improve the persistence of NK cells is to endow them with a memory-like phenotype, for example, by briefly preactivating them with a

![Figure 1.](image-url) NK cell–specific signaling domains incorporated within CAR intracellular domains. Image created in BioRender.com. DAP12, DNAX Activating Protein of 12 KDa; DAP10, DNAX Activating Protein of 12 KDa; ITAM, immunoreceptor tyrosine-based activation motif; ITSM, immunoreceptor tyrosine-based switch motif; YXXM, Y stands for tyrosine, X for any amino acid residue, and M for methionine; TRAF1–3, tumor necrosis factor receptor–associated factors 1, 2, and 3.
cytokine cocktail (IL12, IL15, and IL18) to induce differentiation into cytokine-induced memory-like NK cells (38, 39). Recently these memory-like NK cells were engineered to express a CAR directed against CD19 and showed enhanced responses against NK-resistant B-cell lymphoma in vitro and in vivo (40).

** Trafficking to Tumor Sites **

Rapid homing to tumor beds is critical for the efficacy of adoptive cellular therapy and is regulated by complex interactions between chemokines secreted by NK cells with those secreted by tumor cells (41). Controversy over the effectiveness of NK-cell homing to tumor sites has stimulated efforts to improve this property (42). For example, transfer of the chemokine receptor CCR7 from K562 feeder cells to NK cells via trogocytosis resulted in enhanced homing of NK cells to lymph nodes (43). Similarly, upregulation of CXCR3 on NK cells following ex vivo expansion with irradiated EBV-LCL feeder cells and IL2 resulted in enhanced trafficking and improved antitumor activity in a xenograft mouse model of CXCL10-transfected melanoma (44). Since then, several groups have explored different engineering strategies to improve NK-cell homing. For example, to improve migration of NK cells toward lymph nodes that express the chemokine CCL19, NK cells were electroporated with mRNA coding for the chemokine receptor CCR7 (45). Similarly, NK cells transduced with a CXCR2-encoding viral vector had improved migration to renal cell carcinoma tumors expressing cognate ligands such as CXCL1, CXCL2, CXCL5, CXCL6, and CXCL8 (46). Another strategy applied an NK-cell–recruiting protein-conjugated antibody (NRP-body) that incorporates a cleavable CXCL16 molecule to increase trafficking and infiltration of NK cells into pancreatic tumors (47). This chemokine is cleared by furin, an endoprotease expressed on the surface of pancreatic cancer cells, leading to induction of NK-cell infiltration through RhoA activation via the ERK signaling cascade. This approach was shown to enhance NK-cell–mediated tumor control in a mouse model of pancreatic cancer (47). CAR NK cells are also being engineered to enhance their trafficking to sites of tumor. Muller and colleagues, for example, showed that anti-EGFRvIII CAR NK cells engineered to express CXCR4 conferred specific chemotaxis to CXCL12/SDFα-secretion-glioblastoma cells, leading to improved tumor regression and survival in a mouse model of glioblastoma (48). Finally, NKG2D CAR NK cells engineered to express CXCR1 significantly increased antitumor responses in mice bearing established peritoneal ovarian cancer xenografts (49). Thus, a number of innovative strategies to increase the trafficking of NK cells to the tumor sites have been tested in mouse models; the efficacy of these approaches to improve the success of NK-cell immunotherapy against solid tumors in patients will need to be validated in clinical trials.

** The Immunosuppressive Tumor Microenvironment **

The tumor microenvironment poses major obstacles to successful CAR NK-cell therapy, including immunosuppressive soluble substances, immunosuppressive cells, and an unfavorable milieu for adequate immune cell function. With regard to soluble factors, the tumor microenvironment is rich in immunosuppressive cytokines and metabolites, such as TGFβ, adenosine, indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2), all of which can adversely affect NK-cell function (50). Certain types of cells present in this malignant milieu, including regulatory T cells, regulatory B cells, myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM), platelets, and fibroblasts also induce immunosuppression (51), as do a number of unfavorable metabolic factors, including hypoxia, acidity, and nutrient deprivation (52). Thus, efforts are under way to engineer CAR NK cells so that they can circumvent some of these immunosuppressive factors. One promising approach has been to engineer NK cells to render them resistant to the action of TGFβ. Our group used CRISPR/Cas9 technology to delete the TGFβ receptor 2 gene (TGFβR2) in primary human NK cells, which rendered them resistant to this immunosuppressive growth factor without loss of their effector activity against acute myeloid leukemia (AML; ref. 53). Similarly, NK cells modified to express a dominant-negative TGFβ receptor, a high-affinity non–signal transducing receptor derived from TGFβR2, antagonized the suppressive effects of TGFβ on NK cells and restored their cytotoxicity (54). Another strategy, using an anti-miRNA against miR-27a-5p, a miRNA that is upregulated by TGFβ in NK cells (55), increased NK-cell effector function both in vitro and in vivo (55). Finally, adenosine, an important immunosuppressive metabolite generated from ATP by the ectonucleotidases CD73 and CD39 in response to hypoxia and stress (56), has been targeted by blocking the high-affinity A2A adenosine receptor on NK cells, resulting in more potent antitumor activity in mouse models of breast cancer, melanoma, and fibrosarcoma (57, 58). Checkpoint molecule engagement is another key mechanism by which the tumor microenvironment induces NK-cell dysfunction (59). Gene editing technologies are therefore being used to delete checkpoint molecules within NK cells as a means to enhance their function. For instance, TIGIT knockout was shown to protect against NK-cell exhaustion and improve outcome in tumor-bearing mice (60). Others have targeted NKG2A and reported enhanced cytotoxicity of NKG2A−/− NK cells against HLA-E–expressing tumors (61, 62). Work by our group and others suggests that an effective approach to enhancing NK-cell antitumor activity would be to combine CAR engineering with checkpoint deletion (by targeting CIS, a negative regulator of cytokine signaling; refs. 63–66). Other inhibitory molecules under investigation in NK cells include the classic T-cell checkpoints PD-1 and CTLA4 (67, 68). The application of checkpoint blockade to enhance NK-cell effector function is reviewed in depth by Vivier and colleagues in this issue of Cancer Discovery (69).

The studies discussed above all indicate that the biological limitations of NK cells and the challenges imposed by the tumor microenvironment can be circumvented by innovative engineering techniques and gene editing technologies. Strategies to enhance NK-cell persistence, trafficking to tumor sites, and effector function in a hostile and malignant milieu are likely to transform adoptive therapy with NK cells from a safe treatment with only modest efficacy to a serious contender for a first-line role in cancer immunotherapy.

** EVOLUTION OF CAR NK-CELL THERAPY **

A variety of different cell sources have been used to generate CAR-expressing NK cells. These include peripheral blood,
CAR NK Cells and Other Alternative CAR-Based Platforms

Table 1. Sources of CAR NK cells under investigation in the clinic

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Potential for OTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood</td>
<td>Mature phenotype</td>
<td>Only 5%–10% of PB lymphocytes are NK cells</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Highly functional and cytotoxic</td>
<td>Heterogenous product</td>
<td></td>
</tr>
<tr>
<td>Cord blood</td>
<td>Readily available from global CB banks</td>
<td>Numerically few and therefore requires ex vivo</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>15%–30% of CB lymphocytes are NK cells</td>
<td>expansion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcriptomic profile supports high proliferative potential</td>
<td>Heterogeneous product</td>
<td></td>
</tr>
<tr>
<td>iPSC</td>
<td>High proliferative capacity</td>
<td>Immature phenotype</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Homogeneous product</td>
<td>Low ADCC due to low CD16 expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Long culture condition</td>
<td></td>
</tr>
<tr>
<td>NK cell line (NK-92)</td>
<td>High proliferative capacity</td>
<td>Derived from a patient with NK lymphoma</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Easy to manipulate and engineer</td>
<td>Need for irradiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Homogeneous product</td>
<td>Limited in vivo persistence following irradiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduced sensitivity to freeze/thaw cycles</td>
<td>Low ADCC due to low or absent CD16 expression</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CB, cord blood; iPSC, induced pluripotent stem cells; OTS, off-the-shelf; ADCC, antibody-dependent cellular cytotoxicity.

cord blood, stem cells including hematopoietic or induced pluripotent stem cells, and NK cell lines, each with their unique advantages and disadvantages (Table 1).

Strategies to Combat Hematologic Malignancies

As with CAR-modified T lymphocytes, CAR NK cells were first tested against hematologic malignancies, with preclinical data confirming their activity against leukemia, lymphoma, and myeloma. To treat B-cell malignancies, we engineered NK cells from cord blood using a retroviral vector that expressed a fourth-generation CAR vector (iC9.CAR19.CD28-ζ-IL15; ref. 70) targeting the CD19 antigen and incorporating the IL15 transgene to enhance proliferation and the inducible caspase 9 transgene (iC9) to provide a safety switch (30). The resultant CAR NK cells showed significantly enhanced persistence and antitumor efficacy in a xenograft mouse model of Raji lymphoma, compared with nontransduced NK cells (30). Other groups engineered NK-92 cell lines with an anti-CD19 CAR construct that incorporated a CD3ζ signaling endodomain either alone or with a costimulatory domain (CD28 or CD137), and found that they displayed effective antitumor activity against B-cell malignancies in vitro and in vivo in a Raji lymphoma mouse model (71, 72).

In addition, CAR NK cells could offer an advantage over CAR T cells in the treatment of T-lymphoid cancers because they circumvent the issue of fratricide arising from shared antigens between normal and malignant T cells. Indeed, NK-92 or NK-92MI (engineered to express IL2; ref. 73) cells transduced with a lentiviral vector encoding a second- or third-generation CAR targeting CD5 and a CD3ζ signaling domain containing a CD28, 4-1BB, or 2B4 costimulatory domain produced effective in vitro cytotoxicity against patient-derived T-cell malignancies and improved survival in a xenograft mouse model of Jurkat lymphoma (27, 74). Similarly, NK-92MI cells transduced with anti-CD7 CARs, designed using nanobody technology, had potent in vitro and in vivo activity against T-cell leukemia (75).

Unfortunately, despite the remarkable activity against lymphoid malignancies, CAR-engineered T cells have been much less effective in AML (76). These disappointing results can be attributed, in part, to shared expression of antigen (e.g., CD123) on AML blasts and normal HSCs, and heterogeneous expression of target antigen (e.g., CD33) on blasts (77). Fortuitously, AML blasts are susceptible to NK cell–mediated killing because they express ligands recognized by activating receptors on NK cells (78). Hence, CAR NK cells may possess features that could overcome some of the obstacles related to antigen escape and heterogeneous gene expression that have thwarted efforts to improve the outcome of AML immunotherapy. To meet this challenge, our group and others have demonstrated the antileukemic activity of third-generation (CD28.BBζ) or fourth-generation (CD28ζ-IL15) CAR NK cells targeting CD123 both in vitro and in vivo (79, 80). Others targeted CD4, a T-cell marker that can also be expressed by a subset of AML blasts, using third-generation CAR NK cells (CD28.BBζ), leading to potent antileukemic activity both in vitro and in a xenograft mouse model of CD4+ AML (81). It is important to note that AML stem cells have also evolved mechanisms to evade NK-cell recognition, such as down-regulation of NKG2D ligands, which may, in turn, limit the efficacy of CAR NK-cell therapy (82).

Multiple myeloma may also be especially sensitive to CAR NK-cell therapy, as myeloma cells express multiple ligands for NK receptors (83). In fact, NK-92 cells transduced with a lentiviral vector encoding a first-generation anti-CD138 CAR or a second-generation anti-CS1 CAR.CD28ζ exerted effective in vitro and in vivo activity against myeloma (84, 85).

In summary, the intrinsically favorable biologic features of NK cells and the added specificity afforded by CAR expression, leading to potent in vitro and in vivo activity against various hematologic cancer models provided the impetus...
to initiate phase I and II clinical trials of this therapy and to undertake exploratory studies of CAR NK cells against solid tumors.

**Strategies to Combat Solid Tumors**

In contrast to liquid tumors, solid tumors and their supporting stroma possess the means to evade all but the most innovative immunotherapies. These tactics include the secretion of inhibitory cytokines (e.g., TGFβ) by tumors cells or by cells in the immediate microenvironment (or both), extreme cellular heterogeneity with an abundance of resistant stem-like progenitor cells, and the downregulation of cognate ligands that can stimulate a response from immune effectors such as NK cells. Thus, Kruschinski and colleagues engineered HER-2-specific CAR NK cells (HER-2-CD3ζ-CD28) that mediated effective in vitro and in vivo activity in a mouse model of ovarian cancer (86). To overcome the deficient trafficking of NK cells to the tumor site (86), Han and colleagues administered EGFR-redirected CAR NK cells intracranially and confirmed their safety and efficacy in a mouse model of glioblastoma (87). In efforts to enhance the potency of NK cells and strengthen their CAR-specific signaling, Li and colleagues devised an antisenseoligohelix CAR containing the CD3ζ signaling domain and the 2B4 costimulatory domain, together with the transmembrane domain of the activating NK-cell receptor NKG2D. When transduced with this construct, iPSC-derived NK cells mediated strong antitumor effects in an ovarian cancer xenograft model (26). These successes have not been matched by other investigators using different preclinical solid tumor models. For instance, while NK cells expressing a second- or third-generation GD2-directed CAR (GD2-t2B4ζ, GD2-BBζ or GD2-t2B4.BBζ) could kill several GD2+ cancer cell lines in vitro, they failed to control tumors in a xenograft mouse model (88). This suboptimal activity was subsequently linked to the upregulation of HLA-G (the ligand for the inhibitory KIR2DL4) in studies with Ewing sarcoma cells, leading to NK-cell inhibition and immune escape (88, 89).

One of the major obstacles to reliable preclinical evaluation of CAR therapy for solid cancers is the lack of clinically relevant animal models that recapitulate the complexity of the interactions within the tumor microenvironment. Most studies have relied on transplanted xenografts derived from human tumor cell lines in immune-compromised NOD scid gamma null (NSG) mice that lack a competent immune system. While the extant NSG models are useful for rapid evaluation of CAR effector function and persistence, they fail to establish a clinically relevant tumor microenvironment that would yield an accurate estimate of CAR NK cell function and persistence and allow the exploration of the cross-talk among the different immune cells and the tumor.

**Clinical Evaluation of CAR NK Cells**

Despite growing experimental evidence to support the use of anti-CD19 CAR NK cells in patients with B-cell malignancies, this concept was not tested in the clinic until recently. Using HLA-mismatched NK cells from cord blood, our group led a first-in-human phase I and II clinical trial of CAR NK-cell therapy in patients with relapsed/refractory B-cell hematologic malignancies (ClinicalTrials.gov number NCT03056339). The NK cells were transduced with a retroviral vector encoding (i) a CAR against the CD19 antigen, (ii) IL15 to enhance NK-cell persistence and function, and (iii) inducible caspase 9 (iCas9) as suicide gene safety switch (29). Of the 11 heavily pretreated patients with non-Hodgkin lymphoma (NHL) or chronic lymphocytic leukemia ( CLL), 8 responded, for an overall response rate of 73%, with 7 achieving a complete remission (64%). These responses were rapid and seen at all dose levels (1 × 10⁵ to 1 × 10⁷ per kg). Response durations could not be reliably assessed because patients received other postremission therapies. Importantly, serious toxicities, including CRS, ICANS, or GVHD, did not develop in any of the patients, obviating the need to activate the safety switch in the small group of patients treated to date on this trial (29). In summary, the interim results of this trial show that CAR NK cells can induce complete responses in patients with high-risk CD19+ cancers with relatively few adverse side-effects. Larger multicenter studies are planned to validate the safety and efficacy of this approach in lymphoid malignancies and to ascertain the long-term durability of the response in the absence of maintenance or consolidation therapy. Our group is also working to use this platform to target antigens beyond CD19. Data on the use of CAR NK-cell therapy to target other hematologic and solid malignancies are limited. In a phase I study of NK-92 cells transduced with a third-generation CAR targeting CD33 with CD28 and 4-1BB costimulatory domains, Tang and colleagues treated 3 patients with relapsed or refractory AML (90). The investigators report the safety of this approach, but no durable responses. As pointed out by the authors, one of the disadvantages of using NK-92 cells for cellular therapy is the need for irradiating this cancer-derived cell line, which may have affected the persistence and efficacy of the infused product (90). Among the nearly 20 clinical trials now evaluating CAR NK cells as therapy for a diverse set of cancers (Table 2), 8 are targeting CD19 or CD22 (B-cell malignancies), 1 BCMA (multiple myeloma; NCT03940833), 1 CD33 (AML; NCT02944162), 1 CD7 (T-cell leukemia and lymphoma; NCT02742727), 1 HER2 (glioblastoma; NCT03383978), 1 prostate-specific membrane antigen (PSMA; prostate cancer; NCT03692663), 1 mesothelin (ovarian cancer; NCT03692637), 1 MUC1 (multiple solid tumors; NCT02839954), 1 NKG2D (multiple solid tumors; NCT03415100), and 2 ROBO1 (1 for multiple solid tumors; NCT03940820 and 1 for pancreatic cancer; NCT03941457). With few exceptions, these trials are testing strategies that target single antigens only. It will be interesting to learn whether the risk of immune escape with CAR NK cells will be lower than with CAR T cells owing to the innate ability of NK cells to recognize tumors through their germline-encoded receptors.

**ALTERNATIVE CELL TYPES FOR CAR EXPRESSION**

Just as NK cells have emerged as promising candidates for CAR expression, other cell populations possess specific advantages that may augment the current repertoire of CAR-based therapies (Fig. 2). Indeed, a number of early-phase clinical trials are anticipated or under way to investigate these new immune effectors (Table 2).
## Table 2. Clinical trials of CAR-engineered immune effector cells

<table>
<thead>
<tr>
<th>CAR vehicle NCT identifier</th>
<th>Clinical trial phase</th>
<th>Cancer type</th>
<th>Antigen target</th>
<th>Cell source</th>
<th>Construct/Method</th>
<th>Dose</th>
<th>Status</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT03692767</td>
<td>Early phase I</td>
<td>Refractory B-cell lymphoma</td>
<td>CD22</td>
<td>Unknown</td>
<td>CAR.CD19-CD28-41BB-CD3ζ</td>
<td>50–600×10³/kg</td>
<td>Not yet recruiting</td>
<td>Unknown</td>
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<tr>
<td>NCT03690310</td>
<td>Early phase I</td>
<td>Refractory B-cell lymphoma</td>
<td>CD19</td>
<td>Unknown</td>
<td>CAR.CD19-CD28-41BB-CD3ζ</td>
<td>50–600×10³/kg</td>
<td>Not yet recruiting</td>
<td>Unknown</td>
</tr>
<tr>
<td>NCT03824964</td>
<td>Early phase I</td>
<td>Refractory B-cell lymphoma</td>
<td>CD19</td>
<td>Unknown</td>
<td>CAR.CD19-CD28-41BB-CD3ζ</td>
<td>50–600×10³/kg</td>
<td>Not yet recruiting</td>
<td>Unknown</td>
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<tr>
<td>NCT02892695</td>
<td>Phase I/II</td>
<td>ALL, CLL, follicular lymphoma, mantle cell lymphoma, B-PLL, DLBCL</td>
<td>CD19</td>
<td>NK-92 cell line</td>
<td>CAR.CD19-CD28-41BB-CD3ζ</td>
<td>Unknown</td>
<td>Not yet recruiting</td>
<td>Unknown</td>
</tr>
<tr>
<td>NCT03056339</td>
<td>Phase I/II</td>
<td>ALL, CLL, NHL</td>
<td>CD19</td>
<td>Cord blood</td>
<td>CAR.CD19-CD28-41BB-CD3ζ, iCasp9-IL15</td>
<td>3 dose levels: 10⁵/kg, 10⁶/kg, 10⁷/kg</td>
<td>Phase I portion completed</td>
<td>MD Anderson Cancer Center, Houston, TX</td>
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<tr>
<td>NCT01974479</td>
<td>Phase I</td>
<td>B-ALL</td>
<td>CD19</td>
<td>Haploidentical donor</td>
<td>CAR.41BB-CD3ζ</td>
<td>0.5–5×10⁷/Kg, and up to 1×10⁸/Kg</td>
<td>Suspended for interim review Completed</td>
<td>Singapore</td>
</tr>
<tr>
<td>NCT00995137</td>
<td>Phase I</td>
<td>B-ALL</td>
<td>CD19</td>
<td>Haploidentical donor</td>
<td>CAR.41BB-CD3ζ</td>
<td>Unknown</td>
<td>Recruiting</td>
<td>St Jude Children's Research Hospital, Memphis, TN</td>
</tr>
<tr>
<td>NCT04245722</td>
<td>Phase I</td>
<td>B-cell lymphoma, CLL</td>
<td>CD19</td>
<td>iPSC-derived NK cells</td>
<td>CAR.19-NKG2D-2B4-CD3ζ-IL15RF-hnCD16</td>
<td>Dose escalation, exact doses unknown</td>
<td>Recruiting</td>
<td>University of Minnesota Masonic Cancer Center, Minnesota</td>
</tr>
<tr>
<td>NCT02742727</td>
<td>Phase I/II</td>
<td>AML, preT-ALL, T-PLL, T-cell LGL, PTCL, angioimmunoblastic T-cell lymphoma, extranodal NK/T-cell lymphoma (nasal type), enteropathy-type intestinal T-cell lymphoma, hepatosplenic T-cell lymphoma</td>
<td>CD7</td>
<td>NK-92 cell line</td>
<td>CAR.CD7-CD28-41BB-CD3ζ</td>
<td>Unknown</td>
<td>Unknown</td>
<td>China</td>
</tr>
<tr>
<td>NCT03940833</td>
<td>Phase I/II</td>
<td>Multiple myeloma</td>
<td>BCMA</td>
<td>NK-92 cell line</td>
<td>CAR.CD33-CD28-41BB-CD3ζ</td>
<td>Unknown</td>
<td>Recruiting</td>
<td>China</td>
</tr>
<tr>
<td>NCT02944162</td>
<td>Phase I/II</td>
<td>AML</td>
<td>CD33</td>
<td>NK-92 cell line</td>
<td>CAR.CD33-CD28-41BB-CD3ζ</td>
<td>Unknown</td>
<td>Unknown</td>
<td>China</td>
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(continued)
### Table 2. Clinical trials of CAR-engineered immune effector cells (Continued)

<table>
<thead>
<tr>
<th>CAR vehicle NCT identifier</th>
<th>Clinical trial phase</th>
<th>Cancer type</th>
<th>Antigen target</th>
<th>Cell source</th>
<th>Construct/Method</th>
<th>Dose</th>
<th>Status</th>
<th>Location</th>
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</thead>
<tbody>
<tr>
<td>NCT02839954</td>
<td>Phase I/II</td>
<td>Hepatocellular carcinoma, non-small cell lung cancer, pancreatic carcinoma, triple-negative invasive, breast carcinoma, glioblastoma, colorectal carcinoma, gastric carcinoma</td>
<td>MUC1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>China</td>
</tr>
<tr>
<td>NCT03692663</td>
<td>Early phase I</td>
<td>Castration-resistant prostate cancer</td>
<td>PSMA</td>
<td>Unknown</td>
<td>Unknown</td>
<td>0.5–3 × 10^7/kg</td>
<td>Not yet recruiting</td>
<td>Unknown</td>
</tr>
<tr>
<td>NCT03692637</td>
<td>Early phase I</td>
<td>Epithelial ovarian cancer</td>
<td>PSMA</td>
<td>Unknown</td>
<td>Unknown</td>
<td>0.5–3 × 10^7/kg</td>
<td>Not yet recruiting</td>
<td>Unknown</td>
</tr>
<tr>
<td>NCT03415100</td>
<td>Phase I</td>
<td>Solid tumors</td>
<td>NKG2D ligands</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Not yet recruiting</td>
<td>Unknown</td>
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</tr>
<tr>
<td>NCT03883978</td>
<td>Phase I</td>
<td>Glioblastoma</td>
<td>ROBO1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Recruiting</td>
<td>Germany</td>
<td></td>
</tr>
<tr>
<td>iNKT cells</td>
<td>Phase I</td>
<td>Relapsed/refractory B-cell malignancies</td>
<td>CD19</td>
<td>Allogeneic NKT cells</td>
<td>CAR.19-C28-CD3ζ-IL15</td>
<td>4 dose levels: 1 × 10^7/m^2, 3 × 10^6/m^2, 3 × 10^7/m^2</td>
<td>Recruiting</td>
<td>Baylor-Methodist-Texas Children's</td>
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<tr>
<td>NCT03294954</td>
<td>Phase I</td>
<td>Relapsed/refractory neuroblastoma</td>
<td>GD2</td>
<td>Autologous NKT cells</td>
<td>CAR.GD2-C28-CD3ζ-IL15</td>
<td>4 dose levels: 3 × 10^6/m^2, 1 × 10^7/m^2, 3 × 10^7/m^2</td>
<td>Recruiting</td>
<td>Baylor-Methodist-Texas Children's</td>
</tr>
<tr>
<td>γδ T cells</td>
<td>Phase I</td>
<td>B-cell leukemia and lymphoma</td>
<td>CD19</td>
<td>Allogeneic γδ T cells</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Not yet recruiting</td>
<td>China</td>
</tr>
<tr>
<td>NCT04107142</td>
<td>Phase I</td>
<td>Solid tumors</td>
<td>NKG2D ligands</td>
<td>Unknown</td>
<td>Unknown</td>
<td>3 × 10^2–3 × 10^3 cells</td>
<td>Not yet recruiting</td>
<td>Malaysia</td>
</tr>
</tbody>
</table>

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCMA, B-cell maturation antigen; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; hCD16, high-affinity, noncleavable CD16; IL15RF, IL15 receptor alpha; NHL, non-Hodgkin lymphoma; PLL, prolymphocytic leukemia; PTCL, prolymphocytic T-cell leukemia; PSMA, prostate specific membrane antigen; T-LGL, T-cell large granular leukemia.
iNKT Cells

Invariant NK T cells (iNKT) represent a small subset of specialized T cells that possess unique features of both innate and adaptive immune cells. They can mount a rapid response to antigenic exposure much like innate immune cells, but can also display precise antigen recognition in the manner of adaptive cells (91). Similar to conventional T cells, iNKT cells undergo thymic development and selection and harbor a TCR to recognize antigens; however, unlike conventional T cells, their TCR recognizes lipid antigens presented by CD1d, a monomorphic MHC class 1–like molecule (91). In addition to TCR stimulation, iNKT cells can be activated through a variety of cytokine receptors (e.g., IL12, IL18, IL23, and IL25) similar to innate immune cells such as NK cells and innate lymphoid cells (ILC; ref. 92). Hence, in the tumor microenvironment, upon sensing a breach in tissue integrity via recognition of endogenous lipids, iNKT cells become activated and can mediate effective antitumor immunity via direct tumor lysis or indirectly through secretion of an array of cytokines that can modulate other immune cells in the malignant milieu, thus aiding in the host immune response (93). Another advantage of iNKT cells over conventional T cells is the fact that they are not associated with GVHD (94), making them an attractive option as an allogeneic off-the-shelf cell therapy product.

iNKT cells may also be useful platforms for CAR engineering (95, 96). Heczey and colleagues demonstrated that when transduced with an anti-GD2 CAR, especially one with a third-endodomain (95, 96). Heczey and colleagues demonstrated that when transduced with an anti-GD2 CAR, especially one with a third-endodomain, iNKT cells displayed potent antitumor activity in a metastatic neuroblastoma mouse model, without evidence of GVHD (96). Given a hallmark of iNKT cells is their production of both Th1 (inflammatory) and Th2 (anti-inflammatory) cytokines, the authors showed that a CD3ζ only or a CD28-CD3ζ endodomain skewed the CAR-iNKT cell toward Th2 production, whereas 41BB-CD3ζ or CD28-41BB-CD3ζ polarized the iNKT cells toward a Th1 cytokine profile (96). The same group also demonstrated that coexpression of IL15 with an anti-GD2 CAR (with CD28 or 4-1BB costimulatory domain) enhances iNKT cell persistence and antitumor activity in a neuroblastoma xenograft mouse model (97). These results led to an ongoing phase I clinical trial to evaluate iNKT cells transduced with a vector expressing a GD2-specific CAR and IL15 in children with neuroblastoma (NCT03294954). Early results are encouraging with one of two patients treated to show early clinical results show no GVHD, no CRS, or no ICANS (96).

Macrophage

γδ T Cells

γδ T cells link innate and adaptive immune responses. They represent 1% to 5% of all circulating T cells, but are the predominant lymphocyte at epithelial surfaces (99). Contrary to γδ TCR activation, which requires MHC-bound peptides, γδ TCRs are triggered in an MHC-independent fashion (100). For instance, γδ T cells that are highly prevalent in the adult peripheral blood are activated by aminobisphosphonates, such as the cholesterol precursor isopentylpyrophosphate.
Zoledronic acid (ZOL) interferes with cholesterol synthesis, resulting in accumulation of isopentylpyrophosphate, and therefore can be incorporated into expansion protocols to generate large numbers of these γδ T cells from PBMCs (101). Given that this approach may lead to the preferential amplification of γδ2T cells, several groups have developed alternative protocols to allow for expansion of multiple γδ T-cell subtypes concomitantly. Some of the reported strategies include expansion with the help of feeder cells such as K562 cells engineered to express costimulatory ligands and membrane-bound IL15, the use of an anti-CD2 mAb, or the T-cell mitogen concanavalin A (ConA; ref. 102). Collectively, these protocols enable the production of large numbers of γδ T cells needed for an effective adoptive T-cell therapy.

The unique features described above put γδ T cells in the spotlight as potential platforms for CAR engineering. In the first published report, investigators expanded γδ2T cells from peripheral blood using ZOL, transduced them with a retroviral vector encoding an anti-CD2 or anti-CD19 first-generation CAR, and showed an enhanced antitumor response against Ewing sarcoma cells and B-cell (CD19+) tumor cell lines in vitro (103). Another group showed that CAR-transduced γδ T cells can retain the ability to cross-present the processed peptides from uptaken tumor antigens to responder αβ T cells (104).

Another strategy, designed by Deniger and colleagues, relied on PBMCs electroporated with a sleeping beauty transposase/ transposase system to express a second-generation anti-CD19 CAR with a CD28-CD3ζ endodomain; CARγδ γδ T cells were sort-purified and propagated using K562-based feeder cells (105). The authors showed that these anti-CD19 CAR-transduced γδ T cells can improve tumor control in a CD19+ leukemia (NALM6) xenograft mouse model (105).

Two phase I clinical studies of γδ T cells are anticipated. One will evaluate the safety and efficacy of allogeneic anti-CD19 CAR γδ T cells for the treatment of B-cell hematologic malignancies (NCT02656147), while the other will test escalating doses of haploidentical or allogeneic CAR-transduced γδ T cells targeting the natural killer group 2D ligand (NKG2D) in patients with relapsed or refractory solid tumors (NCT04107142).

Macrophages

Macrophages (Greek for “big eaters”) are specialized innate immune cells responsible for detecting and engulfing cellular debris, pathogens, and cancer cells (106). Unlike T cells and other immune effectors, macrophages have the advantage of being able to readily penetrate solid tumors (107) in response to tumor-derived chemokines such as colony-stimulating factor-1 (CSF-1), CCL2, CCL3, CCL4, CCL5, CCL8, and VEGF (108). Recent preclinical data demonstrate that macrophages can be engineered with a CAR (CAR-Ms) incorporating a CD3ζ intracellular domain to direct their phagocytic activity specifically against tumors (109, 110). The authors of this study used an adenovirus vector (Ad5f35) to transduce macrophages with a CAR and showed that this strategy polarized the macrophages into the M1 immunostimulatory phenotype without reversion to the M2 immunosuppressive phenotype. In a xenograft mouse model of HER2+ ovarian cancer (SKOV3), mice treated with CAR-Ms targeting HER2 had better tumor control and improved survival compared with untreated mice or mice treated with unmodified control macrophages (109, 110). Another group showed that engineering macrophages with a CAR-P (P, phagocytosis) by using intracellular domains from engulfment receptors (MEGF10 and FcRγ) could induce specific engulfment of tumors expressing the antigen to which the scFv is targeted (111). These investigators found that adding a tandem PI3K recruitment domain to the CAR-P intracellular signaling enhanced tumor engulfment further. This directed phagocytosis led to better tumor control in vitro than that induced by unmodified macrophages (111). Preclinical animal data are needed to further test the in vivo antitumor activity of these CAR-P macrophages. Still others, using lentiviral or adenoviral vectors, engineered monocyte-derived human macrophages with a CAR construct (MOTO-CAR) that contains a Toll/IL1 receptor (TIR) signaling domain, demonstrating enhanced phagocytosis (compared with the control) against TK1-expressing non–small cell carcinoma cell lines (112). These encouraging preclinical data suggest that CAR-engineered macrophages have the potential to become useful immunotherapeutic agents, especially in the setting of typically resistant solid tumors.

Dendritic Cells

Dendritic cells (DC) are professional antigen-presenting cells that efficiently process antigens and present them on their surface to activate the adaptive immune system (113). They also possess the unique ability to interact with multiple immune cell subsets and regulate their function, including CD4 and CD8 T cells in lymph nodes, resulting in downstream B-cell activation and antibody secretion, as well as activation of NK cells and phagocytes (114). Because of these unique features, DCs have been used in multiple clinical trials as therapeutic vaccines in the setting of various solid tumors (e.g., prostate cancer, melanoma, glioblastoma multiforme; ref. 115). This clinical experience using DCs as cancer vaccines established their safety as immunotherapeutic agents with some evidence of efficacy. More recently, to demonstrate the feasibility of CAR engineering of DCs, a group of investigators differentiated DCs in vitro using a combination of Flt3-ligand, GM-CSF, and IL4 and then transduced the cells with a lentiviral construct containing a 4-1BB signaling domain (116). This work demonstrated that DCs transduced with a 4-1BB CAR directed against CD33 have higher frequencies of CD141+/Cleq9α+ cells, which play an important role in induction of intratumoral T-cell cytotoxicity, compared with control DCs. The authors of this study demonstrated that combining CAR-DC with CAR T cells can enhance CAR T-cell function and cytotoxicity, leading to improved tumor control in a xenograft mouse model of AML, compared with results with CAR T cells alone. These findings, if validated in future studies, could support the addition of CAR-DCs to the armamentarium of next-generation adoptive cellular therapies.

THE NEXT FRONTIER: OFF-THE-SHELF CELLULAR THERAPY FOR PATIENTS WITH CANCER

Because allogeneic NK cells do not cause GVHD, they offer the opportunity for off-the-shelf therapy with potential advantages, such as large-scale manufacturing and the
production of multiple doses from a single donor, thus, making the therapy cheaper and immediately available for use. However, in contrast to T cells and many other human cells, NK cells are particularly susceptible to cryo-injury, with impairment in their effector function post thaw (117). Thus, before the promise of “on demand” off-the-shelf CAR NK-cell therapy can be fulfilled, the development of optimized cryopreservation protocols for efficient freezing and banking is critical. Factors that could influence cryopreservation outcomes include the metabolic state of the cells, cell culture density, cytokine-driven versus nonstimulated cultures, and cell age, to name a few. While to date, progress in the cryopreservation of NK cells has been modest, recent advancements in the basic science of cryobiology and its application, the development of novel cryoprotectants and optimization of GMP protocols for rate-controlled cooling and thawing will likely ensure efficient manufacturing and banking of these cellular therapeutics for off-the-shelf therapy.

CONCLUSIONS AND FUTURE PROSPECTS

The cellular therapy field has undergone a shift in its guiding paradigm since the advent of CAR engineering. As the first example of CAR-engineered immune effector cells to yield promising results in the clinic, CAR T cells have set the pace for future developments in CAR-based immunotherapy. Despite this success, CAR T cells possess distinct shortcomings that have catalyzed studies of other immune effector cells, both in the innate and adaptive compartments, as alternative platforms for CAR engineering. Although definitive clinical trials of these new strategies are still under way, the results to date carry the promise of an improved safety profile without loss of efficacy and the feasibility of an off-the-shelf CAR-engineered therapeutic product. While a number of immune cell subsets have properties and activities that clearly support their candidacy as novel platforms for CAR expression, NK cells appear especially attractive given the strength of their intrinsic antitumor lytic activity and their relative lack of toxic side-effects. In the near future, we are likely to see a plethora of strategies combining CAR engineering with other approaches, such as checkpoint blockade or gene editing technologies, to further enhance the function of immune effector cells, especially in solid tumor microenvironments that are fraught with obstacles to successful immunotherapy.

Disclosure of Potential Conflicts of Interest

M. Daher, K. Rezvani, and The University of Texas MD Anderson Cancer Center (MDACC) have an institutional financial conflict of interest with Takeda Pharmaceutical for the licensing of the technology related to the CAR-NK cell research mentioned here. MD Anderson has implemented an Institutional Conflict of Interest Management and Monitoring Plan to manage and monitor the conflict of interest with respect to MDACC’s conduct of any other ongoing or future research related to this relationship. No other potential conflicts of interest were disclosed.

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CAR NK Cells and Other Alternative CAR-Based Platforms


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Outlook for New CAR-Based Therapies with a Focus on CAR NK Cells: What Lies Beyond CAR-Engineered T Cells in the Race against Cancer

May Daher and Katayoun Rezvani

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