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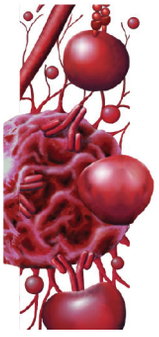

'Atypical' CAR T cells: NKG2D and Erb-B as examples of natural receptor/ligands to target recalcitrant solid tumors

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Chimeric antigen receptor (CAR) T-cell therapy has recently been recommended for approval for certain B-cell malignancies bringing the approach closer to mainstream cancer treatment. This rapid rise to prominence has been driven by impressive clinical results and the means to successfully commercialize the approach now being actively pursued. The current success of CAR T cells in B-cell malignancies relies upon the absolute lineage specificity of the CD19 antigen. CARs can also be targeted using non-antibody approaches, including the use of receptors and ligands to provide target specificity that have different specificities and binding kinetics. The specific examples of NKG2D and Erb-B are used that provide different characteristics and target profiles for CAR T-cell therapy of cancer.

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Although chimeric antigen receptor (CAR) T-cell therapy has emerged into mainstream understanding only within the last 5–6 years, the potential of the approach has ignited a frenzy of academic and commercial activity in the area. This has largely been driven by a single target – CD19 – and clinical responses seen in patients with advanced refractory B-cell leukemia and lymphoma, having received CD19-specific CAR T cells.

The early clinical testing of CAR T cells failed to ignite the collective imagination primarily due to the absence of clinical responses aligned with the perceived practical difficulties around the production and clinical delivery of the engineered T cells [1,2]. Importantly, these early trials involved 'first-generation' CARs that possessed a single T-cell signaling domain and also aimed high, focusing on solid tumors. Improving the functional capacity of the CAR construct proved relatively straightforward, involving the addition of further signaling domains that enabled the CAR to deliver both primary activation and co-stimulatory signals [3,4].

A change of emphasis to target CD19 and hematologic cancer has proven to be pivotal. CD19 is a protein that is involved in B-cell signaling and is present on all B cells, including malignant cells from the pro-B cell stage of development. As a target profile, the strong attraction of CD19 is this restricted range of expression to the B-cell lineage, with no expression at a multi- or pluri-potent precursor level. Although targeting of CD19 would be likely to result in loss of $CD19^+$ B cells, this is not generally considered life-threatening since it can be mitigated with the judicious application of immunoglobulin replacement therapy [5]. Since CD19 is highly expressed on a broad range of B-cell leukemias and lymphomas, this underscores the fact that a single CAR vector could be exploited to tackle an array of lineage-specific malignancies. Moreover, B cells tend to reside in similar anatomical regions to T cells, implying that access to tumor is likely to be less of a hurdle that is the case with solid tumors, where T cells would need to egress from the peripheral circulation and enter tissue. Additionally, B cells are good targets for T cells since they are antigen presenting cells that express co-stimulatory ligands, further enhancing the activity of CAR T cells [6].

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Together, this combination of traits suggests that CD19 is close to being the optimal tumor associated antigen for targeting using CAR T cells. Current clinical evidence supports this conclusion with many reports of clinical responses in patients receiving CD19 CAR T-cell therapy in differing B-cell indications and with differing combinations of vector, CAR structure and clinical design [7]. However, the therapy is not without its limitations, most notably severe treatment-related toxicity that has been seen in a significant number of patients. Nonetheless, the risk-benefit analysis is strongly in favor of the therapeutic approach, given the high level of objective responses observed in patients with refractory tumors. The challenges associated with this specific area are now well documented and have been excellently discussed elsewhere [2,8–10]. For the CAR engineer, CD19 provides a strong clinical proof of concept for CAR T-cell immunotherapy. However, it also raises questions concerning the ability to translate this platform technology beyond B-cell malignancies, where tumor associated antigens are expressed more broadly albeit at much lower levels on nonmalignant healthy tissues. These challenges and how they may be circumvented using ligand-targeted CAR T-cell immunotherapy are the focus of this review.

The 'standard' CAR format

The term 'standard' is used here to capture the engineering approach that has largely been explored to date in most pre-clinical and clinical CAR T studies. The authors accept that the term 'standard' also implies a long history of validation and general acceptance but this is not truly relevant to CARs, given that the first descriptions of engineering T-cell lines to target cell surface proteins in a HLA non-restricted manner emerged less than 30 years ago [11]. Indeed, the field remained very much under the radar until 2011 when initial clinical reports of CD19 CAR T-cell therapy were presented and captured more widespread attention [12,13]. Hence, despite the connotations associated with the term 'standard', it is used here to describe CARs that use antibody-based targeting modules, tethered to a transmembrane domain that anchors the receptor to the T cell in the correct orientation and is fused to either single or multiple intracellular signaling domains. From an engineering perspective, optimization of the intracellular signaling domain(s) has been the subject of greatest efforts. First generation receptors utilizing a single T-cell activating domain (source of signal one) functioned well *in vitro* but have proven sub-optimal in the clinical setting. Subsequent work showed that inclusion of the signaling domain of CD28 within the receptor enabled the CAR to initiate signal one and a co-stimulatory signal upon ligand binding, resulting in enhanced IL-2 release [14] and more potent initiation and prolongation of T-cell effector responses [4]. Over time, further co-stimulatory receptor signaling domains including CD137 [15] and others all show promise (together classified as 'second-generation' receptors) and enhanced potency of CAR T-cell responses. Perhaps surprisingly, CAR T cells employing either CD28 or CD137 co-stimulatory domains appear to drive equivalent levels of clinical response, at least when evaluated at relatively short time points after treatment. Whether differences in receptors bearing different signaling domains will be found in terms of durability of response remains to be seen, given the lack of head to head studies in this arena. However, the Biotech company Juno has recently halted a key trial using CD19 CAR T cells armed with a CD28-CD3ζ signaling domain while continuing with CD137-CD3ζ versions proposing that the dynamics of cytokine production underlie the observed toxicities. Nonetheless, other companies continue with CD28-based CARs while reporting no similar level of toxicity suggesting that there may be multiple factors at play here, including issues that pertain to the specific targeting moiety included in the CAR.

Further engineering of the cytoplasmic domains by the incorporation of additional co-stimulatory domains (third-generation [16]) and separate CAR inducible transgenes enabling the production of immune modulatory cytokines when the CAR is engaged (fourth generation) or the continuous co-expression of CAR and cytokine (armored CAR [17]) are at advanced preclinical or early clinical testing, albeit too early to draw definitive conclusions relating to the efficacy of these approaches. Together, these observations indicate that the field is at a very early stage of development with still limited understanding of the interface of CAR signaling domains with clinical trial design. Atypical CARs exploiting natural receptor or ligand exploit some of these modular concepts through the engineering of natural binding partners with varying signaling domains. These will be discussed in the context of NKG2D and Erb-B based receptors that show differential levels of engineering. The key element of such an atypical CAR approach is the target binding domain.

ScFv antigen binding & extracellular spacer domains: critical for CAR function

The diversity of targeting domains employed in CAR engineering has been recently reviewed [18]. For atypical CARs, the comparator is effectively the standard CAR format that has largely evolved based upon the exploitation of antibody-type recognition technology, most commonly using single chain variable antibody fragments (scFv)

to achieve target specificity. These elements represent an attractive option due to their relatively small size (23–25 kDa) and their amenability to protein engineering, while maintaining virtually identical binding characteristics to the parental antibody of origin. Moreover, while many scFv used in CAR constructs have been derived from well characterized monoclonal antibodies, phage display and other library selection technologies mean that scFv constructs can be generated against virtually any cell surface protein or nonprotein target [19].

Somewhat surprisingly, there appears to be little restriction relating to the ability of scFv to function in the context of a T cell expressed CAR. However, achieving maximal T-cell effector function is frequently dependent on the affinity and spatial location of the scFv in relation to the T cell and target cell membrane. In the context of first generation CARs, there is reasonable evidence suggesting that the relative position of the scFv epitope binding site on the target antigen could impact upon effector T-cell function and that this could be managed by the inclusion or exclusion of an extracellular spacer domain. For example, scFv targeting epitopes at a distance from the target cell membrane including carcino-embryonic antigen or CD19 induced highest levels of cytokine responses *in vitro* when the CAR had a minimal extracellular domain. Conversely, scFv targeting epitopes close to the target cell membrane in 5T4 and neural cell adhesion molecule proteins induced optimal antigen-specific cytokine responses when a larger spacer domain was included into the CAR [20]. Subsequent studies performed by other groups validate and indeed extend these observations and support two potentially complementary views that relate to achieving optimal CAR T-cell function [21–23]. The first of these proposes that the optimization of the precise distance between T cell and target cell is required for maximal function. Second, the ability of scFv to access buried epitopes may require reach and/or flexibility, which may be provided by an extracellular spacer domain and/or hinge [20,24]. While definitive answers to determine the relative role of distance against flexibility in CAR T-cell activity are lacking, it is noteworthy that the concept of an optimal distance for T-cell effector function aligns well with the kinetic segregation theory of T-cell activation and is the basis of higher order control systems currently being developed for CAR T cells.

The affinity of scFv for antigen would be expected to play a major role in defining CAR T-cell activity. Indeed, there is evidence that high affinity scFv enable CAR driven T-cell effector responses against targets expressing relatively lower levels of antigen [25]. Furthermore, the relative level and location of the CAR on the T-cell surface is also likely to have a major impact upon effector function. In the first-generation CAR setting, incorporation of a CD3ζ-based CAR into the T-cell receptor/CD3 complex was associated with enhanced responsiveness to target antigen while mutation of transmembrane residues that facilitated homo-dimerization and interaction with the T-cell receptor resulted in the CAR failing to be incorporated within the TCR/CD3 complex and subsequent reduction in sensitivity to antigen [26]. Beyond first generation CARs, there is very little detailed understanding relating to the structural biochemistry of second, third and further generation of CARs. Indeed, CAR constructs employ diverse transmembrane, extracellular and intracellular domains, differing scFv even against the same target antigen, different vectors and different methods to generate the final T-cell product. Despite this heterogeneity and poorly understood structural biochemistry, most scFv enable CARs to function, endowing T cells with HLA-independent effector function and potential clinical applicability. It is highly conceivable that an improved understanding of the biochemical nature of CARs and definition of binding will improve the specificity of CAR activity. Indeed, the concept of using scFv possessing reduced affinity for target antigen is being examined in order to avoid on-target, off-tissue targeting of healthy, nonmalignant tissue expressing low levels of the target antigen. Further engineering of CARs using multiple scFv (e.g., Tan-CARs) and co-expression of multiple chimeric receptors that deliver split T-cell activation and co-stimulation [27,28] will be dependent upon a greater understanding of CAR protein structure and biochemistry for success.

Overall, the standard scFv-based CAR approach has proven unexpectedly successful in the treatment of B-cell malignancy. However, beyond CD19 CAR T-cell therapy, standard CARs have failed to deliver significant clinical responses as yet, questioning the generic applicability of the approach. Additionally, the single antigen-binding specificity of scFv may be problematic given the observation of tumor antigen-loss variants in many patients receiving CD19 CAR T-cell therapy. Furthermore, it is increasingly appreciated that some scFv-based CARs exhibit tonic signaling leading to acquisition of an exhausted phenotype [29], an occurrence that has been linked to antigen-independent scFv clustering [30]. Dealing with these questions may require consideration of alternative systems to enable CAR T cells to engage cognate target(s) on the tumor cell surface.

Exploiting natural binding partners for CAR T-cell therapy

An alternative strategy to direct the specificity of CARs involves harnessing of natural receptor/ligand interactions that are commonly dysregulated in transformed cell types. The concept here is to engineer either a ligand or receptor as the targeting moiety within the CAR, thereby providing specificity that is directed by the ligand/receptor interaction. The key upside of this approach is that should the ligand/receptor bind multiple targets, this expands the potential range of the CAR, thereby reducing the likelihood of antigen-loss escape variants. However, the downside is the real possibility of widespread target antigen expression and the danger of on-target, off-tumor toxicity. The questions around CAR engineering, safety testing and clinical delivery differ for this approach but the ability to exploit natural binding partners offers potential advantages beyond that of breadth of specificity.

The range of receptors, ligands and other moieties that can potentially be used to target CARs is potentially broad and diverse including cytokines (e.g., IL-13) and receptor/ligands (e.g., CD70, PD-1/PD-L1). This diversity of targeting domains has recently been reviewed in this journal [18]. In this treatise, two specific examples will be discussed that captures the essence of atypical CAR binding while comparing the mechanisms of action that lead to specific clinical trial designs that differ from that currently employed by standard CAR T-cell therapy. The first entails the use of NKG2D as a CAR targeting moiety, exemplifying the exploitation of naturally occurring stress receptors for this purpose. The second approach involves CAR targeting using a promiscuous ErbB ligand named T1E that engages eight of the nine ErbB homo- and hetero-dimeric receptors, an integrated network that is dysregulated in several solid tumors.

Exploiting the natural killer receptor NKG2D to target CAR T cells

The control of natural killer (NK) cell effector function involves the complex and dynamic signaling interaction between activating and inhibitory receptors, thereby determining the net response of an individual NK cell. The Natural Killer Group 2D (NKG2D) protein is a member of the NK activating panel of receptors that binds eight known stress inducible ligands (MICA, MICB, ULBP1–6 in humans) [31]. At the RNA level, these ligands are broadly expressed throughout the tissues and organs of the body but cell surface protein expression is tightly controlled through multiple mechanisms. As a result, there is very little cell surface expression of NKG2D ligands in normal tissues [31]. However, these proteins are rapidly expressed at the cell surface under conditions of stress, flagging the cell in question for NK cell targeting and potential elimination. Importantly, transformed cells across a breadth of hematologic and solid tumors tend to be 'stressed' with high activity of DNA repair pathways, leading to expression of NKG2D ligands [31–33]. Why tumors avoid NK mediated elimination due to this upregulation of NKG2D ligands remains unclear but the multiplicity of mechanisms employed by tumors to blunt immune surveillance may help to explain this. Nonetheless, the presence of NKG2D ligands on such a range of tumors is attractive for a generic therapeutic targeting approach.

Various groups have shown that T cells armed with NKG2D receptors can target antigen-expressing target cells. In particular, ovarian tumor cells could be effectively targeted using T cells armed with NKG2D binding domains fused to 4–1BB/CD3ζ signaling domains with activity enhanced by treatment of target cells with inhibition of histone deacetylation [34]. Furthermore, T cells armed with a NKG2D binding domains fused to CD28/CD3ζ CAR could also target Ewing sarcoma family of tumors. Interestingly, this work suggested that the expression system used impacted upon CAR T-cell function since CAR expression from a lentiviral vector resulted in consistent, prolonged *in vitro* T-cell activity while receptor downregulation was observed in mRNA transfected T cells expressing the same receptor during target cell engagement [35]. The most extensive work in the area has been performed by Professor Charles Sentman based at Dartmouth College, USA. His concept appears straightforward at first sight, given that the CAR simply consisted of a fusion of NKG2D with a CD3ζ intracellular signaling domain [36,37]. However, there are subtleties to the design that provide this CAR construct with activity that extends well beyond that of a single signaling domain or first-generation CAR. For instance, NKG2D associates with the adaptor protein DAP-10 in T cells [38] which, upon ligation with NKG2D ligand, delivers a co-stimulatory signal like that provided by CD28 [36,37]. Thus, the NKG2D CAR acts like a second-generation CAR upon antigen binding. Interestingly, NKG2D is a type II transmembrane protein in which the carboxy-terminus of the protein is located within the extracellular space. This represents the reverse orientation of that seen with most standard CARs which tend to be expressed as type I transmembrane proteins (Figure 1). Furthermore, NKG2D itself is expressed in sub-sets of T cells where it may play a co-stimulatory function, but antigen-engagement is not thought to be sufficient to induce T-cell effector function [39].

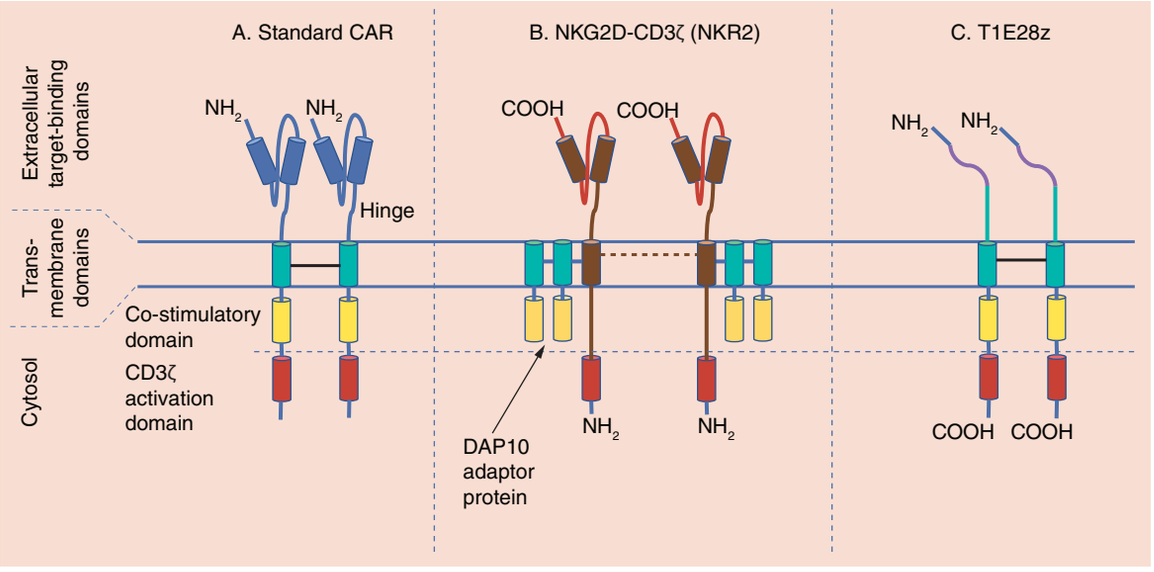

Figure 1. Standard, NKG2D-CD3ζ and T1E28z chimeric antigen receptor structures. (A) Overview of a second-generation chimeric antigen receptor (CAR) construct. These typically type I transmembrane proteins, consist of an extra-cellular scFv domain that is generally fused to a transmembrane domain by means of an extracellular hinge region. For a second-generation CAR, the signaling domains generally consist of a single co-stimulatory signaling domain (e.g., CD28, CD137) fused to the CD3ζ activation domain. Dependent upon the receptor used, CAR dimerization is driven by interchain di-sulfide bonding and ionic interactions mediated by extracellular and transmembrane domains. However, since most CARs employ 'natural' transmembrane domains such as CD28 or CD8α, these may also drive heterodimerization with the relevant endogenous receptors. **(B)** The NKG2D-CD3ζ CAR consists of the straightforward fusion of NKG2D with the CD3ζ activation domain. NKG2D is a type II protein with the carboxy-terminus of the protein residing within the extracellular compartment. NKG2D homodimerizes through interactions within the transmembrane domain while each NKG2D monomer also associates with two DAP10 monomers through further ionic transmembrane interactions. Binding of NKG2D ligand results in T-cell activation with the CD3ζ domain and a CD28-like co-stimulatory signal driven through the DAP10 adaptor protein signaling domain. Hence, the NKG2D-CD3ζ CAR functions in a manner similar to a CD28-based second generation CAR. **(C)** The T1E28z CAR utilizes a chimera involving the amino terminal seven amino-acid peptide from TGF-α fused to the carboxy terminal 48 amino acids of EGF. This polypeptide is fused to a truncated CD28 receptor fused to CD3ζ. As with the standard CAR construct, this type I transmembrane protein homodimerizes through the extracellular and transmembrane domains. Heterodimerization through the natural binding partners of this domains is also potentially possible.

Mouse T cells bearing the NKG2D CAR have been shown to effectively challenge the growth of pre-established syngeneic tumors in a diversity of preclinical mouse models [40–43]. Moreover, animals in long term remission reject further challenge with the original tumor but succumb to different tumors. At the time of re-challenge, no CAR T cells could be detected but CD4 and CD8 T-cell responses against the original tumor could be identified, suggesting that NKG2D CAR T-cell treatment had resulted in the generation of a tumor specific adaptive immune response [44]. Furthermore, analysis of tumors shortly after NKG2D CAR T-cell infusion demonstrated a reduction in regulatory T-cell number. This was accompanied by alterations in the myeloid compartment indicative of a general switch away from the typical immunosuppressive environment seen in tumors excised from animals in which control T cells had been administered [45]. Further studies using established B16F10 melanoma tumors indicated that NKG2D CAR T cells may also target tumor endothelial cells that express NKG2D ligands, presenting a further indirect and putatively antiangiogenic mode of action against the tumor [43].

A key feature of these experiments is the fact that the animals received no preconditioning to aid engraftment and function of the NKG2D CAR T cells. Preconditioning, usually in the form of chemotherapy, is thought to be an essential part of the standard CAR T-cell approach for multiple reasons. These include direct impact on the tumor, beneficial alteration of the immune suppressive tumor microenvironment, attainment of increased 'space' through elimination of competing white blood cells and the induction of homeostatic cytokines that aid the *in vivo* expansion of the infused CAR T cells. Unlike the standard CAR T approach where a single infusion of cells is given, multiple infusions of NKG2D CAR T cells were given over a period of a few weeks. These differences require

slightly more consideration as each is important to the overall design of the CAR itself. Single infusions of standard CAR T cells (i.e., CD19 specific CAR T cells) are given soon after the preconditioning chemotherapy to take advantage of the factors listed above. Split doses of CD19 CAR T cells have been given but these are usually given at short time intervals in order to provide some control but also seeking to exploit the short window of opportunity afforded by preconditioning. Furthermore, preconditioning also abrogates the immune response against the CAR construct itself which commonly uses murine derived scFv and, given the foreign nature of the CAR construct itself, may generate anti-CAR responses even with humanized scFv and extracellular spacer domains. Thus, for this combination of reasons, preconditioning is currently essential for standard CD19 CAR T-cell therapy and enables the rapid expansion of CAR T cells postinfusion resulting in the observed rapid and highly impressive antitumor responses. It is also likely that the tendency toward substantial *in vivo* expansion and potent antitumor response fuels the development of cytokine release syndrome and neurotoxicity in many patients.

By contrast, in the preclinical setting, NKG2D CAR T cells are infused with lengthy times between infusion leading to limited evidence of *in vivo* expansion and persistence of only a few weeks. To date, there has been no evidence of an antitransgene response against the NKG2D CAR, most likely due to the absence of engineered foreign protein sequences within the CAR. Indeed, the only foreign sequence present is the intracellular junctional sequence between NKG2D and CD3ζ.

A critical issue for all CARs is on-target, off-tissue toxicity. This issue is magnified for receptors exploiting NKG2D due to the multiligand targeting and may be further magnified dependent upon the engineering of the CAR itself. VanSeggelen *et al*. were the first to raise this question when using NKG2D-based CARs. In this study, engineering of the CAR through increased expression of the adaptor protein DAP10 or engineering of the NKG2D binding domain to a standard second-generation CAR configuration (in this case CD8 transmembrane domain fused to CD28 and CD3ζ signaling domains) resulted in acute toxicity in mice receiving the T cells [46]. The least engineered receptor involving NKG2D fused to CD3ζ resulted in the least toxicity in naive animals; however, combining cyclophosphamide preconditioning with any of the NKG2D CAR configurations resulted in significant acute toxicity. Importantly, this toxicity was strain dependent with Balb/c mice showing much increased levels of sensitivity to the effect of NKG2D as compared to C57 bl/6 mice [46]. This aligns with earlier observations made using CAR T cells targeting the murine CD19 in a syngeneic setting where Balb/c mice suffered acute toxicity after receiving CAR T cells while C57bl/6 mice showed no adverse response [47]. However, in the NKG2D CAR study, there was no report of chronic toxicity as was observed in Balb/c mice receiving CD19 -specific second-generation CAR T cells.

By contrast, a dosing study could show acute toxicity in the C57 bl/6 model when naive animals received high doses of NKG2D-CD3ζ CAR T cells which was strongly related to cytokine release [48]. In this report, animals receiving 20 million CAR T cells showed rapid weight loss and were sacrificed days after adoptive T-cell transfer. However, the study also showed that 10 million cells could be given over three separate doses with no obvious adverse effects implying that the maximum tolerated dose for NKG2D CAR T-cell infusion lay between 10 and 20 million CAR T cells per dose [48]. Thus, the toxicity resulting from the adoptive transfer of NKG2D CAR T cells appears to be dependent upon strain, preconditioning and cell dose. The relevance of the observations of strain-dependent toxicity in preclinical models has been recently discussed [49]. However, the observation of potential toxicities needs to be considered and was in the design of the first clinical trial testing NKG2D-CD3ζ CAR T cells. The trial focused upon patients with advanced malignant myeloma and acute myeloid leukemia (MM/AML) using low doses of CAR T cells (maximum dose 30 million CAR T cells in a single dose) adoptively transferred in the absence of preconditioning chemotherapy (NCT02203825). Initial reports presented at the Annual Society of Hematology meeting in Dec 2016 suggested no toxicity was associated with the therapy and some very early indicators of 'unexpected' benefit [50]. A follow-up trial has recently opened testing higher and multiple doses of NKG2D-CD3ζ (now called NKR2) without preconditioning chemotherapy in hematological (MM/AML) and several solid tumors (pancreatic, colorectal, triple negative breast cancer, bladder and ovarian); this trial is now recruiting (THINK trial; NCT03018405). The results of these early phase clinical studies are expected during the next 18 months.

Targeting of the extended ErbB network using ligand targeted CAR T cells

CAR T cells engage native cell surface targets, reminiscent of the actions of several successful monoclonal antibodies (moabs) with anticancer activity. Consequently, cancer targets that have proven favorable for moabs also warrant consideration for the development of experimental CAR T-cell immunotherapeutic approaches. In the solid tumor

setting, the most successful tumor-associated targets for moabs are members of the extended ErbB network, most notably EGFR and HER2 (ErbB2). Nonetheless, it is well recognized that tumors acquire resistance to such precisely targeted therapies, commonly through increased expression and/or activation of nontargeted ErbB family members [51]. Consequently, it is logical to develop targeted therapeutic approaches that are directed across the entire network of ErbB homo- and heterodimers.

To achieve this, a second generation CAR named T1E28z was designed [52] in which the signaling endodomain contained a CD28 module, placed upstream of CD3ζ [4]. Targeting was achieved using a chimeric polypeptide named T1E in which the N-terminal 7 amino acids from TGF-α were joined to the C-terminal 48 amino acids of EGF. Like TGF-α and EGF, T1E binds with high affinity to ErbB1-based homo- and hetero-dimers. Importantly, however, T1E also binds ErbB2/3 heterodimers and to all ErbB4 containing dimers [53]. Using a panel of 32D hematopoietic cells that had been engineered to express all known ErbB dimer species, it was confirmed that the T1E28z CAR could mediate T-cell activation and cytokine release upon encounter with each of these eight distinct ErbB dimer species [52].

Safe implementation of ErbB targeted CAR T-cell immunotherapy requires a cautious and considered approach, given the fact that these receptors are naturally expressed at low levels throughout many healthy organs in the body. Underscoring this, a patient with metastatic HER2$^+$ colorectal cancer succumbed to a fatal serious adverse event due to the intravenous infusion of CAR T cells that had been re-directed against this target [54]. The cause of death has been ascribed to on-target off-tumor toxicity that resulted from HER2 binding to the pulmonary parenchyma [54] or (as seems more probable) to the microvasculature of the pulmonary circulation [55].

Trafficking studies undertaken in immune compromised mice indicate that human CAR T cells remain within the lungs for several hours after intravenous delivery, a factor that is likely to have marked significance in the context of the suspected unexpected severe adverse reaction described above. Thereafter, CAR T cells redistribute to liver, spleen, intestine and lymph nodes [56]. This pattern of migration is seen even when CAR T cells are directed against human targets that are not present in the murine hosts. By contrast if CAR T cells are injected using the intratumoral or intraperitoneal (ip.) routes, minimal absorption to other sites occurs prior to the natural clearance of the cells from the mouse [56,57].

These observations led investigators to hypothesize that CAR T cells directed against ErbB receptors might be safely administered using regional delivery strategies such as intratumoral or intracavitary infusion. Head and neck squamous cell carcinoma was selected as the first disease model to test this approach, given the fact that locally advanced or locally recurrent tumor formation represents the main unmet clinical need. Furthermore, 5-year survival statistics have only marginally improved over the past 50 years and patients who are deemed unsuitable for conventional medical management have a 100% mortality rate within 30 weeks [58]. Although human head and neck cancer (HNSCC) cells express a diverse repertoire of ErbB receptor profiles, all of 13 human HNSCC tumor cell lines were susceptible to *in vitro* destruction by ErbB re-targeted CAR T cells. Using xenograft models of established HNSCC, ErbB re-targeted CAR T cells also achieve sustained disease control [52]. Human T1E28z CAR T cells can recognize mouse ErbB orthologs, enabling the destruction of mouse ErbB expressing HNSCC cells. Moreover, mouse pulmonary endothelium expresses ErbB receptors when actively growing in culture, rendering these cells susceptible to destruction by human T1E28z$^+$ T cells [57]. This point requires emphasis since it recapitulates *in vitro* the suspected unexpected severe adverse reaction elicited by HER2 re-targeted CAR T cells, as described above. Nonetheless, human ErbB re-targeted CAR T cells did not elicit any clinical or histologic toxicity in immune compromised mice, either when delivered using the intratumoral or intravenous routes. By contrast, these T-cells could elicit dose-dependent cytokine release syndrome when administered using the ip. route to SCID Beige mice, a finding that reflects the ability of the human CAR T cells to interact with endogenous mouse macrophages, leading to IL-6 release [57]. Such intense toxicity was not required for efficacy when these cells were delivered ip., causing regression of ovarian [59] or mesothelioma tumor xenografts [60].

These preclinical data supported the initiation of a Phase I trial of intratumoral injection of T1E28z$^+$ CAR T cells in patients with locally advanced or locally recurrent HNSCC. Traditionally, leukapheresis is used as starting material to manufacture CAR T-cell products. However, this procedure is costly, invasive and is inconvenient for patients. A key differentiating feature of this trial is that a blood draw is collected as starting material. This is feasible since CAR T cells are engineered to co-express a chimeric cytokine receptor in which IL-4 receptor α ectodomain has been fused to the shared β chain used by receptors for IL-2 and IL-15 [61]. By this means, IL-4 is converted into a potent and selective mitogen for the CAR-engineered T cells during cell product manufacture. Although lymphodepletion is potentially beneficial as indicated above, it is also toxic and may not be tolerated by infirm

individuals with advanced malignancy. Consequently, it has been avoided in this study design. Ten patients have been treated thus far in a dose escalation protocol in which up to 300 million ErbB re-targeted CAR T cells are administered as a single dose, injected at multiple points within the tumor [62]. No dose-limiting toxicities have been observed, while disease control has been achieved in six patients by RECIST (Response Evaluation Criteria In Solid Tumors) assessment at 6 weeks [63]. Despite lymphopenia in all but one patient, batches of between 2 and 7.5 billion CAR T cells have been produced in every case, derived from a 40–120 ml blood draw. Further development post Phase I testing is likely to involve intracavitary delivery in patients with mesothelioma and/or high grade serous ovarian cancer [64].

Conclusion

The underlying rationale of the Chimeric Antigen Receptor is the targeted re-direction of T-cell specificity against virtually any cell surface molecule of interest. Antibody technology is highly powerful, providing exquisite specificity. However, exploiting other receptor/ligand interactions provides potential advantages including the exploitation of different binding affinities and potential multiple target binding. The examples discussed above expand this concept of the generic application of CAR technology. Indeed, the NKG2D approach alone potentially enables the targeting of more than 80% of all tumors given the broad breath of NKG2D ligand expression [33].

NKG2D and Erb-B clearly just represent two examples of atypical CARs. Many others of course exist with the IL-13 zetakine CAR currently the leading example that has reported early clinical proof of principle [65]. The IL-13 zetakine is based upon the use of a mutated membrane-bound IL-13 that specifically binds to IL-13Rα2 that is highly expressed upon tumors including glioblastoma and has much reduced binding to the more broadly expressed IL-13Rα1 [66,67]. Initial testing in three patients treated with IL-13 zetakine $CD8^+$ T-cell clones showed transient evidence of antiglioma response [68]. Moreover, a recent case study reported significant clinical responses in one patient that lasted for 7.5 months after repeated intracranial infusions of IL-13 zetakine CAR T cells [65]. It is important to consider that this case study reported observations during the early stages of an on-going clinical trial with this patient reported as one of seven having been treated at the stage of the report. Thus, albeit early in clinical development, such reports of encouraging clinical responses provide support to the development of the concept.

Naturally, the benefits of such potential targeting are large but this requires balance against the challenges of avoiding toxicity driven by on-target, off-tissue targeting. To achieve this will require careful receptor design and increasing knowledge concerning the biology of the receptors and ligands being targeted. Together, the use of 'atypical' CARs provides an additional level of capability to this technology and broadens the scope for CARs to be targeted against an increasingly wide and diverse array of targets for cancer and also beyond into other disease areas.

Future perspective

The ability to endow T cells with artificial target specificity is now emerging as an important paradigm in cancer. Antibody, ligand or receptor-based targeting methodologies are now providing an increasing diversity to the CAR approach. Given the challenge of tacking increasingly recalcitrant tumors, this diversity of targeting will become ever more important. Indeed, combining targeting approaches is likely to provide additional power but also increasing specificity for CAR T-cell therapy.

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Executive summary

- The success of chimeric antigen receptor (CAR) T-cell therapy of B-cell malignancies raises a question concerning the wider utility of this therapy against cancer. Whether CAR T-cell therapy has the potential to tackle highly recalcitrant solid tumors that have developed many effective mechanisms to avoid immune detection is now a major question.
- The methods used to target CARs to tumor are critical in this respect. CARs targeting B-cell malignancies employ antibody-based specificity to target the CD19 antigen.
- While the high affinity and excellent mono-antigen specificity are prime advantages of such antibody-based technology, exploiting natural receptor—ligand interactions may provide an alternative approach that could allow for multiple antigen-targeting and -binding kinetics that differ from the 'standard' CAR approach.
- Two examples focusing upon NKG2D and ErbB are described to exemplify the exploitation of a natural receptor-based CAR and a ligand-based CAR-targeting approach.
- These examples indicate that differing strategies may be exploiting to enhance the potency and specificity of CARs to fight a wider array of cancers.

References

1. Cheadle EJ, Gornall H, Baldan V, Hanson V, Hawkins RE, Gilham DE. CAR T cells: driving the road from the laboratory to the clinic. *Immunol. Rev.* 257(1), 91–106 (2014).
2. Jackson HJ, Rafiq S, Brentjens RJ. Driving CAR T-cells forward. *Nat. Rev. Clin. Oncol.* 13(6), 370–383 (2016).
3. Finney HM, Akbar AN, Lawson AD. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. *J. Immunol.* 172(1), 104–113 (2004).
4. Maher J, Brentjens RJ, Gunset G, Riviere I, Sadelain M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta/CD28 receptor. *Nat. Biotechnol.* 20(1), 70–75 (2002).
5. Kochenderfer JN, Wilson WH, Janik JE *et al.* Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* 116(20), 4099–4102 (2010).
6. Cheadle EJ, Rothwell DG, Bridgeman JS, Sheard VE, Hawkins RE, Gilham DE. Ligation of the CD2 co-stimulatory receptor enhances IL-2 production from first-generation chimeric antigen receptor T cells. *Gene Ther.* 19(11), 1114–1120 (2012).
7. Maher J. Clinical immunotherapy of B-cell malignancy using CD19-targeted CAR T-cells. *Curr. Gene Ther.* 14(1), 35–43 (2014).
8. Holzinger A, Barden M, Abken H. The growing world of CAR T cell trials: a systematic review. *Cancer Immunol. Immunother.* 65(12), 1433–1450 (2016).
9. Jaspers JE, Brentjens RJ. Development of CAR T cells designed to improve antitumor efficacy and safety. *Pharmacol. Ther.* doi:10.1016/j.pharmthera.2017.03.012 (2017) (Epub ahead of print).
10. Lim WA, June CH. The principles of engineering immune cells to treat cancer. *Cell* 168(4), 724–740 (2017).
11. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc. Natl Acad. Sci. USA* 86(24), 10024–10028 (1989).
12. Kalos M, Levine BL, Porter DL *et al.* T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci. Transl. Med.* 3(95), 95ra73 (2011).
13. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N. Engl. J. Med.* 365(8), 725–733 (2011).
14. Finney HM, Lawson AD, Bebbington CR, Weir AN. Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product. *J. Immunol.* 161(6), 2791–2797 (1998).
15. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* 106(1), 376–383 (2005).
16. Choi BD, Suryadevara CM, Gedeon PC *et al.* Intracerebral delivery of a third generation EGFRvIII-specific chimeric antigen receptor is efficacious against human glioma. *J. Clin. Neurosci.* 21(1), 189–190 (2014).
17. Yeku OO, Brentjens RJ. Armored CAR T-cells: utilizing cytokines and pro-inflammatory ligands to enhance CAR T-cell anti-tumour efficacy. *Biochem. Soc. Trans.* 44(2), 412–418 (2016).
18. Bezverbnaya K, Mathews A, Sidhu J, Helsen CW, Bramson JL. Tumor-targeting domains for chimeric antigen receptor T cells. *Immunotherapy* 9(1), 33–46 (2017).
19. Bridgeman JS, Hawkins RE, Hombach AA, Abken H, Gilham DE. Building better chimeric antigen receptors for adoptive T cell therapy. *Curr. Gene Ther.* 10(2), 77–90 (2010).
20. Guest RD, Hawkins RE, Kirillova N *et al.* The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens. *J. Immunother.* 28(3), 203–211 (2005).

21. Hombach AA, Schildgen V, Heuser C, Finnern R, Gilham DE, Abken H. T cell activation by antibody-like immunoreceptors: the position of the binding epitope within the target molecule determines the efficiency of activation of redirected T cells. *J. Immunol.* 178(7), 4650–4657 (2007).
22. Hudecek M, Lupo-Stanghellini MT, Kosasih PL *et al.* Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. *Clin. Cancer Res.* 19(12), 3153–3164 (2013).
23. Hudecek M, Sommermeyer D, Kosasih PL *et al.* The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for *in vivo* antitumor activity. *Cancer Immunol. Res.* 3(2), 125–135 (2015).
24. Wilkie S, Picco G, Foster J *et al.* Retargeting of human T cells to tumor-associated MUC1: the evolution of a chimeric antigen receptor. *J. Immunol.* 180(7), 4901–4909 (2008).
25. Weijtens ME, Willemsen RA, Valerio D, Stam K, Bolhuis RL. Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. *J. Immunol.* 157(2), 836–843 (1996).
26. Bridgeman JS, Hawkins RE, Bagley S, Blaylock M, Holland M, Gilham DE. The optimal antigen response of chimeric antigen receptors harboring the CD3zeta transmembrane domain is dependent upon incorporation of the receptor into the endogenous TCR/CD3 complex. *J. Immunol.* 184(12), 6938–6949 (2010).
27. Wilkie S, Van Schalkwyk MC, Hobbs S *et al.* Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. *J. Clin. Immunol.* 32(5), 1059–1070 (2012).
28. Alvarez-Vallina L, Hawkins RE. Antigen-specific targeting of CD28-mediated T cell co-stimulation using chimeric single-chain antibody variable fragment-CD28 receptors. *Eur. J. Immunol.* 26(10), 2304–2309 (1996).
29. Frigault MJ, Lee J, Basil MC *et al.* Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells. *Cancer Immunol. Res.* 3(4), 356–367 (2015).
30. Long AH, Haso WM, Shern JF *et al.* 4–1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat. Med.* 21(6), 581–590 (2015).
31. Spear P, Wu MR, Sentman ML, Sentman CL. NKG2D ligands as therapeutic targets. *Cancer Immun.* 13, 8 (2013).
32. Sentman CL, Barber MA, Barber A, Zhang T. NK cell receptors as tools in cancer immunotherapy. *Adv. Cancer Res.* 95, 249–292 (2006).
33. Sentman CL, Meehan KR. NKG2D CARs as cell therapy for cancer. *Cancer J.* 20(2), 156–159 (2014).
34. Song DG, Ye Q, Santoro S, Fang C, Best A, Powell DJ Jr. Chimeric NKG2D CAR-expressing T cell-mediated attack of human ovarian cancer is enhanced by histone deacetylase inhibition. *Hum. Gene Ther.* 24(3), 295–305 (2013).
35. Lehner M, Gotz G, Proff J *et al.* Redirecting T cells to Ewing's sarcoma family of tumors by a chimeric NKG2D receptor expressed by lentiviral transduction or mRNA transfection. *PLoS One* 7(2), e31210 (2012).
36. Zhang T, Barber A, Sentman CL. Generation of antitumor responses by genetic modification of primary human T cells with a chimeric NKG2D receptor. *Cancer Res.* 66(11), 5927–5933 (2006).
37. Zhang T, Lemoi BA, Sentman CL. Chimeric NK-receptor-bearing T cells mediate antitumor immunotherapy. *Blood* 106(5), 1544–1551 (2005).
38. Garrity D, Call ME, Feng J, Wucherpfennig KW. The activating NKG2D receptor assembles in the membrane with two signaling dimers into a hexameric structure. *Proc. Natl Acad. Sci. USA* 102(21), 7641–7646 (2005).
39. Barber A, Sentman CL. NKG2D receptor regulates human effector T-cell cytokine production. *Blood* 117(24), 6571–6581 (2011).
40. Barber A, Meehan KR, Sentman CL. Treatment of multiple myeloma with adoptively transferred chimeric NKG2D receptor-expressing T cells. *Gene Ther.* 18(5), 509–516 (2011).
41. Barber A, Zhang T, Demars LR, Conejo-Garcia J, Roby KF, Sentman CL. Chimeric NKG2D receptor-bearing T cells as immunotherapy for ovarian cancer. *Cancer Res.* 67(10), 5003–5008 (2007).
42. Zhang T, Barber A, Sentman CL. Chimeric NKG2D modified T cells inhibit systemic T-cell lymphoma growth in a manner involving multiple cytokines and cytotoxic pathways. *Cancer Res.* 67(22), 11029–11036 (2007).
43. Zhang T, Sentman CL. Mouse tumor vasculature expresses NKG2D ligands and can be targeted by chimeric NKG2D-modified T cells. *J. Immunol.* 190(5), 2455–2463 (2013).
44. Barber A, Zhang T, Sentman CL. Immunotherapy with chimeric NKG2D receptors leads to long-term tumor-free survival and development of host antitumor immunity in murine ovarian cancer. *J. Immunol.* 180(1), 72–78 (2008).
45. Barber A, Rynda A, Sentman CL. Chimeric NKG2D expressing T cells eliminate immunosuppression and activate immunity within the ovarian tumor microenvironment. *J. Immunol.* 183(11), 6939–6947 (2009).
46. VanSeggelen H, Hammill JA, Dvorkin-Gheva A *et al.* T cells engineered with chimeric antigen receptors targeting NKG2D ligands display lethal toxicity in mice. *Mol. Ther.* 23(10), 1600–1610 (2015).
47. Cheadle EJ, Sheard V, Rothwell DG *et al.* Differential role of Th1 and Th2 cytokines in autotoxicity driven by CD19-specific second-generation chimeric antigen receptor T cells in a mouse model. *J. Immunol.* 192(8), 3654–3665 (2014).

48. Sentman ML, Murad JM, Cook WJ *et al.* Mechanisms of acute toxicity in NKG2D chimeric antigen receptor T Cell-treated mice. *J. Immunol.* 197(12), 4674–4685 (2016).
49. Kalaitsidou M, Kueberuwa G, Schutt A, Gilham DE. CAR T-cell therapy: toxicity and the relevance of preclinical models. *Immunotherapy* 7(5), 487–497 (2015).
50. Nikiforow S, Werner L, Murad J *et al.* Safety data from a first-in-human Phase 1 trial of NKG2D chimeric antigen receptor-T cells in AML/MDS and mMulitple myeloma blood. *Blood.* 128, 4052 (2016).
51. Wheeler DL, Huang S, Kruser TJ *et al.* Mechanisms of acquired resistance to cetuximab: role of HER (ErbB) family members. *Oncogene* 27(28), 3944–3956 (2008).
52. Davies DM, Foster J, Van Der Stegen SJ *et al.* Flexible targeting of ErbB dimers that drive tumorigenesis by using genetically engineered T cells. *Mol. Med.* 18, 565–576 (2012).
53. Wingens M, Walma T, Van Ingen H *et al.* Structural analysis of an epidermal growth factor/transforming growth factor-alpha chimera with unique ErbB binding specificity. *J. Biol. Chem.* 278(40), 39114–39123 (2003).
54. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol. Ther.* 18(4), 843–851 (2010).
55. Heslop HE. Safer CARS. *Mol. Ther.* 18(4), 661–662 (2010).
56. Parente-Pereira AC, Burnet J, Ellison D *et al.* Trafficking of CAR-engineered human T cells following regional or systemic adoptive transfer in SCID beige mice. *J. Clin. Immunol.* 31(4), 710–718 (2011).
57. Van Der Stegen SJ, Davies DM, Wilkie S *et al.* Preclinical *in vivo* modeling of cytokine release syndrome induced by ErbB-retargeted human T cells: identifying a window of therapeutic opportunity? *J. Immunol.* 191(9), 4589–4598 (2013).
58. Jeannon JP, Ofu E, Balfour A, Bowman J, Simo R. The natural history of untreated squamous cell carcinoma of the head and neck: how we do it. *Clin. Otolaryngol.* 36(4), 384–388 (2011).
59. Parente-Pereira AC, Whilding LM, Brewig N *et al.* Synergistic chemoimmunotherapy of epithelial ovarian cancer using ErbB-retargeted T cells combined with carboplatin. *J. Immunol.* 191(5), 2437–2445 (2013).
60. Klampatsa A, Achkova DY, Davies DM *et al.* Intracavitary 'T4 immunotherapy' of malignant mesothelioma using pan-ErbB re-targeted CAR T-cells. *Cancer Lett.* 393, 52–59 (2017).
61. Wilkie S, Burbridge SE, Chiapero-Stanke L *et al.* Selective expansion of chimeric antigen receptor-targeted T-cells with potent effector function using interleukin-4. *J. Biol. Chem.* 285(33), 25538–25544 (2010).
62. Van Schalkwyk MC, Papa SE, Jeannon JP, Urbano TG, Spicer JF, Maher J. Design of a Phase I clinical trial to evaluate intratumoral delivery of ErbB-targeted chimeric antigen receptor T-Cells in locally advanced or recurrent head and neck cancer. *Hum. Gene Ther. Clin. Dev.* 24(3), 134–142 (2013).
63. Papa S, Adami AA, Metoudi M *et al.* Online Proceedings of the Annual Meeting of the American Association for Cancer Research. www.abstractsonline.com/pp8/#!/4292/presentation/12333 (2017).
64. Papa S, Van Schalkwyk M, Maher J. Clinical evaluation of ErbB-targeted CAR T-cells, following intracavity delivery in patients with ErbB-expressing solid tumors. *Methods Mol. Biol.* 1317, 365–382 (2015).
65. Brown CE, Alizadeh D, Starr R *et al.* Regression of glioblastoma after chimeric antigen receptor T-cell therapy. *N. Engl. J. Med.* 375(26), 2561–2569 (2016).
66. Kahlon KS, Brown C, Cooper LJ, Raubitschek A, Forman SJ, Jensen MC. Specific recognition and killing of glioblastoma multiforme by interleukin 13-zetakine redirected cytolytic T cells. *Cancer Res.* 64(24), 9160–9166 (2004).
67. Stastny MJ, Brown CE, Ruel C, Jensen MC. Medulloblastomas expressing IL13Ralpha2 are targets for IL13-zetakine+ cytolytic T cells. *J. Pediatr. Hematol. Oncol.* 29(10), 669–677 (2007).
68. Brown CE, Badie B, Barish ME *et al.* Bioactivity and safety of IL13Ralpha2-redirected chimeric antigen receptor CD8+ T cells in patients with recurrent glioblastoma. *Clin. Cancer Res.* 21(18), 4062–4072 (2015).