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Redirecting T cells to treat solid pediatric cancers

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Abstract
The capacity of single-agent therapy with immune checkpoint inhibitors to control solid cancers by unleashing preexisting local antitumor T cell responses has renewed interest in the broader use of T cells as anticancer therapeutics. At the same time, durable responses of refractory B-lineage malignancies to chimeric-receptor engineered T cells illustrate that T cells can be effectively redirected to cancers that lack preexisting tumor antigen-specific T cells, as most typical childhood cancers. This review summarizes strategies by which T cells can be modified to recognize defined antigens, with a focus on chimeric-receptor engineering. We provide an overview of candidate target antigens currently investigated in advanced preclinical and early clinical trials in pediatric malignancies and discuss the prerequisites for an adequate *in vivo* function of engineered T cells in the microenvironment of solid tumors and intrinsic and extrinsic limitations of current redirected T cell therapies. We further address innovative solutions to recruit therapeutic T cells to tumors, overcome the unreliable and heterogenous expression of most known tumor-associated antigens, and prevent functional inactivation of T cells in the hostile microenvironment of solid childhood tumors.

Keywords Chimeric antigen receptors · Childhood cancer · T cell therapy · T cell engineering

1 Introduction

The treatment of solid childhood cancers today combines various cytotoxic agents with surgery and radiotherapy. The systematic improvement of such multimodal treatment regimens in randomized multi-center trials performed by large international study groups has resulted in an increase in relapse-free survival for patients with localized tumors [1]. However, metastatic and relapsed disease remains fatal in many patients [2, 3]. Survival curves have now reached plateaus, and further increasing the intensity of current regimens is unlikely to provide additional benefit. Driven by an increased understanding of the molecular mechanisms of cancer, novel agents have been developed that selectively target aberrant signaling molecules [4]. For instance, inhibitors of tropomyosin receptor kinase (TRK) proteins, which are driver mutations in rare sarcomas [5], have had significant clinical success.

T cells are highly potent effector cells that can control otherwise life-threatening infections. Their antigen-specific memory function provides sustained, often lifelong, protection against re-exposure to pathogenic agents. T cells may thus be a novel cancer therapeutic for the eradication of residual disease and prolongation of relapse-free survival. The promise of T cell immunotherapy has been documented by the successful use of checkpoint inhibitors for solid tumors in adults [6]. Checkpoint inhibitors are inhibitory antibodies that bind immune-inhibitory receptors or their ligands, such as programmed death receptor 1 (PD-1) and PD ligand 1 (PD-L1). They act by invigorating preexisting but suppressed local T cell responses to tumor-associated antigens at tumor sites [7, 8]. Predictors of the efficacy of PD-1 checkpoint inhibitors include a high tumor mutational burden and a T cell inflammatory gene expression profile with high expression of PD-L1 [9]. Childhood tumors typically exhibit a low mutational burden [10, 11], and fail to express neoantigens recognizable by T cells. In addition, there is a striking absence of infiltrating T cells in the tumor microenvironment of the majority of pediatric solid tumors [12]. Thus, tumor growth and spread in pediatric cancers are largely ignored by the cellular immune system. Consequently, checkpoint inhibitors have been found to have limited efficacy in pediatric tumors, as assessed by clinical trials [13, 14]. Efficient T cell–based immune targeting involves recruiting T cells to the tumor, specifically redirecting them to tumor-associated targets, and creating conditions that favor their antitumor activity.

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Attempts have been made to expand naturally occurring T cells with native specificity for either tumor-associated fusion peptides [15] or self-antigens with tumor-restricted expression [16, 17] from the T cell repertoire of solid cancer patients *in vitro*, for subsequent adoptive transfer. While refined *in vitro* stimulation techniques now allow to overcome tolerance against these antigens and expand even rare T cell populations with low avidity receptors [18, 19], the resulting T cell products are often not functional against tumor cells endogenously expressing the antigen [20]. This is explained by central T cell tolerance of shared self-antigens, resulting in low T cell receptor (TCR) avidity. A solution could be therapeutic engineering of T cells for recognition of defined antigens selectively overexpressed in tumors.

2 T cell receptor engineering

By engineering with selected T cell receptor (TCR) genes, T cell products with defined and optimized recognition of intracellular proteins, presented as peptides on the major histocompatibility complex (MHC), can be generated (Fig. 1a). This requires selection of antigen-specific CD8+ T cells and isolation of TCRα and β genes and their validation. This is followed by viral gene transfer for expression in T cells and *in vitro* expansion of these genetically modified T cells to reach therapeutic numbers [21]. Advances in molecular engineering in the genetic modification of TCRs have enabled the enhancement and optimization of T cell activity [22].

In pediatric cancer patients, adoptive transfer of TCR-engineered T cells has recently shown promise in the treatment of tumors expressing the cancer testis antigen NY-ESO-1 [23]. Autologous T cells were genetically modified with an affinity-enhanced TCR recognizing an HLA-A2-restricted peptide shared by two cancer testis antigens, NY-ESO-1 and LAGE-1, and administered via infusion to 12 patients with metastatic or locally advanced NY-ESO-1-expressing synovial sarcomas following lymphodepleting, myeloablative chemotherapy. Tumor shrinkage over several months was observed in 6 patients, including one complete and 5 partial responses, along with *in vivo* T cell expansion and persistence for at least 6 months in all responders. The most common treatment-related toxicities were cytopenias and febrile reactions attributed to cytokine release. Although all tumors that were not resected eventually progressed, the study demonstrated that adoptive transfer of autologous antigen-specific T cells mediates clinical antitumor effects in solid tumors.

Strategies that rely on TCR-mediated T cell recognition are limited by the ineligibility of many patients for these therapies due to variation in HLA types, downregulation of major histocompatiblity antigens (MHC), and defects in the antigen-processing machinery in tumor cells [24]. Moreover, while NY-ESO-1 appears to be a safe target, unpredicted organ-specific off-target toxicities have been reported with the use of affinity-enhanced TCRs in the adult population [25]. Since mispairing of native and engineered α and β TCRs can generate novel specificities, disruption of endogenous TCRs by gene-editing techniques may improve the safety of TCR engineering [26].

3 Antibody-based retargeting of T cells

Alternative strategies redirect T cell activation in response to target tumor antigens by employing the epitope-binding moieties of monoclonal antibodies. For instance, bispecific T cell engagers such as blinatumomab engage CD3 on T cells and CD19 on leukemic cells [27] (Fig. 1b). Continuous infusions of blinatumomab are effective to induce deep, molecular remissions in a proportion of both pediatric and adult patients with refractory acute lymphoblastic leukemias (ALL) [28] and are now investigated as new, immunological treatment modality in ALL frontline therapies. Bispecific T cell engagers are additionally under development for use in several other cancers, including G_{D2} in neuroblastoma [29]. Clinical studies will be needed to demonstrate whether these small molecules can effectively redirect circulating T cells to eliminate tumor cells within the tumor microenvironment.

Another promising approach is the adoptive transfer of T cells genetically engineered to express a chimeric antigen receptor (CAR), an artificial transmembrane protein (Fig. 1c). The extracellular domain of a CAR determines the antigen specificity of the engineered T cell and is a single-chain Fv molecule derived from a monoclonal antibody or other recognition domain. CARs that are currently used in T cell therapies additionally contain a transmembrane domain and signaling components of costimulatory receptors (typically 4-1BB or CD28) and of the T cell receptor complex [30, 31]. The first generation of CARs, which did not yet utilize co-stimulation, was first described in 1993 by Zelig Eshhar [32]. For CAR T cell therapy, leukapheresis is performed to obtain T cells from patients as starting material for CAR T cell manufacturing, then T cells are activated *in vitro*, gene-modified by viral gene transfer to express the CAR transgene and expanded to therapeutic numbers under GMP conditions [33]. Effective CAR T cell therapy requires a combination of adoptive transfer and a lymphodepleting conditioning regimen, usually including cyclophosphamidse and fludarabine and starting 5 days prior to CAR T cell transfusion [34]. Lymphodepleting chemotherapy serves to optimize *in vivo* conditions for CAR T cell expansion and functional persistence as memory cells.

The potency of CAR-engineered T cells to eliminate even high burdens of malignant disease has been demonstrated in patients with refractory B cell cancers [35–38], leading to approval and routine use of CAR T cells directed against the B cell lineage antigen CD19. *In vivo* cytokine release leading

to systemic inflammatory responses and neurotoxicity and on-target interactions with normal cells are the major toxic side effects of CAR T cell therapy. Besides these potentially life-threatening toxicities, the major limitation of CAR T cell therapy in CD19-positive cancers is disease relapses caused either by clonal acquisition of mutations leading to loss of CD19 surface expression or by insufficient functional persistence of CAR T cells in patients. However, single infusions of autologous CAR T cells have been effective to repress CD19-expressing leukemias or lymphomas in certain patients for several years, creating a plateau of disease-free survival and possibly cure in a highly refractory patient population [35].

These studies have elevated expectations for the use of CAR-engineered T cells in solid cancers. A first-in-human study in neuroblastoma with CAR T cells targeting G_{D2} had been performed prior to the development of CD19-specific T cells [33]. However, the observed antitumor activity of CAR T cells in this study and subsequent studies on neuroblastoma was modest [39, 40]. More recently, encouraging individual objective responses were reported from dose-escalation studies with CAR T cells in pediatric neuroblastomas and sarcomas [41–43]. With further technical and strategic refinements, this modality may play an important role in advanced treatment regimens for childhood cancers in the future. However, there remain challenges that have yet to be sufficiently addressed in CAR T cell therapy development. These include limited target antigens that allow for selective and comprehensive targeting of the malignant clone while avoiding antigen-negative escape, and strategies that ensure tumor invasion, local expansion, and continued fitness of CAR T cells in the presence of immune-inhibitory cells and tumor stromal components.

4 CAR target antigens in pediatric cancers

A critical prerequisite for the use of CAR T cell therapy in pediatric cancers is aberrant (over)expression of cell surface antigens that facilitate T cell discrimination between normal and malignant cells. CAR targets exclusively and homogenously expressed on cancer cells have not yet been discovered. However, the B-lineage antigens CD19, CD20, and CD22 are potential targets since expression on healthy cells is limited to cell populations that are dispensable, namely B cells and their precursors. Moreover, on-target toxicity of CAR T cells by B cell depletion can be compensated by immunoglobulin administration to effectively prevent immunodeficiency. B cell antigens remain the only feasible targets for CAR T cell therapy, despite an abundance of other immune cell-restricted antigens. For instance, recent evidence found antigen-negative immune escape limits the curative potential of CD19-specific CAR T cells in B lymphoid cancers [44].

Current therapy development focuses on characterizing additional potential target antigens in pediatric solid cancers, some of which have reached advanced stages of (pre)clinical investigation. A non-protein antigen ganglioside G_{D2} was the first therapeutic CAR target evaluated in a childhood cancer [33]. G_{D2} is associated with immature neural crest tissue and consistently overexpressed in neuroblastoma, a tumor originating from G_{D2}-positive neuroectoderm [45, 46]. Co-expression on normal tissues is restricted to low levels on neuronal cell bodies in the central nervous system (CNS). Treatment of patients with refractory neuroblastomas with G_{D2}-redirected CAR T cells was safe in a first-in-human clinical trial [33, 40, 47], followed by further early-phase clinical studies using costimulatory signal-enhanced CARs and lymphodepleting regimens to enhance activity [39, 41]. Antitumor responses in these studies were moderate and only temporary. A recent trial has demonstrated dose-dependent clinical activity of a G_{D2}-specific CAR T cell product, associated with cytokine release syndrome and tumor lysis in individual patients [41]. Peripheral neurotoxicity with pain side effects characterizing treatment with G_{D2}-specific monoclonal antibodies was not observed, likely due to the lack of complement activation in the absence of the antibody Fc domain in the CAR [48]. Since CAR T cells effectively cross the blood-brain-barrier, supported by cytokines released in the periphery and causing capillary leaks, CNS toxicity is the predominant safety concern for the use of T cells against G_{D2}. In preclinical work, T cells expressing a G_{D2}-specific CAR caused neurotoxicity in one mouse model [49], but not in others [20, 21]. No on-target off-tumor toxicities in the CNS were observed in any of the clinical studies, including dose levels and designs with first evidence of activity [33, 40, 41]. Thus, a safe threshold for G_{D2} antigen recognition by CARs above the expression level on normal neural cells may exist and prevent neurotoxicities in humans. This supports the use of G_{D2}-redirected CAR T cells even in cancers of the CNS that also highly and uniformly express G_{D2}, including retinoblastoma [50, 51] and diffuse intrinsic pons glioma [52]. Mesenchymal childhood cancers of the bone and soft tissues, such as osteosarcoma, Ewing sarcoma, rhabdomyosarcoma, and desmoplastic small round cell tumors, can also contain G_{D2}-overexpressing tumor cell populations [53–57], though at more variable expression levels both among and within individual tumors [54, 55]. Ongoing G_{D2}-specific CAR T cell trials often include G_{D2}-positive sarcomas.

The transmembrane proteoglycan chondroitin sulfate proteoglycan 4 (CSPG4), also called neuron-glial antigen 2 (NG2), was first found to be overexpressed in melanomas [58], and subsequently in other solid tumors including pediatric glioblastomas [59, 60], rhabdomyosarcomas [61], osteosarcomas [62], and neuroblastomas [63]. It can be expressed on both tumor cells and angiogenic vasculature [60]. CSPG4 knockdown experiments in melanoma and glioblastoma

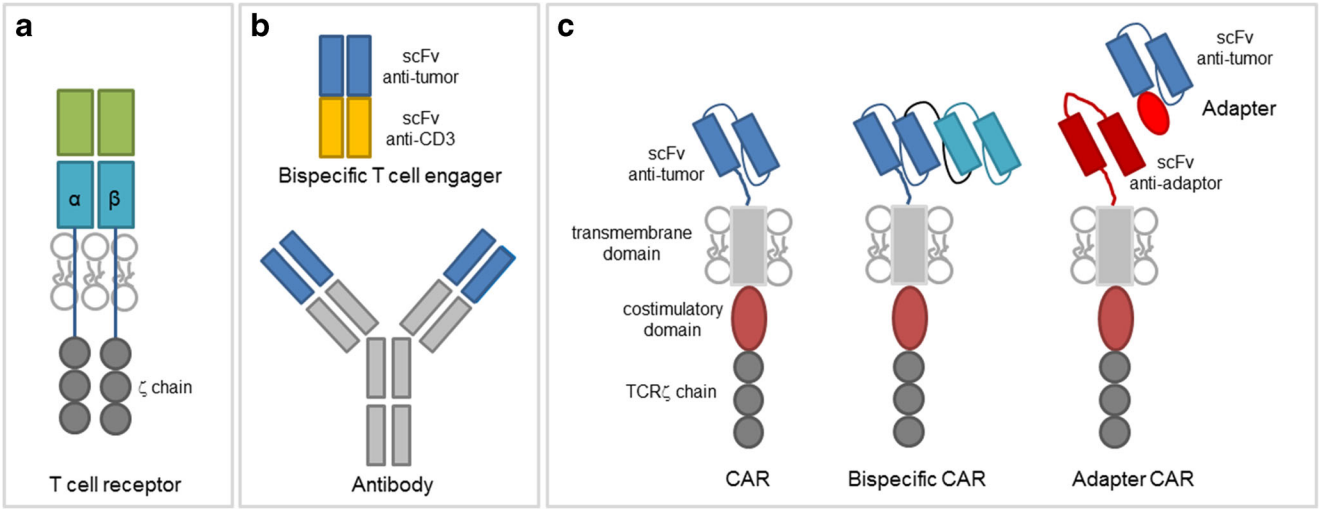

Fig. 1 Schematic presentation of strategies for redirecting T cells to recognize cancer cells. **a** T cell receptors with defined αβ recognition domains for tumor-associated peptides presented on MHC class I can be expressed in T cells. **b** Bispecific engagers of both tumor cells and T cells are generated by linking scFv domains of monoclonal antibodies directed against a tumor-associated surface antigen and against CD3, respectively. **c** CARs consist of extracellular scFv domains for antigen recognition, linked to costimulatory molecules and the ζ signaling domain of the T cell receptor complex. Bispecific CARs co-engage two antigens by single combined extracellular domains. Adapter CARs recognize tumor-associated antigens only in the presence of a tumor antigen-specific adapter molecule

demonstrated a functional role in tumor growth and angiogenesis [64], and expression in solid tumors is associated with poor survival [60]. Expression in non-malignant tissues is limited to low levels on vessels during wound healing. These findings together support the use of CSPG4 as a therapeutic target. In glioblastoma-derived neurospheres, CSPG4 was found to be further upregulated by tumor necrosis factor-α (TNF-α) released from microglia cells [60]. Thus, cytokine-inducible expression beyond constitutive levels may contribute to effective and selective T cell targeting of glioblastomas and prevent antigen-negative immune escape. CAR T cells against CSPG4 were generated from various monoclonal antibodies and by several groups and found to effectively interact with antigen-expressing tumor targets *in vitro* [60, 63, 65]. In a xenograft model, intra-tumoral administration of CSPG4-specific CAR T cells effectively controlled the growth of glioblastomas, without antigen escape [60]. Beyond solid tumors, CSPG4-specific CAR T cells are also under development for MLL-rearranged ALL where high expression was found to be associated with leukemic dissemination, migration, and seeding at extramedullary sides [66].

Another proteoglycan, Glypican-2 (GPC2), also called cerebroglycan, is densely surface-expressed in neuroblastoma [67, 68], medulloblastoma [67], and retinoblastoma [67]. In normal tissues, GPC2 is very restricted to low levels in peripheral nerves and skin. Expression in neuroblastoma is associated with molecular risk features, including *MYCN* amplification, and with a worse prognosis. GPC2 depletion in neuroblastoma cells resulted in apoptosis and growth inhibition *in vitro*, supporting its pro-tumorigenic function. GPC2-specific CAR T cells showed promising antitumor activity in a neuroblastoma mouse model [68], motivating further investigation of this target as a potential CAR target antigen in neuroblastoma.

Transmembrane receptors and oncogenic driver kinases such as epithelial growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and human epidermal growth factor receptor 2 (HER2) are overexpressed in many childhood cancers. While pharmacologic antibody or small molecule inhibitors of receptor tyrosine kinases in pediatric tumors have had very limited effectiveness [69], aberrant surface expression could serve to redirect T cells against cancer cells.

The most clinically advanced CAR target of this class is HER2. It is overexpressed in many osteosarcomas [70], though without amplification and at substantially lower levels than in breast cancers, limiting effective therapeutic targeting by monoclonal antibodies [71]. HER-2 is further expressed by some rhabdomyosarcomas [72] and medulloblastomas [73]. Clinical development of HER2-specific CAR T cells slowed after causing fatal respiratory failure in an adult colon cancer patient receiving a high dose of anti-HER2 CAR T cells derived from the monoclonal antibody trastuzumab [74]. A dose-escalation study of an alternative second-generation CAR in patients with osteosarcoma, starting with very low CAR T cell doses without lymphodepleting chemotherapy, could establish a safe dose of T cells that trafficked to tumor sites but did not expand within tumors, with limited clinical benefit [42]. In a subsequent trial, $1 \times 10^8/m^2$ autologous HER2-specific CAR T cells were administered following lymphodepletion to 10 patients with refractory or metastatic pediatric-type sarcomas [75]. CAR T cells expanded *in vitro* and remained detectable in all patients for at least 6 weeks. No

high-grade systemic or on-target toxicities occurred. Objective clinical benefit was reported in some patients, including a patient with rhabdomyosarcoma who achieved a complete remission. Thus, HER2 is a safe and potentially effective CAR target in at least a proportion of childhood cancers.

In glioblastoma, two widely expressed tumor-associated antigens are a genetic variant of EGFR, EGFRvIII, and the interleukin (IL)-13 receptor α2 (IL-12Rα2). Mutated EGFRvIII renders the protein constitutively active and thereby drives the malignant phenotype. This EGFR variant is expressed in 20–30% of newly diagnosed glioblastomas [76, 77]. Whereas EGFR surface expression is too broadly distributed among healthy and malignant cells to allow for safe tumor targeting, mutated EGFRvIII contains a tumor-specific epitope within the extracellular domain and is thus a potential CAR T cell target [78]. In a phase I study investigating single-dose intravenous infusion of T cells lentivirally engineered with an EGFRvIII-specific second-generation CAR without previous lymphodepletion in 10 adult glioblastoma patients, toxicities attributable to cross-reactivity with EGFR, as rashes, diarrhea, or pulmonary symptoms, were not observed and no neurotoxicities clearly attributable to CAR T cell transfer were reported [77]. Post-therapeutic biopsies confirmed CAR T cell trafficking to sites of active tumor in the brain, but no tumor regressions were seen. A likely reason was diminished antigen EGFRvIII expression in post-therapeutic tumors, indicative of immune escape of tumor cells expressing low-level EGFRvIII. No objective responses were seen also in an alternative phase I pilot trial investigating anti-EGFRvIII CAR T cell therapy in adult patients with glioblastoma, despite the use of lymphodepletion, post CAR T cell IL-2 support, and third-generation CAR design with both CD28 and 4-1BB co-stimulation, although dose-limiting toxicities and *in vivo* persistence of CAR T cells were seen [79].

IL13Rα2 is overexpressed in a majority of glioblastomas but is undetectable in normal brain tissue [80]. The receptor was found to specifically interact with EGFRvIII to promote glioblastoma cell proliferation by aberrant tyrosine kinase signaling [81]. Michael Jensen's group has generated a CAR against IL13Rα2 using a membrane-tethered IL-13 molecule as an extracellular recognition domain, with an incorporated mutation for selective binding to the α2 receptor. Following a pilot clinical study in which 3 patients with recurrent glioblastoma were safely treated with up to 12 consecutive intracranial doses of T cells expressing this first-generation IL13Rα2-specific CAR [82], a second-generation CAR was used along with an HSV1-TK reporter gene for non-invasive monitoring of CAR T cell viability and trafficking [83]. Up to 12 doses per patient were administered intratumorally or into the resection cavities at increasing doses. Imaging studies confirmed CAR T cell viability at sites of active disease. In one of the 7 patients, complete regression of all intracranial and spinal tumors was found [84]. Even though this patient was the only responder, and initial response was followed by progressive disease at other sites, this report supports the concept that CAR T cell therapy can mediate profound antitumor responses against solid malignancies.

The receptor tyrosine kinase anaplastic lymphoma kinase (ALK) is constitutively activated in pediatric anaplastic large cell lymphoma (ALCL) as a consequence of the characteristic ALK and nucleophosmin gene fusion. ALK is also overexpressed in advanced neuroblastomas [85]. Downstream signaling by activated ALK can drive oncogenesis and tumor cell survival. A small-molecule inhibitor, crizotinib, is tested in early clinical trials in both cancers, but acquisition of resistant kinase mutations can limit activity, and overexpression does not always reflect activated ALK signaling [86]. CAR T cells can target overexpressed ALK independent of kinase domain mutations and activation status. But in a preclinical study, high surface expression of ALK, exceeding the antigen density found on most neuroblastoma cells, was required for optimal ALK-specific CAR T cell function both *in vitro* and *in vivo* [87]. The function of ALK-specific CAR T cells was further limited by rapid and complete antigen-induced internalization of the CAR. For effective tumor targeting via ALK, combination strategies will be needed that enable reliable and adequate surface expression of this antigen on tumor cells.

Another example for a tyrosine kinase target is fibroblast growth factor receptor 4 (FGFR4). FGFR4 is expressed during myoblast development towards skeletal muscle [88]. In rhabdomyosarcoma, FGFR4 is found on the cell surface in all subtypes. It further is a direct target of the disease-defining translocation PAX3-FKHR in alveolar rhabdomyosarcoma [89]. Dysregulated FGFR4 signaling by mutations in the kinase domain found in a proportion of tumors [90] can act as a driver of tumor growth [91, 92]. CAR T cells against surface FGFR4 in rhabdomyosarcoma are in preclinical development [93].

B7-H3 (CD276), a member of the B7-CD28 family of immunoregulatory proteins, has recently emerged as an ideal candidate for immune targeting of pediatric cancers. B7-H3 consists of an extracellular paired V-C Ig domain and a short intracellular tail with no known signaling motif. Expression is human tissues is ubiquitous on mRNA level, but constitutive protein expression is restricted by a posttranslational mechanism to low levels on fibroblasts, endothelial cells, and osteoblasts, along with inducible expression in dendritic cells, monocytes, NK cells, B cells, and activated T cells. The biological function of B7-H3 is not understood. A first binding partner has recently been identified, the IL-20 receptor subunit α [94]. B7-H3 was identified as the target of a monoclonal antibody, 8H9, described by Nai-Kong B7-H3 (CD276), a member of the B7-CD28 family of immunoregulatory proteins, has recently emerged as an ideal candidate for immune

targeting of pediatric cancers. B7-H3 consists of an extracellular paired V-C Ig domain and a short intracellular tail with no known signaling motif. Expression is human tissues is ubiquitous on mRNA level, but constitutive protein expression is restricted by a posttranslational mechanism to low levels on fibroblasts, endothelial cells, and osteoblasts, along with inducible expression in dendritic cells, monocytes, NK cells, B cells, and activated T cells. The biological function of B7-H3 is not understood. A first binding partner has recently been identified, the IL-20 receptor subunit α [94]. B7-H3 was identified as the target of a monoclonal antibody, 8H9, described by Nai-Kong Cheung's group and highly reactive with cells from human brain tumors, childhood sarcomas, and neuroblastomas [95, 96]. Immunohistochemistry studies confirmed limited membrane staining on healthy tissues, in contrast to tumor cells from various pediatric solid cancer types and also tumor-associated vasculature [97–101]. On a tissue microarray including 388 pediatric tumor samples, a majority (84%) expressed B7-H3 [99]. Especially Ewing sarcomas, rhabdomyosarcomas, neuroblastomas, and brain tumors had consistently high staining intensities. Fc-enhanced monoclonal antibodies, antibody-drug conjugates, and radiolabeled antibodies targeting B7-H3 are under clinical development, including studies in neuroblastoma [102, 103]. Two independent groups reported that T cells expressing a B7-H3 specific CAR have significant preclinical *in vivo* activity in various mouse models [99, 104, 105]. Adoptive transfer of B7-H3 CAR T cells mediated regression of osteosarcoma, Ewing sarcoma and neuroblastoma xenografts, and eradicated medulloblastoma and glioblastoma xenografts from the CNS [99, 104, 105]. The CAR T cells preferentially targeted tumor cells with high B7-H3 expression, creating a therapeutic window, and no antigen loss or downregulation was found in these experiments [99]. B7-H3-specific CAR T cells cross-reactive with murine B7-H3 did not cause toxicity in a syngeneic mouse model [104]. Based on these promising preclinical data, B7-H3 specific CARs are now moving towards clinical trials in childhood cancers.

5 Target recognition: overcoming heterogeneity of antigen expression

With rare exceptions, adoptive T cell therapy for the treatment of solid cancers has been unsuccessful to date. One of the greatest obstacles to developing effective antibody-redirected T cell therapies for solid tumors is the intertumor and intratumor heterogeneity of antigen expression. With the exception of G_{D2} in neuroblastoma, none of the above-described antigens is expressed reliably and at high densities on the cell surface of pediatric tumors. Since many CARs require high target antigen expression to fully activate T cells [87, 106–108], CAR T cell therapy selects for tumor cells with low-level antigen expression which then reconstitute the disease.

A potential solution is pre-treatment or combination of CAR T cell therapy with agents that selectively upregulate antigen expression on tumor cells. Our group recently reported that treatment with an enhancer of zeste homolog 2 (EZH2) inhibitor at non-toxic doses upregulates the CAR target G_{D2} exclusively on Ewing sarcoma cells and sensitizes these cells to effective cytolysis by G_{D2}-specific CAR gene-modified T cells [109]. Other examples, though in hematological cancers, are the protein kinase C modulator bryostatin which upregulates CD22 on the cell surface of leukemic precursor B cells [110] and γ-secretase inhibitors which increase B cell maturation antigen (BCMA) on the cell surface of multiple myeloma cells [111]. Combination therapies with upregulating agents may extend application of current CAR T cells to tumors expressing the respective targets at low or heterogenous levels.

Antigen-negative immune escape by loss of function mutations under continuous pressure of persisting target-specific T cells has been observed in many responders to CD19-specific CAR T cell therapy. It illustrates that approaches targeting single antigens may not be sufficient for durable long-term antitumor responses, and this will be even more relevant in solid cancers with less ideal target antigens. More than a single antigen may have to be targeted simultaneously. Options are to deliver CAR T cell products with two or more distinct specificities, or to co-express different receptors in one cell [112]. T cells can even be engineered to recognize a complex expression pattern unique to the tumor site, including both tumor-associated antigens and stroma components [113]. When several CARs are co-expressed in one T cell, the distance of the target epitopes from the tumor cell membrane must be comparable for both antigens, or the length of the spacer must be individually adjusted. Advanced T cell engineering techniques now allow to co-target two antigens with a single CAR construct. Bispecific CARs with two antigen-binding domains, also called "tandem CARs," co-engage two antigens together in a bivalent immune synapse [114, 115]. Encounters with either of the antigens alone induce T cell activation and target cytolysis, thus extends CAR T cell reactivity to tumor targets expressing only one of two antigens and overcomes loss of antigen expression as long as the second antigen is preserved. Technical challenges of generating dual- or even multi-specific CARs are adequate protein folding of scFv molecules upon linear assembly and the stability of multi-scFv domains. More simply structured single-domain antibody mimics may provide alternative CAR recognition domains for the targeting of multiple antigens [116]. Still, the number of individual antigens targeted by a single engineered T cell will remain limited.

One innovative solution is the engineering of CAR T cells to secrete bispecific T cell engagers to an antigen broadly

expressed on the tumor cells, to allow for bystander cytolysis of tumor cells that fail to express the CAR antigen in the vicinity of antigen-expressing tumor cells. An example is secretion of a T cell engager against wild-type EGFR by EGFRvIII-specific CAR T cells [117]. On-target toxicities by lack of tumor restriction of the second target could be avoided by the local delivery of the engager in the tumor microenvironment.

An alternative approach involves modular constructs in which antigen recognition is dissociated from the signaling domain of the CAR to allow flexible targeting of various antigens. Such CARs, called universal CARs or adapter CARs, are redirected to a molecule not normally present in the human body, representing one of the two components of a bispecific adapter which cross-links CAR-expressing T cells to a tumor-associated target antigen [118–122]. Published adapter CARs have extracellular antigen recognition domains composed of avidin for binding to biotinylated antigens, or derived from monoclonal antibodies against a nuclear autoantigen, La-SS-B to recognize La-SSB-scFv fusion proteins, or against fluorescein (FITC) to engage FITC-labeled antibodies or conjugates [118, 119]. The antigen specificity of adapter-redirected CAR T cells is determined by the adapter molecule, and their function is limited to the time period in which an adapter is present in the blood stream. Sequential or concomitant administration of various bispecific adapters directed against individual tumor targets combined with adoptive transfer of the CAR T cell product can broaden the reactivity of the T cells to tumors with heterogenous target expression [118, 120, 121]. And due to the short half-lives of the adapters, on-target toxicities of adapter-redirected CAR T cells can be rapidly abrogated by stopping administration of the adapter. Thus, this strategy allows to target tumors with heterogeneous antigen expression while including a safety mechanism. Whether or not, and to what extent, adapter-redirected CAR T cells can persist *in vivo* in the absence of antigen and become reactivated by administration of adapters to maintain remissions remains to be studied.

The choice of targetable surface antigens in childhood cancers remains limited. Combinatorial antigen recognition could facilitate safe targeting of imperfect antigens more broadly expressed on healthy tissues. This can be achieved by modular designs employing synthetic Notch receptors which fuse an extracellular scFv to a Notch cleavage domain and an intracellular transcription factor expressed in T cells [123]. Antigen engagement of these receptors does not directly activate the T cell but induces transcriptional activation of a gene encoding for a CAR against a second antigen. Consequently, T cell activation and lysis is restricted to target cells co-expressing both antigens or expressing the two antigens in close co-localization [124]. In this way, T cells could engage with a heterogeneously expressed tumor cell-restricted antigen, e.g., G_{D2} in Ewing sarcoma, while simultaneously expressing a CAR that recognizes a homogenously expressed antigen on both malignant and non-malignant cells and thereby induce bystander lysis of G_{D2}-negative tumor cells.

6 Local action of redirected T cells at solid tumor sites: overcoming extrinsic and intrinsic mechanisms of dysfunction

Besides reliable antigen recognition, additional issues exist that prevent the optimal function of adoptively transferred T cells in solid tumors. This becomes evident by the lack of consistent therapeutic activity of G_{D2}-redirected CAR T cells even in neuroblastomas with their homogenous high-density expression of G_{D2}. Major issues are barriers against CAR T cells at solid tumor sites and their local inactivation and dysfunction by both extrinsic and intrinsic mechanisms.

While in hematological cancers, CAR T cells administered by the bloodstream can reach their targets in blood, bone marrow, lymph nodes, and at extramedullary sites and beyond the blood-brain-barrier, CAR T cells directed against solid cancers often fail to traffic to tumor sites and penetrate local stroma barriers. Co-expression of enzymes that degrade components of the extracellular matrix, such as heparanase, has led to improved tumor invasion in *in vivo* models [125], but concerns are tissue damage and supportive effects on tumor growth. Once they reach the tumor site, adoptively transferred T cells are exposed to a hostile vascular and cellular microenvironment, including myeloid-derived suppressor cells, tumor-associated macrophages and neutrophils, and regulatory T cells (reviewed in [126]), and to cytokines that polarize inflammatory cells towards immune suppression, such as vascular endothelial growth factor (VEGF), IL-4, and transforming growth factor (TGFβ). Immunosuppressive myeloid cell populations were found to suppress the homing, proliferation, and expansion of CAR-engineered effector cells in *in vivo* tumor models of Ewing sarcoma [55] and neuroblastoma [127]. Moreover, metabolic conditions in the tumor microenvironment challenge the T cells to survive in a hypoxic, acidic environment with low glucose levels and under oxidative stress [128].

Therapeutic blockade of a single factor that protects tumors against immune attack is unlikely to enable redirected T cells to gain activity in solid tumors. Conceptual advances are needed that simultaneously overcome essential barriers. *In vivo* lymphodepletion using both fludarabine and cyclophosphamide has been found to be a key prerequisite for optimal CAR T cell activity even in hematological malignancies [129], but this alone is insufficient in solid tumors. One approach is the use of CAR-engineered T cells as vehicles to produce and locally release a transgenic pro-inflammatory cytokine, e.g., IL-12, within the targeted tumor niche [130, 131]. Locally released IL-12 recruits additional immune

effector cells like NK cells to act against antigen-negative cancer cells and re-programs stroma-associated immune suppressor cells and dysfunctional T cells to overcome local immune suppression. A first-in-human trial performed by Stephen Rosenberg's group using tumor-infiltrating lymphocytes engineered to secrete IL-12 in an inducible manner upon antigen-specific T cell activation led to dose-limiting toxicities attributed to systemic IL12 [132]. An alternative cytokine with a favorable safety profile in humans, IL-18 [133], may be a more attractive candidate [134–136]: IL-18 secreted by CAR T cells creates a pro-inflammatory environment that enhances proliferation and cytolytic activity of CAR T cells and prevents their exhaustion. In addition, it recruits and activates bystander effector cells to broaden the antitumor response and avoid escape. IL-18-secreting CAR T cell targeting carcinoembryonic antigen had potent activity against solid tumors refractory to standard CAR T cells in both syngeneic and xenograft mouse models [134]. A third cytokine under clinical investigation for engineered augmentation of the function of both NK cells and (CAR) T cells is IL-15. Driven by the hypothesis that G_{D2}-specific CAR T cells in neuroblastoma do not meet their target sufficiently early after administration to receive their antigen-induced costimulatory survival signals, an IL-15 gene was introduced along with the CAR gene to enhance T cell survival in the absence of antigen, and in the immune-inhibitory neuroblastoma environment [137]. Clinical development of cytokine-enhanced CAR T cells requires safe and effective co-expression of the two genes. To prevent systemic side effects, cytokine expression by CAR T cells can be placed under control of the NFAT(6)-IL2 minimal promoter that initiates transcription upon antigen-induced T cell activation [134]. Further refinement of the strategy will require methods to define and control the quantities of local cytokine release. The principle of using CAR T cells as micropharmacies may be extended to the local production and release of antitumor molecules acting by other, non-immunological, mechanisms.

Immunization strategies could also be a powerful means to restore the function of CAR T cells in the tumor microenvironment *in vivo*. For this purpose, T cells with native TCR specificities for viral antigens, e.g., of Epstein-Barr Virus (EBV) [47] or Varicella-Zoster Virus (VZV) [138, 139], were proposed as CAR-engineered effector cells. This allows *in vivo* reactivation of CAR T cells by viral vaccination [140]. More recently, boosting of CAR T cells via their CAR was found feasible by the use of amphiphilic polymers. These polymers were designed to carry CAR target antigens to lymph nodes where they are integrated into the cell surface of antigen-presenting cells [141]. Vaccine boosting of CAR T cells was found to effectively rescue CAR T cells in preclinical models, and a clinical trial combining G_{D2}-specific CAR T cells with a VZV vaccine in patients with neuroblastoma is ongoing (NCT01953900). An alternative suggestion is the use of oncolytic viruses as combination partners for CAR T cells [142]. Pro-inflammatory danger signals by oncolytic viruses, along with their direct cytolytic properties, could be an elegant tool to increase T cell infiltration and change the tumor microenvironment for facilitating CAR T cell function [143].

Beyond exogenous parameters that affect the quality of CAR T cell responses to antigen, intrinsic signaling by the individual CARs requires careful consideration. Clinical studies in lymphoid malignancies have clearly established that the presence and type of costimulatory signal determines *in vivo* proliferation and persistence of CAR T cells [35, 36, 140]. Whereas CD19-specific CAR T cells with endogenous CD28 co-stimulation are characterized by especially rapid and strong *in vivo* expansion, continued detection of CAR T cells in peripheral blood beyond a few weeks is exclusively found in patients receiving products with 4-1BB derived costimulatory signaling domains. This translates into higher continued remission rates in patients with refractory acute lymphoblastic leukemia receiving 4-1BB-costimulated CAR T cells as their final therapy [35, 36, 144], but not in patients with lymphoma who fare at least equally well with both types of CAR T cell therapy [38, 145]. Thus, whether CAR T cells after their initial *in vivo* expansion have to persist for several days, weeks, or months to allow eradication of the disease may vary with the kind of malignancy. Experimental data confirm that the two clinically established types of CAR-mediated co-stimulation, CD28 and 4-1BB, have specific effects on T cell metabolism and the fate of antigen-induced T cell activation [146]. Immunostimulatory cytokines, e.g., IL-7, add important additional signals for optimal T cell activation. CAR T cells expressing a constitutively active IL-7 receptor unable to respond to cytokine were found to have superior performance both *in vitro* and *in vivo*, while T cell expansion remained strictly antigen-dependent [147].

Optimal costimulatory and cytokine receptor signals for ensuring CAR T cell function in the local tumor microenvironment of solid cancers have not yet been determined and may vary with different CARs and targets. Importantly, intrinsic signaling by CARs not only drives antigen-induced activation of T cells but can also contribute to their rapid exhaustion and dysfunction. Compared with the natural TCR immune synapse, signaling via CD28ζ CARs is stronger, more rapid, and of shorter duration [148]. In a G_{D2}-specific CAR, the CD28 costimulatory domain was found to mediate tonic signaling which promoted exhaustion of the engineered T cells [149]. 4-1BB co-stimulation was superior in this model, but others found that high retroviral expression of 4-1BBζ CARs in T cells can also induce tonic signaling, and along proaptotic pathways [150]. Signaling analysis of CD28-costimulated CAR T cells by mass cytometry at single cell

resolution revealed the CD3ζ component of the CAR along with its downstream canonical T cell receptor signaling as source of tonic signaling, leading the authors to investigate alternative CAR designs on the basis of γδ T effector cells [151]. Even extracellular spacer domains can contribute to tonic signaling [152], and this may vary with individual recognition domains and their avidities.

For more sustained CAR-mediated T cell activation against solid tumors, it will be important to select individual configurations and expression systems which together avoid tonic and/or death receptor signaling. Michel Sadelain's group found that editing the types and numbers of the activation motifs of CAR signaling domains can allow to calibrate the strength of the signal and tailor optimized effector/memory programs for enhanced stress resistance in *in vivo* models [153]. An alternative solution could be the use of the above-described modular system in which CAR-mediated T cell activation relies on the presence of adapters. Discontinuous, sequential administration of adapters may avoid or even revert exhaustion by continuous CAR signaling [118, 119, 121]. Protection against overstimulation and thus preservation of a more adequate phenotype may also be achieved by inducible gene-expression systems [154] or by pharmaceutical agents, as the tyrosine kinase inhibitor dasatinib, that interfere with CAR signaling and allow to induce a reversible OFF state [155]. At the same time, such strategies that allow to switch CAR T cell activity on and off in a controlled manner can act as emergency safety tools in cases of severe cytokine-mediated or on-target toxicities.

7 Conclusions

Redirecting cytotoxic T cells to pediatric cancers by engineering with natural or chimeric antigen receptors was found feasible in clinical studies. For the strategy to achieve their full potential, further improvements will be necessary. Consideration has to be given to (a) adequate target antigens and their combined recognition, (b) the nature and design of the signaling components of artificial receptors, to avoid exhaustion and premature cell death, and (c) strategies that augment both antigen recognition and T cell activity in the tumor microenvironment. Since all existing *in vivo* models have substantial flaws, only clinical studies can ultimately identify the most effective tools and combinations. Still, in view of the impressive activity of CD19-specific CAR T cells against refractory B cell cancers, including bulky disease at extramedullary sites and sanctuaries, the development of redirected T cell therapy for additional childhood cancers appears worth pursuing.

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