Chimeric Antigen Receptors for Cancer: Progress and Challenges

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Abstract Chimeric antigen receptors (CARs) genetically link an antigen-binding domain with cell-signaling domains to redirect immune cell specificity toward antigens expressed on the surface of cancer cells. Progress in CAR engineering over the last two decades has elucidated fundamental principles impacting CAR potency, and today CARs can be readily generated toward essentially any cell surface target on cancer cells. Efficacy thus far has been most impressive using CD19-CAR expressing T cells to treat B cell lymphoblastic leukemia, although clear activity has also been observed using CD19-CARs in patients with chronic lymphocytic leukemia and B cell lymphoma. Much less data is available regarding CAR efficacy for solid tumors. Here, we summarize current concepts of CAR design, with a focus on the relationship between structure and function, a review of the clinical results reported thus far, and the challenges to be addressed in future studies.

Keywords Adoptive immunotherapy · Chimeric antigen receptors · Genetic engineering · T cells · B cell malignancies · Acute lymphoblastic leukemia

Introduction

Chimeric antigen receptors (CARs) are hybrid immune receptors that directly bind to antigen and initiate immune cell activation, degranulation, and cytokine release. Although CARs theoretically can be expressed on essentially any immune cell, the vast majority of work using CARs to redirect specificity has focused on T cells. In this context, the fundamental goal of CAR engineering is to link T cell signaling domains with MHC-independent antigen-binding domains. Thus, CARs eliminate the MHC-restriction traditionally associated with T cell recognition, while maintaining T cell potency and the capacity for long-lived immune responses. Optimizing these “designer receptors” to yield greater potency and specificity continues to be a major focus of investigation. This review will discuss fundamental aspects of CAR design, providing both a historical perspective as well as a vision toward the future. We will also review clinical results of CAR-based T cell immune therapies for the treatment of cancer and comment on challenges facing this nascent, but promising field.

Principles of CAR Design

Antigen-Binding Domains

The first chimeric receptor was reported in 1989 by Eshhar et al., who developed a receptor linking the variable domains of a monoclonal antibody (mAb) targeting trinitrophenyl to the constant regions of the TCR alpha and beta chains, which

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endowed transfectants with MHC-independent antigen specificity [1, 2]. CAR potency was significantly increased in 1991 by incorporation of the TCR zeta chain as the signaling moiety in the receptor, in lieu of the TCR alpha or beta chains [3]. The first “modern CAR” was reported in 1993, which linked the potent TCR zeta signaling domain with a single chain variable fragment (scFv) as the antigen-binding moiety [4]. scFvs are fusion proteins derived from mAbs that covalently link heavy and light chain variable regions (V_H and V_L) via a flexible peptide linker. Thus, scFvs impart antigen specificity provided by both antibody heavy and light chains, but as a single peptide construct. Today, most CARs under study incorporate a scFv as the antigen-binding domain, though some investigators have also utilized ligands for receptors overexpressed by tumor cells as antigen-binding domains, most notably IL13 to target IL13R α 2⁺ brain tumors [5–11].

Several properties of the antigen:antigen-binding domain interaction have been demonstrated to impact CAR efficacy including affinity to antigen [12–15], target epitope location [15–17], and target epitope density [12, 17]. In general, a threshold level of affinity is required for CAR activity. However, the relationship between affinity and efficacy is less clear for CARs than it is for soluble mAbs, as higher-affinity scFvs do not always provide improved CAR efficacy [14, 15]. Regarding target epitope location, several studies demonstrate that membrane-proximal epitopes are more effectively targeted via CARs than membrane-distal epitopes [15–17], again a distinction from principles of mAb-based protein therapeutics. With regard to target epitope density, it is clear that antigen density above a minimum threshold is required for effective CAR activity. However, prediction of a universal threshold across antigens remains difficult, since the importance of other factors such as scFv affinity and CAR expression levels is also impacted with changes in target epitope density. As discussed later, an improved understanding of the impact of target epitope density is critical for optimizing CAR therapy, both in terms of understanding the risks associated with low-level antigen expression on normal tissues and in addressing the problems of tumor resistance associated with antigen downregulation.

Recently, oligomerization of scFvs has been identified as a critical factor that can diminish the efficacy of CAR T cells through the induction of antigen-independent, tonic signaling [18•]. Long et al. compared the efficacy of two distinct CARs, one targeting CD19 and a second targeting the GD2 ganglioside, against an engineered osteosarcoma line expressing both GD2 and CD19 antigens. Both CARs demonstrated equivalent killing *ex vivo*, whereas only the CD19-CAR reduced tumor size *in vivo*. T cells expressing the ineffective GD2-CAR in this study developed evidence of exhaustion early after transduction, which was not observed in T cells transduced with the CD19-CAR. Further studies revealed that the scFv incorporated into the GD2-CAR spontaneously aggregated on the surface of the transduced T cells, leading to

antigen-independent signaling. Early exhaustion was also observed in T cells expressing CARs targeting Her2Neu and CD22, but was distinctly absent in T cells expressing the CD19-CAR. These results demonstrate that some cell-associated scFvs spontaneously aggregate, consistent with similar observations made historically by investigators studying scFv proteins [19–21]. Similar findings were also recently reported in CARs targeting c-Met and mesothelin, where constitutive signaling was associated with diminished functionality in xenograft models [22]. Thus, an emerging paradigm holds that a propensity for individual scFvs to induce spontaneous CAR signaling can adversely affect CAR function, and therefore such properties should be screened for early during CAR development.

Modulation of “spacer domains” represents another area of CAR design where emerging science demonstrates impressive effects on functionality. Spacer domains are frequently incorporated between the antigen-binding domain and transmembrane segment in order to improve accessibility to target antigens. Frequently, spacer domains take the form of immunoglobulin hinge and constant regions, but can also comprise extracellular domains from CD28, CD8 α , or other proteins [23•]. An increasing number of reports suggest that the CAR spacer domains can significantly impact functionality of these synthetic receptors. Effective targeting of some antigens requires that CAR spacer length be optimized in order to obtain efficient lysis and cytokine production upon exposure to target cells. This is thought to be due to a need to achieve an ideal distance between the target cell and T cell membranes [13, 17, 24, 25]. However, the importance of optimizing spacer domain length does not appear to be universally required [15, 17]. Furthermore, recent reports have demonstrated that immunoglobulin-based spacer domains can negatively impact CAR efficacy in xenograft models [26, 27•]. In these studies, the Fc γ R1 receptor on Ly6C⁺ myeloid cells in immunodeficient mice cross-linked CARs incorporating an IgG₄ CH₂CH₃ spacer domain, leading to CAR T cell activation-induced cell death *in vivo* and poor antitumor efficacy. These findings may have significant implications regarding the future design of CAR spacer domains, though the extent to which this phenomenon occurs in the immunocompetent host where Fc receptors are likely pre-occupied by circulating immunoglobulins remains to be seen.

Costimulatory Signals

T cells expressing “first generation” CARs, which incorporate a primary signaling domain without a costimulatory domain, showed some efficacy in early studies [28–36], though cytokine production and *in vivo* persistence of these CAR T cells were modest [32, 37–39]. Thus, “second generation” CARs were developed, which incorporate a costimulatory domain into the primary receptor, thereby providing the second signal

necessary for complete T cell activation upon antigen binding. The most common costimulatory domains incorporated into second generation CARs have been CD28 or 4-1BB [40], though OX40 [41, 42], CD27 [43], ICOS [44] and FcεRIγ [23•] have also been described. To date, no comprehensive study has systematically compared the efficacy of CAR expressing T cells bearing the entire range of known costimulatory domains, but numerous studies have shown that the addition of a costimulatory domain significantly enhances CAR T cell persistence and cytokine production in preclinical [45–51] and clinical trials [52•]. Thus, second generation CARs are the most common form currently being tested.

A recent study provided insight into qualitative distinctions between the CD28 and 4-1BB costimulatory domains on CAR T cell function. Using a GD2-specific CAR, which developed early exhaustion due to tonic, antigen-independent signaling, investigators observed that GD2-CAR T cells expressing the TCR zeta and the CD28 costimulatory domain had greater levels of T cell exhaustion than those incorporating TCR zeta alone [18••]. In contrast, CAR T cells expressing TCR zeta and the 4-1BB costimulatory domain showed diminished exhaustion compared to GD2-CARs expressing CD28 endodomains. This study was the first to identify a potent anti-exhaustion effect of 4-1BB costimulation, which resulted in greater persistence of both CARs rendered exhausted due to tonic signaling (e.g., GD2-CARs), as well as those rendered exhausted by high levels of antigen (e.g., CD19-CARs transferred into leukemia bearing hosts). Thus, emerging concepts hold that investigators can selectively design CARs to be more persistent by incorporating a 4-1BB costimulatory domain, whereas CARs designed for short-term effects might be better suited with a CD28 costimulatory domain.

Some groups have also developed “third generation” CARs, which incorporate two costimulatory domains, though the data remains unclear as to whether additional costimulation enhances efficacy of CAR therapies [15, 41, 42, 53, 54]. Indeed, some studies have demonstrated that excessive costimulation such as that provided by the “third generation” CARs is associated with diminished efficacy. Consistent with this model, recent work by Kunkle et al. demonstrated that augmentation of T cell activation via CAR signaling either by modifying spacer lengths or costimulatory domains may ultimately diminish CAR functionality due to the induction of activation-induced cell death [55]. Together, the data suggest a “goldilocks” phenomenon for CAR T cell activation, where underactivation or overactivation is equally undesirable, and where optimal functionality likely requires intermediate levels of activation.

Next Generation CAR Engineering

Tumor heterogeneity is a fundamental principle in oncology, and a corollary is that any therapeutic focused on one target is

likely to ultimately be met with resistance due to selection of cells lacking the target of interest. CAR-based immunotherapy is no exception to this rule, and emerging data emanating from studies of CD19-CARs for the treatment of B cell acute lymphoblastic leukemia have demonstrated a substantial incidence of CD19 immune escape [56•, 57•]. Similar results have also been observed following treatment with blinatumomab, the CD19/CD3 antibody-based bispecific engager [58]. Loss of expression of EGFRvIII has also been described on glioblastoma targeted with CAR T cells to that antigen. Thus, the development of effective cancer immunotherapies will likely require targeting of more than one antigen, and studies are underway to determine the best way to accomplish this in the context of CAR-based therapies.

The simplest approach to designing CAR therapies against two antigens is to utilize two populations of cells, each with specificity for one antigen. Proof-of-principle for this approach comes from studies by Hedge et al., wherein investigators demonstrated significant heterogeneity of Her2Neu and IL13Rα2 expression among glioblastoma multiforme tumors at the time of resection, which was followed by the development of antigen loss variants following immune pressure mediated by CAR-modified T cells. Such evolution could be inhibited by simultaneous exposure to CARs targeting Her2 and IL13Rα2 [59•]. Alternatively, gene constructs can be engineered to express two CARs on the surface of an individual T cell, and while this is feasible, expression of each CAR in this setting may not be optimal. Recently, tandem CARs have been reported, which incorporate two scFv antigen-binding domains into one receptor using Gly-Ser linkers [59•, 60•]. Given the prevalence of tumor heterogeneity and the emerging importance of antigen loss/immune escape in the context of immunotherapy, it is anticipated that studies of bivalent CARs and regimens incorporating CARs targeting multiple antigens will be a focus of intense study in the coming years.

Many groups are also focusing on novel strategies to enhance specificity of CARs toward cancer rather than normal tissues, in an effort to minimize on-target, off-tumor toxicity. To this end, inhibitory CARs (iCARs) have been recently described, which incorporate signaling domains of inhibitory receptors such as PD-1 and CTLA-4 [61]. Such models hold that T cells co-transduced with CARs specific for tumor-antigens and iCARs specific for normal-tissue antigens would theoretically activate and degranulate only in the absence of normal tissue antigen, enhancing specificity for tumor. Other studies have attempted to restrict CAR T cell killing to cells concurrently expressing two tumor antigens. To date, this has been proposed by separating the CD3ζ and costimulatory domains across two different CARs, in hopes the presence of both antigens will be required for full CAR T cell effector function [62•, 63]. Proof-of-principle studies have been reported, though it appears that risk remains if “inefficient”

CARs with CD3 ζ signaling are rescued through exposure to costimulatory signaling within the inflamed tumor microenvironment. Furthermore, it also remains unclear to what extent such dual CAR T cells could be “licensed” at the tumor site where both antigens are present, and subsequently mediate on-target off-tumor toxicity in other compartments.

CAR T Cells for B Cell Malignancies

Clinical Results

The first clinical report of CD19-CAR T cells was published in 2010 and described a case of lymphoma regression and B cell depletion following infusion of CAR T cells [64]. Since that time, several groups have demonstrated activity of CD19-directed CAR T cell therapies against a variety of B cell malignancies [56•, 57•, 65, 66, 67•]. The most impressive responses have occurred in children and adults with B cell acute lymphocytic leukemia (B-ALL), where complete response rates of 70–90 % have been reported [56•, 57•, 67•]. Studies in adult B cell lymphoma and chronic lymphocytic leukemia (CLL) trials have posted lower but still impressive response rates [65, 68]. Specific response rates reported in these early trials should be interpreted with caution however, since most studies have excluded patients whose engineered CAR product does not meet pre-established criteria for release, and excluded subjects whose disease progressed during the interim between apheresis and product generation. One study provided a true intent-to-treat complete response rate of 70 % in refractory B-ALL [56•]. This trial also demonstrated 90 % feasibility of generating and delivering the prescribed dose of CD19-CAR T cells in heavily pretreated patients with refractory B-ALL, many of whom had already undergone one or two hematopoietic stem cell transplants [56•]. Thus, while these early impressive results make it clear that CD19-CAR therapy provides potent therapeutic effects against an array of B cell malignancies, results of formal phase II studies of CD19-CAR therapy are needed in order to accurately measure true response rates to CD19-CAR therapy across diseases and across platforms.

Most CD19-CAR T cell protocols administer relatively low cell doses, in the range of 10^7 – 10^8 CAR T cells, which undergo dramatic expansion in vivo and can eradicate large disease burdens. Indeed, the typical CD19-CAR T cell dose range is approximately two logs lower than the T cell doses administered in the context of adoptive immunotherapy using tumor infiltrating lymphocytes [69] and substantially lower than the dose of T cells administered in clinical trials using genetically engineered, affinity enhanced T cell receptors [70]. The difference in dose necessary for effective expansion in vivo likely relates to the fact that modern CARs incorporate a costimulatory domain, which enhances in vivo expansion

and therefore enhances potency [52•]. Indeed, administration of 10^{10} CAR-transduced T cells with specificity for Her2Neu and incorporating both a CD28 and a 4-1BB endodomain was fatal, likely due to massive cytokine release syndrome [71]. Thus, on a cell per cell basis, the potency of CARs incorporating costimulatory domains is vastly greater than the potency of other adoptive cell therapies studied thus far. The lower total dose of T cells needed for efficacy has had substantial practical implications for simplifying and shortening the processes needed to generate CAR T cell grafts compared to other adoptive cell therapy products.

Animal studies clearly demonstrate the importance of lymphodepletion in supporting expansion of adoptively transferred T cells through the induction of elevated levels of homeostatic factors such as IL-7, which support the infused CAR T cells [72, 73]. In addition, such regimens transiently diminish regulatory T cells, may augment innate immunity as a result of damage to the gut epithelium, may alter myeloid suppressor populations, and may allow enhanced penetration into the tumor bed, especially in the setting of solid tumors [74, 75]. Numerous uncontrolled clinical studies provide data consistent with a beneficial effect of lymphodepletion prior to adoptive cell therapy for cancer. For example, three CLL patients treated with CD19-CAR T cells without a preparative regimen had no response, while three of four subsequently treated patients who received cyclophosphamide prior to cell infusion had significant responses [76]. Similar results have been reported in studies of adoptive immunotherapy using tumor infiltrating lymphocytes for melanoma [77]. Lymphodepleting regimens have varied widely across trials and it remains unknown whether one regimen is superior to another, or whether increasing dose intensity in the lymphopreparative regimen impacts response rate to CD19-CAR therapy. Relatively low doses of chemotherapy appear sufficient for hematologic malignancies [56•]; however, higher doses of chemotherapy have often been administered in the setting of CAR therapy for lymphoma [68]. It is important to note that short-term response rates in clinical trials incorporating a dose-intensive lymphopreparative regimen should be interpreted with caution, since agents utilized in most preparative regimens are also directly active against B cell malignancies and could impact response rates directly.

All but two clinical trials of CD19-CAR T cells to date have utilized T cell grafts engineered from autologous T cells, regardless of whether the recipient has previously undergone allogeneic hematopoietic stem cell transplantation (HSCT). However, it is well recognized that patients relapsing after an allogeneic HSCT often have dysfunctional and few T cells, potentially diminishing the efficacy of CAR therapy in this setting. A trial by Cruz et al. administered donor-derived, viral-specific T cells engineered to express a CD19-CAR incorporating the CD28 costimulatory domain post-transplant without a lymphopreparative regimen. Objective responses

were observed in two of six patients with evaluable disease at the time of treatment and there was no evidence for graft-versus-host disease (GVHD) [78]. A second trial administered polyclonal donor-derived CD19-CAR T cells to allogeneic HSCT recipients post-relapse, also in the absence of a lymphodepleting preparative regimen. Despite the fact that potentially alloreactive, non-tolerized T cells of unknown specificity were infused directly to the recipient, no significant GVHD occurred, and three of ten patients showed evidence of an objective response [79]. These results suggest that donor-derived CD19-CAR therapy may provide an option for patients following allogeneic HSCT, although it remains unclear whether the relatively low response rates observed in these series relates to the absence of a lymphopreparative regimen, and whether efficacy and/or safety might be modulated by administering such a regimen in this setting.

Impact of Costimulatory Domain on CAR Persistence

In clinical results reported thus far, response rates appear similar regardless of whether the CD19-CAR transgene is inserted via a lentiviral versus a retroviral vector, and similar response rates have been observed using CD19-CARs incorporating the FMC63 scFv [56•, 57•, 80] versus the SJ25C1 scFv [67•]. In contrast, emerging clinical results have identified distinctions in behavior between CD19-CARs incorporating a CD28 costimulatory domain (e.g., CD19.28.z) compared to those incorporating a 4-1BB domain (CD19.BB.z). In general, CD19.28.z undergo dramatic early expansion, often associated with early cytokine release syndrome and high response rates [56•, 67•, 80]. However, such CARs rarely persist long-term and recovery of normal B cells within 2–3 months is typically observed. CD19.BB.z CARs incorporating the 4-1BB endodomain also undergo dramatic expansion, although the time to peak expansion appears slower and peak levels measured by PCR appear to be lower than observed in clinical trials using the CD19.28.z CARs [57•]. Furthermore, CD19.BB.z CAR T cells often persist for months to years, resulting in profound and prolonged B cell aplasia [57•]. Thus far, clinical results are not mature enough to determine whether the prolonged persistence of the CD19.BB.z CAR translates into improved cancer control in the long-term and whether this varies with disease. Such questions are critical to address in the coming years.

Toxicity of CD19-CAR Therapy

The most notable and frequent adverse event of CD19-CAR T cell therapy is cytokine release syndrome (CRS), which may range from a simple fever and constitutional symptoms to difficult-to-control hypotension requiring multiple vasopressors, respiratory failure, end-organ damage, or death. In typical cases, multiple inflammatory cytokines are elevated during

the peak of symptoms, with levels of IL-6 and IFN γ correlating with severity [56•]. The syndrome appears to be effectively treated with immunosuppression, most commonly tocilizumab (an anti-IL6R antibody) or corticosteroids [57•, 66, 67•, 81]. Current concepts hold that it is important to scale interventions to the severity of the syndrome, to minimize risks of excessive toxicity while maximizing the chance for therapeutic benefit from the CAR therapy. Several groups have demonstrated an important role for tumor burden in increasing the risk for severe cytokine release syndrome [56•, 57•], raising the prospect that incorporation of CD19-CAR therapy into regimens where disease burdens are lower at the time of treatment may abrogate this toxicity altogether. Because multiple different grading systems have been used to assess severity of cytokine release syndrome, it has been difficult to compare the incidence and severity of this syndrome across CAR platforms. Recently, a multi-institutional consensus paper was published with the goal of establishing a standardized grading system linked to a treatment algorithm [81]. Harmonized grading systems for this novel toxicity would allow results of treatment interventions to be compared across institutions and clinical trials, and help to ensure the safety of this therapy as it is exported to larger numbers of institutions.

Neurotoxicity is another notable adverse event of CD19-CAR T cell therapy. Visual hallucinations, dysmetria, ataxia, temporary aphasia, and seizures have been reported [56•, 57•, 68], all of which are typically fully reversible within hours to days. The basis for the toxicity remains unclear. While low-level CD19 expression has been reported on neuronal cells by immunohistochemistry [82], surface expression has not been validated. Furthermore, on-target, off-tumor T cell cytotoxicity of neuronal cells appears unlikely given the general reversibility of the neurologic symptoms. Alternatively, high levels of inflammatory cytokines could account for these symptoms. Elevated levels of inflammatory cytokines are present in the CSF of patients with CRS following CD19-CAR therapy [81] and IL-6 has been directly implicated in neurotoxicity [83, 84].

Challenges and Opportunities

Multiple Dosing Strategies and CAR Immunogenicity

Experience with cell-based therapy for melanoma has demonstrated that effective antitumor immune responses often manifest over the course of many months [85]. Similarly, while impressive anti-leukemic effects of CD19-CAR therapy are often observed within 28 days of treatment, many investigators believe that long-term disease control and/or cure require long-term immune pressure. One potential approach to apply persistent immune pressure utilizing CAR T cells is administration of multiple doses. However, several groups have

measured T cell-mediated anti-CAR immune responses following CAR therapy, and such responses may preclude the effectiveness of multiple dosing regimens [56•, 86–88]. Indeed, results with multiple dosing strategies using CAR expressing T cells have been disappointing thus far. In the report of Lee et al., three patients were treated with a second dose of CD19-CAR T cells for residual or recurrent CD19+ ALL following an initial dose of CD19-CARs and none responded [56•]. In another report, mesothelin-CARs were administered repeatedly and one patient experienced anaphylaxis following the third dose [89]. Many of these patients were highly immunocompromised, suggesting that CAR T cell therapies are sufficiently immunogenic to induce anti-CAR immunity even in such a setting. The molecular basis for immunogenicity of CAR T cells has not been systematically mapped thus far; however, most current CARs under study utilize a murine-based scFv, which is a likely candidate for the target of anti-CAR T cell immunity. For this reason, novel CARs currently being developed are often utilizing humanized or fully human binders [15]. Alternatively, junctions between the costimulatory domain and CD3 ζ , or the scFv and the transmembrane domain, also represent “neoantigens” and could contribute to immunogenicity.

CARs for Solid Tumors: Target Selection

A major factor in the success of CAR therapy for B cell malignancies is that normal tissue expression of CD19 is limited to B cells. CAR-induced B cell depletion appears to be adequately managed, even in the long-term, with replacement immunoglobulin therapy [57•]. Unfortunately, most cell surface molecules that are highly expressed on solid tumors are also expressed to varying degrees on normal tissues, and thus do not have as favorable of expression profiles as CD19. A primary challenge facing CAR therapy for solid tumors will be the identification of antigens that can be safely targeted. Pertinent to this issue is a deficit in our understanding of the degree to which low level antigen expression on normal tissues poses a risk in the context of CAR therapy. Emerging data from several sources provide increasing insights into this important problem.

On-target toxicity related to CAR therapy is most well documented from studies of a CAR targeting carbonic anhydrase IX (CAIX), which is highly expressed on renal cell carcinoma as well as normal bile duct epithelium. An initial report of three patients treated with high doses (1×10^9) of first generation CAIX-CAR T cells reported reversible, severe (grade 3–4) hepatitis following CAR infusion [90]. A follow-up study provided histologic evidence of CAR infiltration at sites of inflammation and also demonstrated that pre-treatment with an unconjugated anti-CAIX mAb prevented liver toxicity in patients receiving similar doses of CAIX-CAR T cells [86]. As discussed above, anti-CAR immune responses were observed

in patients in this trial; however interestingly, such immune responses did not occur in patients pretreated with anti-CAIX mAb [86].

Studies targeting GD2, a ganglioside expressed on neuroblastoma, some sarcomas and melanomas, and on peripheral nerves at low levels [91], have yielded a different experience. Treatment of children with anti-GD2 mAb therapy typically results in severe, temporary, neuropathic pain, presumably resulting from complement activation at the site of peripheral nerves [92]. In contrast, administration of a first generation GD2-CAR T cells without lymphodepletion demonstrated a favorable safety profile with no evidence for neurotoxicity. Eleven children with high risk or refractory neuroblastoma were treated with a first generation CAR targeting the disialoganglioside GD2. Half of eight evaluable patients had objective responses with one complete response [31], with GD2-CAR detected in surviving patients up to 192 weeks after infusion [93•]. Clinical trials of a third generation GD2-CAR for osteosarcoma and neuroblastoma administered following lymphodepletion are underway (NCT02107963, NCT01822652, NCT01953900), and no evidence for neurotoxicity has been reported.

A third scenario, as discussed above, involved administration of very high doses of CAR T cells expressing a third generation Her2Neu-CAR following lymphodepletion, which resulted in fatal toxicity with onset of symptoms within 30 min of infusion [71]. Initial reports implicated low-level antigen expression on normal tissues in the toxicity observed. However, a subsequent interpretation was that this toxicity represented cytokine release syndrome related to the high T cell dose, rather than on-target, off-tumor toxicity. On this basis, a follow-up study was performed using a second generation Her2Neu-CAR with careful dose escalation. Results of this recently published study demonstrate that administration of second generation anti-Her2Neu CAR T cells at doses up to $1 \times 10^6/\text{m}^2$ resulted in significant expansion and persistence without evidence of toxicity [94]. Studies are ongoing to determine whether this favorable safety profile observed using the Her2Neu-CAR persists following a lymphodepleting preparative regimen. In summary, while there is clear evidence that normal tissue expression of a CAR target (e.g., CAIX) can result in substantial toxicity, some targets expressed at low levels on normal tissues have not resulted in autoimmune toxicity. More work is necessary to better understand the degree to which low-level expression of target antigens, on either normal or malignant tissues, serve as effective targets for CAR therapy.

Regional Delivery and Approaches to Enhance Tumor Trafficking

One approach to potentially prevent systemic off-tumor, on-target cytotoxicity of solid tumor CARs, and to enhance

exposure within the tumor microenvironment, is to deliver CARs regionally. An increasing number of groups have begun investigating such an approach. Her2Neu is highly expressed in glioblastoma and other malignant CNS tumors, and an ongoing study of intracranial injection of Her2Neu-CAR T cells for glioblastoma is underway (NCT02442297). Another group is investigating intratumoral delivery of Her2Neu-CAR T cells that expand preferentially with IL-4 [95] for recurrent head and neck squamous cell carcinoma (NCT0181323). Intrapleural delivery of mesothelin CAR T cells in mesothelioma has shown promise in preclinical models [96] and is currently being tested in a clinical trial [75]. Regional delivery may require significantly lower doses of cells and result in less systemic exposure, thereby minimizing toxicity while maximizing antitumor response.

Other groups have attempted to improve tumor trafficking by modulating chemokines and/or incorporating chemokine receptors into CAR vectors. For example, neuroblastoma and other tumors secrete CCL2. Tumor cell infiltration by GD2-CAR T cells in pre-clinical models increased dramatically upon co-expression of CCR2, the receptor for CCL2, and mediated increased cytotoxicity [97]. Thus, exploiting tumor-secreted factors, many of which are classically thought to be inhibitory, by incorporating their receptors into the CAR construct is another way to potentially augment CAR T cell function.

Conclusions

Adoptive T cell immunotherapy with CD19-directed CAR expressing T cells has shown unprecedented response rates as a single therapeutic maneuver in patients with refractory B lymphoblastic leukemia. Impressive response rates have also been observed in other B cell malignancies. This success resulted from over 20 years of optimizing the composition of such receptors, though our understanding of the optimal approach to engineering these receptors continues to evolve. Numerous challenges remain in this field, as investigators seek to expand the repertoire of effective CAR therapies to other hematologic malignancies and to solid tumors. Ultimately, the full potential of this field can only be realized by careful basic and clinical studies that can identify optimal methods for CAR engineering, safe and effective antigens for targeting, and clinical approaches to deliver the therapy most safely and most effectively.

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Compliance with Ethical Standards

Conflict of Interest Adrienne H. Long and Daniel W. Lee declare that they have no conflict of interest.

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- Of major importance

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