International Immunology, Vol. 28, No. 7, pp. 355–363
doi:10.1093/intimm/dxw018
Advance Access publication 28 March 2016

Chimeric antigen receptor-modified T cells strike back

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Received 17 February 2016, *accepted* 22 March 2016

REVIEW

Abstract

Chimeric antigen receptors (CARs) are engineered molecules designed to endow a polyclonal T-cell population with the ability to recognize tumor-associated surface antigens. In their simplest form, CARs comprise a targeting moiety in the form of a single-chain variable fragment from an antibody connected to various intracellular signaling domains allowing for T-cell activation. This powerful approach combines the specificity of an antibody with the cytotoxic ability of a T cell. There has been much excitement since early phase trials of CAR-T cells targeting CD19 expressed on B-cell malignancies demonstrated remarkable efficacy in inducing long-term, stable remissions in otherwise relapsed/refractory disease. Despite these successes, we have just begun to understand the intricacies of CAR biology with efforts underway to utilize this platform in the treatment of other, previously refractory malignancies. Challenges currently include identification of viable cancer targets, management strategies for potentially severe and irreversible toxicities and overcoming the immunosuppressive nature of the tumor microenvironment. This review will focus on basic CAR structure and function, previous success and new approaches aimed at the broader application of CAR-T-cell therapy.

Keywords: cellular immunotherapy, chimeric antigen receptor, clinical immunology, gene therapy, T-cell therapy

Introduction: origins of chimeric antigen receptors

The idea that the immune system could be harnessed to treat cancer was demonstrated as far back as 1893 by William Coley's use of Coley's toxin, a mixture of heat-killed bacteria, to elicit regression of inoperable sarcomas. Although his experiments were likely the first demonstration of Janeway's 'dirty little secret', the immune system's true role in fighting malignancy remained highly contentious throughout the first half of the 20th century. It wasn't until 1967 when Frank Macfarlane Burnet described the possibility of immunological surveillance and tumor-associated antigens (TAAs) that we began to understand the true potential of the immune system in cancer therapy (1). Since these first descriptions, our understanding of the role of the immune system in cancer has grown considerably along with the technology to purify and manipulate specific immune cell types with the goal of treating cancer.

The concept of a chimeric antigen receptor (CAR) was first described by Gross *et al.* in 1989 (2). These early experiments recombined the variable heavy and light chain (V_H and V_L) regions of antibodies recognizing a model antigen (a hapten, 2,4,6-trinitrophenyl) with the constant (C)-region gene segments of the α or β chains from TCRs (Fig. 1) transfected via somatic fusion into T-cell hybridoma cell lines. The power of such an approach was recognized by the senior author, Zelig Eshhar, who even hypothesized the therapeutic use of these early chimeric receptors. This was followed shortly thereafter with the descriptions of chimeric receptors encoding CD4, CD8 or CD25 linked to intracellular signaling domains (ICDs) that could replicate much of the TCR signaling necessary for activation (3–5).

Some of the earliest clinical work demonstrating the use of CAR-modified T cells (CAR-T cells) was performed in patients with HIV. Between 1998 and 2005 three clinical trials evaluated the use of CD4ζ (i.e. CD4 or CD8 extracellular and transmembrane domains linked to the intracellular portion of CD3ζ responsible for T-cell signal transduction) CARs (Fig. 1) expressed in autologous CD4 and CD8 T cells (clinicaltrials.gov NCT01013415) (6, 7). These studies did not show significant efficacy; however, they did demonstrate the long-term safety of retrovirally modified peripheral T cells. In a subsequent analysis, CD4ζ CAR-T cells were detected in 98% of samples tested for at least 11 years after infusion at frequencies exceeding the average T-cell levels observed with most vaccine approaches. Furthermore, of the >500 patient-years of follow-up, no transformational events were noted emphasizing the safety of T cells modified by retroviral gene transfer in clinical application (8).

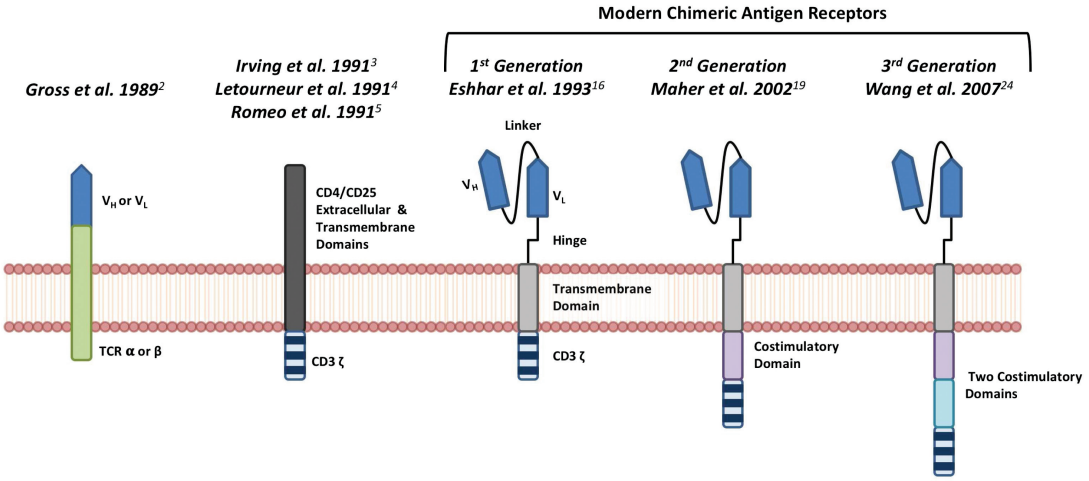

Fig. 1. The evolution of CARs. Early CARs were molecules composed of ectodomains from immunoglobulin V_H or V_L or from various receptors, fused with the TCR α or β chain. Modern CARs are composed of an extracellular antigen-binding domain usually derived from an immunoglobulin scFv, an extracellular spacer or 'hinge', a transmembrane domain and various ICDs required for T-cell activation.

Fundamentals of modern CARs

In its simplest form, a modern CAR is composed of a targeting moiety linked by a hinge region to its transmembrane and ICDs. Antigen specificity is obtained through inclusion of a single-chain variable fragment (scFv) composed of cloned antibody V_H and V_L regions in various orientations connected by a short-linker peptide (Fig. 1) (9). These can be derived from mouse, humanized or fully human antibodies, with some evidence suggesting that the presence of nonhuman scFvs may lead to decreased CAR-T-cell persistence, if not frank anaphylaxis upon repeated exposure (10). The nature of the interaction between a CAR and its ligand differs from traditional TCR recognition in that CARs recognize cell surface proteins in an MHC-independent manner.

The scFv component is joined through a hinge region, commonly derived from CD8α or IgG4, to the transmembrane domain and ICD. The length of the hinge region may influence the quality of interaction with scFvs; targeting more membrane-proximal epitopes may require longer hinge regions (11, 12). Additionally, some constructs have demonstrated deleterious antigen-independent effects (13). In one instance, the IgG F_c domain of an IgG1 hinge mediated unintended binding of T cells to IgG F_c receptors ($F_c\gamma$Rs) on innate immune cells resulting in off-target T-cell activation (14). Jonnalagadda *et al.* also demonstrated that CD19-specific CARs containing IgG4-F_c spacers were able to bind soluble $F_c\gamma$Rs *in vitro.* Mutating or deleting the CH2 region resulted in decreased $F_c\gamma$R binding, improved persistence and anti-tumor efficacy in NSG mice (which lack T cells, B cells and NK cells) (15).

First-generation TCRs included only the ICD of the CD3ζ chain with subsequent second- and third-generation molecules adding additional co-stimulatory support (Fig. 1) (16). In transgenic mouse models, first-generation CARs lacked significant *in vivo* efficacy (17, 18). Second-generation CARs introduced additional co-stimulatory domains such as those from CD27, CD28, CD134, 4-1BB (CD137), CD244 and ICOS. The addition of co-stimulation significantly augmented CAR signaling, improving cytokine production and T-cell proliferation, differentiation and survival (19). As expected, the effects of each co-stimulatory domain on T-cell function differs (20). CD28ζ (i.e. domains from CD28 and the CD3ζ chain) CARs display higher functionality as seen by increased IL-2 production and cytotoxicity, whereas 41BBζ CARs demonstrate comparable efficacy with improved persistence *in vitro* and in early mouse models (21, 22). Whether the use of third-generation CARs containing multiple co-stimulatory domains will have added benefit is unclear (22–24). It is conceivable that different clinical settings may require different combinations of scFv, hinge, transmembrane, stimulatory and co-stimulatory domains with no 'universal CAR' construct to be found (25).

In addition to the generation of modern constructs, delivery and persistent expression within a desired T-cell population is critical for clinical success. Adoptive cell transfer of CAR-T cells involves the isolation, stimulation, expansion, transduction and ultimately reinfusion of human T lymphocytes (Fig. 2). Peripheral blood mononuclear cells are usually isolated via leukapheresis with or without flow sorting or column separation to obtain the desired cell population, including T-central memory-like and/or T-stem cell memory-like phenotypes (26). This population can then be stimulated using a bead-based or artificial antigen-presenting cell (APC) approach with or without the addition of exogenous cytokines (27). The stimulation method and choice of cytokines allow for additional polarization of the final T-cell product, that is, T_h1, T_h2 versus T_h17 (28).

The transduction strategies currently being utilized in the clinical setting include transposon, gammaretrovirus-based or lentivirus-based transduction systems with or without the addition of a nonretroviral promoter (29, 30). The final product is then infused back into the patient, ideally following lymphodepleting pre-conditioning to optimize CAR-T-cell expansion and engraftment.

The CD19 experience

The most clinical data to date involve CARs targeting CD19, which is highly and uniformly expressed on B cells starting in

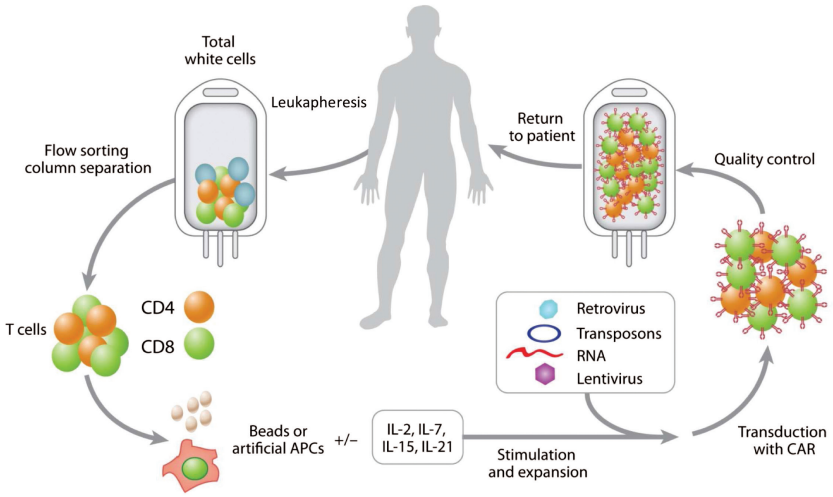

Fig. 2. Overview of CAR adoptive cell transfer. T cells are collected via apheresis, expanded *ex vivo* and genetically modified to express a desired CAR construct before they are infused back into the patient. Reproduced with permission of Annual Review of Medicine, Volume 65 Copyright © 2014 by Annual Reviews, http://www.annualreviews.org.

early development and throughout all mature stages except plasma cells (31). It is therefore an ideal target for B-cell malignancies including B-cell acute lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphomas (NHL) (32). Given its isolated expression on B-cell lineages, its 'on-target, off-tumor' toxicities have been relatively isolated and will be discussed later. This review will focus on important aspects of the most mature studies to date (Table 1).

Chronic lymphocytic leukemia/non-Hodgkin's lymphoma

There are approximately 15 000 new cases of CLL and 70 000 cases of NHL diagnosed each year in the USA. NHL comprises a diverse set of both indolent and aggressive diseases with varying biology and clinical outcomes (46). In some instances, initial chemotherapy may offer the chance for cure; however, in both NHL and CLL, relapsed and aggressive disease is associated with significant mortality (47).

Some of the first studies examining the utility of CAR-T cells in refractory CLL were performed by the group from the University of Pennsylvania (UPenn, Philadelphia, Pennsylvania, USA). In the initial UPenn series, three extensively pre-treated patients with unfavorable cytogenetics were treated with the CTL019 CAR-T product containing an mCD19_41BBζ (murine scFv against CD19 with 41BB co-stimulation and CD3ζ chain domains) construct following cytoreduction (34, 35). Most remarkable was the observed T-cell proliferation, marked trafficking to bone marrow (BM) and inflammatory cytokine profiles. Subsequent accrual of 11 additional subjects reported in 2015 demonstrated a combined overall response rate (ORR) of 57% with functional CAR-T cells persisting beyond 4 years (33). Given the early success of CTL019, it was expanded to relapsed/refractory (R/R) $CD19^+$ NHLs with preliminary work presented by Schuster *et al.* at the 57th American Society of Hematology Annual Meeting (ASH 2015, December 5–8, 2015, Orlando, Florida, USA) (48). Of the 22 evaluable patients, the ORR at 3 months was 68% with a progression-free survival of 62% at a median follow-up of 11.7 months.

A group from the Memorial Sloan-Kettering Cancer Center (MSKCC, New York, USA) reported in 2011 on a similar population of heavily pre-treated refractory CLL patients; however, they utilized an mCD19_CD28ζ (murine scFv against CD19 with CD28 co-stimulation and CD3ζ chain domains) construct. In their initial cohort of three patients, no preconditioning therapy was given and no objective responses were seen. The decision was then made to pre-treat the second cohort of four patients with lymphodepleting chemotherapy ultimately improving the ORR to 25%, emphasizing the importance of patient preconditioning. No CAR-T cells were observed to persist past 30 days (36).

A second mCD19_CD28ζ construct was utilized by a group from the National Cancer Institute (NCI, Bethesda, Maryland, USA) and has resulted in three published trials to date. The initial cohort of patients included four CLL and four NHL lymphodepleted patients treated with CAR-T cells and exogenous IL-2. Of the four CLL patients treated, three had a measurable response—one complete response (CR) and two partial responses (PRs)—whereas three PRs were noted among the NHL patients. T-cell persistence was noted as far out as 132 days post-infusion (37). A second trial from the NCI utilized the same CAR construct in 10 patients with refractory disease post-allogeneic hematopoietic stem cell transplantation (allo-HSCT) without preconditioning or exogenous IL-2. Of the 10 patients treated, measurable response was noted in only three patients (1 CR and 2 PRs) with no CAR-T cells persisting beyond 1 month (38). The third and most recent study treated 15 patients who had either CLL or NHL following lymphodepletion; 13 of the 15 patients were eligible for evaluation demonstrating 8 CRs, 4 PRs and 1 SD. T cells persisted for upward of 60 days (39).

Cruz *et al.* from the Baylor College of Medicine (Houston, Texas, USA) utilized mCD19_CD28ζ virus-specific T cells (VST cells) in post allo-HSCT patients with relapsed B-ALL and CLL. Donor-derived allogeneic VST cells were generated against EBV antigens and transduced with the CD19 CAR construct. The premise behind these experiments was to include the added benefit of T cells that not only targeted a

Table 1. Compiled results from major CD19-targeted CAR studies

CLL/NHL	Preconditioning?	*n*	Treatment response				
			CR	PR	SD	No response/PD	Persistence
Porter *et al.*, (UPenn, 2015) (33)	(+)	14					>4 years
Includes 3 patients from 2011 cohorts (34,35)		14 CLL	4/14	4/14	–	6/14	
mCD19_41BBζ construct							
Brentjens *et al.*, (MSK 2011) (36)		8 CLL					Up to 30 days
mCD19_CD28ζ construct	None	4 CLL (1 passed from sepsis)	–	–	–	4/4	
	(+)	4 CLL	–	1/4	2/4	1/4	
Kochenderfer *et al.* (NCI, 2012) (37)	(+) & IL-2	8					Up to 132 days
mCD19_CD28ζ construct		4 CLL	1/4	2/4	1/4	–	
		3 FL (1 s treatement, 1 passed from flu)	–	2/3	–	–	
		1 SMZL	–	1/1	–	–	
Kochenderfer *et al.* (NCI, 2013) (38)	None	10					Up to 30 days
mCD19_CD28ζ construct		4 CLL	1/4	–	1/4	2/4	
		2 DLBCL	–	–	2/2	–	
		4 MCL	–	2/4	2/4	–	
Kochenderfer *et al.* (NCI, 2015) (39)	(+)	15					Up to 60 days
mCD19_CD28ζ construct		4 CLL	3/4	1/4	–	–	–
		1 SMZL	–	1/1	–	–	
		4 PMBCL (1 Death)	2/4	–	1/4	–	
		5 DLBCL (1 lost to follow-up)	2/5	2/5	–	–	
		1 Low-grade NHL	1/1	–	–	–	
Cruz *et al.* (NCI, 2013) (40)	None	8					Up to 84 days
mCD19_CD28ζ construct in VSTs		4 CLL	–	1/4	1/4	2/4	
B-ALL	**Preconditioning?**	***n***	**Treatment response**				
			MRD-	MRD+	SD	No response/PD	Persistence
Cruz *et al.* (Baylor, 2013) (40)	None	4 Adult ALL					Up to 84 days
mCD19_CD28ζ construct in VSTs		4 (2 in CR at the time of treatment)	1/4	–	–	1/4	
Davila *et al.* (MSK 2014) (41)	(+)	16 Adult ALL					Up to 3 months
Includes Brentjens *et al.* (2011 Cohort) (36)		8 with Refractory BM blasts	7/8	–	–	1/8	
Includes Brentjens *et al.* (2013 Cohort) (42)		5 MRD+ following salvage chemotherapy	4/5	1/5	–	–	
mCD19_CD28ζ construct		2 MRD$^-$ following salvage chemotherapy	2/2	–	–	–	
		1 with Extramedullary disease	–	–	–	1/1	
Maude *et al.* (UPenn 2014) (43)	(+)	30 (25 Pediatric, 5 Adult)					Up to 12 months
Includes Grupp *et al.* (2013 Cohort) (44)		27 Patients with morphologic CR (25 assessed for MRD)	23/25	2/25	–	–	
mCD19_41BBζ construct							
Lee *et al.* (NCI 2015) 45)	(+)	20 Pediatric and young adult					Up to 42 days
mCD19_CD28ζ construct		20 ALL with Measurable Disease	12/20	2/20	3/20	3/20	

FL: follicular lymphoma; MCL: mantle cell lymphoma; PMBCL: primary mediastinal large B-cell lymphoma; SD: stable disease; SMZL: splenic marginal-zone lymphoma.

B-cell malignancy but also provided protection against viral infections that commonly occur post-HSCT—EBV, cytomegalovirus and adenovirus. Of the four CLL patients treated, only one PR was noted. Additionally four ALL patients were included in this cohort, with one of the four achieving a brief CR with eventual relapse (40).

More recently, data presented at ASH 2015 by the group of Turtle *et al*. (Seattle, Washington, USA) examined the use of differing preconditioning regimens with an mCD19_41BBζ construct in R/R NHL and CLL. Of the 28 NHL patients, the first 12 received cytoxan-based lymphodepletion without fludarabine, whereas the remaining received cytoxan plus fludarabine (49). In the first 12 NHL patients pre-treated with cytoxan alone, the ORR was 50% with a CR rate of 8%—one patient with diffuse large B-cell lymphoma (DLBCL). Interestingly, a CD8-mediated immune response was detected against the murine scFv in this first cohort. The subsequent addition of fludarabine to preconditioning increased the ORR and CR to 67% and 42% respectively. Higher peak CAR-T-cell levels were also noted with longer persistence, suggesting that a detrimental anti-murine scFv immune response may be occurring.

B-Acute lymphoblastic leukemia

ALL comprises a set of B- and T-cell malignancies with approximately 6000 new cases reported per year in the USA resulting in nearly 1500 deaths (50). The survival rate of childhood ALL approaches 90%; however, with increasing age survival rates as low as 10% have been observed (51). Additionally, in adults who relapse after initial remission, the median survival is only approximately 6 months (52). Given that B-ALL almost universally express CD19, CAR-based therapies have become increasingly exciting options for R/R disease.

One of the first studies to demonstrate the efficacy of CAR-based ALL therapy came from the group at MSKCC as reported by Brentjens *et al*. (36). Subsequent cohort-expansion studies culminated in a paper by Davila *et al*. describing 16 adult ALL patients treated with salvage chemotherapy, followed by mCD19_CD28ζ CAR-T cells (41). Of these 16 patients, 14 had refractory BM blasts or had minimal residual disease (MRD^+) at the time of treatment, with 13 ultimately achieving MRD^- status. All patients had few to undetectable CAR-T cells by 2–3 months after infusion. This study also identified a possible deleterious effect of high-dose steroids administered in the setting of cytokine-release syndrome (CRS, discussed subsequently) as steroid administration in all three patients led to ablation of CAR-T cells with eventual disease relapse (41, 42).

The MSKCC cohort results were updated at ASH 2015 by Park *et al*. describing a total of 44 patients, 43 of whom were evaluable; 36 patients (84%) achieved a CR after CAR-T cell infusion including 29 (83%) who achieved MRD^- status (53). The median overall survival (OS) for all patients versus patients who achieved an MRD^- CR was 8.5 months and 10.8 months, respectively, with two known cases of $CD19^-$ disease relapse. Interestingly, allo-HSCT after achieving CR with CAR-T-cell infusion did not seem to affect survival rates at 6 months. These findings are being further investigated with a currently ongoing multicenter, phase 2 trial in adult patients with R/R ALL.

The UPenn group first reported their experience with CTL019, an mCD19_41BBζ CAR construct and R/R ALL in 2013. In the initial report by Grupp *et al*., two children with R/R ALL were treated with CAR-T cells with both obtaining an MRD^- CR (44). In both patients, CTL019 T cells expanded to a level that was more than 1000 times as high as the initial engraftment level, and the cells were identified in BM and cerebrospinal fluid (CSF). Of the two patients treated, one remains in CR without allogeneic SCT, whereas the second unfortunately relapsed with a $CD19^-$ clone.

This initial case series was important for three reasons. First, it identified tocilizumab, an anti-IL-6 mAb, as a successful treatment for severe CRS without detrimental effects on CAR-T efficacy. Second, it identified a $CD19^-$ clone escape mechanism. Lastly, and most importantly, it demonstrated the possibility of long-term engraftment and persistence as demonstrated by ongoing detection of CAR-T for upward of 12 months, something not seen before in the CD28-containing constructs.

Maude *et al*. later reported an expanded pediatric cohort of 30 children treated with CAR-T cells engineered with CTL019. Of the 30 children with R/R ALL, CR was achieved in 27 (90%), including 2 patients with blinatunomab-refractory disease (blinatunomab is a bispecific antibody that binds CD3 and CD19; see below), and 15 who had previously undergone allo-HSCT. Of the 27 patients, 22 achieved MRD^- status including two patients in whom blasts were detected in the CSF at the time of infusion. Of the 27 patients who achieved a CR, 7 relapsed, 3 of whom were with $CD19^-$ disease. Sustained remissions were achieved with a 6-month OS rate of 78% (43).

Updated results were presented by Maude *et al*. at ASH 2015, reporting on the 53 patients treated to date, 50 of whom had achieved a CR following CAR-T-cell infusion including 8 of the 12 CRs among patients with known central nervous system (CNS) involvement (54). This update also described repeat treatment with hCD19_41BBζ (fully humanized scFv) CAR T cells in 14 of the 50 patients as indicated by B-cell recovery and MRD^+ status or undetectable CAR-T cells by peripheral flow with successful re-induction of B-cell aplasia in one of the four patients and conversion of MRD^+ to MRD^- in one of the two patients.

The NCI recently reported on 20 patients treated with R/R ALL in children and young adults utilizing an mCD19_28ζ CAR construct following chemotherapy pre-conditioning. This cohort also included one patient with DLBCL [progressive disease (PD) following therapy] who is not included in this summary. CR was seen in 14 patients (70%), with 12 of the 14 obtaining MRD^- status. Of the responders with adequate samples, 11 of the 17 had evidence of CAR-T cells within the CSF and both of those with known CNS disease had documented clearance. CAR-T-cell persistence was noted for up to 42 days (45). These data were recently updated by Lee *et al*. at ASH 2015 with the addition of 18 new patients. Of the 20 patients achieving an MRD^- CR, the median leukemia-free survival was 17.7 months, with 17 of the 20 patients undergoing SCT (55).

Despite these impressive response rates, the true long-term outcome of patients treated with these various constructs is unclear. We have already seen the development

of CD19⁻ escape variants; however, additional mechanisms of ALL recurrence and resistance are coming to light. Sotillo *et al.* recently described a unique mechanism in which B-ALL cells utilize alternative splicing mechanisms to maintain intracellular CD19 expression while evading CAR-T targeting. These alternative splice variants result in deletion of the specific epitope targeted by the FMC63 mAb from which some CAR-T scFvs were derived (56). Gardner *et al.* also recently described another novel mechanism, where patients with mixed lineage leukemia (MLL) gene-rearranged B-ALL achieved a CR following mCD19_CD28ζ CAR therapy relapsed with acute myeloid leukemia containing the same clonal MLL rearrangement. They comment on *in vitro* data identifying IL-6 as a key factor in driving myeloid differentiation of an MLL-rearranged B-ALL cell line. Collectively, these data suggest that the high serum cytokine levels during CRS may play a role in myeloid differentiation of an MLL-rearranged lymphoid clone and/or outgrowth of a myeloid leukemic clone (57).

Toxicities

Given the extensive trials to date involving CD19, we have gained a much better understanding regarding possible toxicities that can be divided into on-target/off-tumor versus systemic inflammatory responses. The three most heavily described in recent CD19 trials are B-cell aplasia, CRS and a diverse array of neurological toxicities (58).

B-cell aplasia is considered an on-target/off-tumor result of CD19-directed therapies. Given its isolated effect of hypogammaglobulinemia, it is easily managed with γ-globulin replacement therapy. In previous studies, ongoing B-cell aplasia has served as a functional marker of CAR-T-cell persistence, with long-term aplasias noted in the absence of detectable CAR-T cells (43). The importance of CAR-T-cell persistence, and thereby ongoing tumor surveillance, is currently under investigation with construct-dependent differences emerging.

Whereas B-cell aplasia can be easily managed, other on-target/off-tumor toxicities may not be as tolerable. An example of such a case is ERBB2, a marker found on both breast and colon cancers. It is also identified at low levels in several normal tissues including heart and the pulmonary vasculature. In 2010, a single patient with ERBB2⁺ metastatic colon cancer was treated with an ERBB2_CD28_41BBζ third-generation CAR construct. Within 15 min of infusion, the patient experienced respiratory distress with development of new lung infiltrates on imaging. She was intubated and despite intensive medical management died 5 days after from CRS and CAR-T-cell targeting of ERBB2 on lung epithelium (59).

Another major concern observed during the recent CD19 trials is that of severe CRS. CRS has been observed in all CD19-targeted CAR constructs, as well as blinatumomab, a bispecific T-cell-engaging antibody against CD19 and CD3ζ (BiTE®) (60). In most patients, CRS symptoms are mild and flu-like with isolated fevers and myalgias. Some patients, however, experience a severe inflammatory response syndrome involving hypotension, vascular leak, hypoxia and coagulopathy resulting in multisystem organ failure. CRS is an inflammatory process related to exponential T-cell proliferation and marked elevation in inflammatory cytokines including IL-10, IL-6 and IFN-γ as well as IL-2R, MCP-1 and MIP1-β (44). This profile also mimics that of macrophage activation syndrome/hemophagocytic lymphohistiocytosis (MAS/HLH) often with associated hyperferritinemia (>10 000 ng ml^{-1}), hepatomegaly/splenomegaly and hypofibrinogenemia with evidence of hemophagocytosis on BM biopsy (61). Although most responding patients have had some degree of CRS, the severity of CRS does not correlate with response.

Current theories regarding the relation of CRS and MAS/HLH involve IFN-γ production in the setting of rapid T-cell activation and cytotoxicity. In children with primary HLH, abnormally activated CD8 T cells produce significant levels of IFN-γ which in turn stimulates uncontrolled macrophage activation and the inflammatory profile observed in HLH (62). As IFN-γ is likely critical for the efficacy of CAR-T cells, it is therefore an unfavorable target when attempting to ameliorate CRS toxicities. IL-6, however, is a potent inflammatory cytokine often elevated in infection and patients with MAS/HLH. Unlike IFN-γ, it did not appear to be critical for CAR-T cell efficacy in pre-clinical xenograft mouse models, suggesting it as a driver of the observed inflammatory response without being critical for CAR-T-cell function (61).

Currently CRS is monitored with clinical indices as well as C-reactive protein, a biomarker with some evidence correlating elevated levels (>20 mg dl^{-1}) with severe CRS and its associated cytokine profile. Management of CRS involves the intensive care unit (ICU) and supportive care with the use of either anti-IL-6-directed therapy, such as tocilizumab, and/or high-dose steroids with some evidence suggesting deleterious effects on CAR-T-cell efficacy with use of the latter (42, 63) (see earlier).

Neurologic toxicities appear to be unique to CD19-targeted therapies and have been observed with nearly all CD19 CAR constructs as well as the CD19 BiTE® blinotumumab. Manifestations of neurologic toxicity include confusion, obtundation, seizures, hallucinations and/or aphasia. They often correlate with any degree of CRS but have also been noted to occur independently and/or following the resolution of CRS. They appear to be self-limited, resolving after several days without long-term sequelae. Interestingly, they do not resolve following tocilizumab, and severe cases are treated with steroids, anti-epileptics and supportive care. The mechanism behind such toxicities has yet to be identified, with further studies underway (58, 61).

Hurdles of CAR therapy in solid tumors and beyond

Despite nearly universal expression of CD19 on CLL, NHL and ALL, there are notable differences in disease response and treatment outcomes. It has been suggested that a chemotherapy-related or tumor-induced defect within the T-cell compartment may explain these differences. In fact, a small-scale test expansion is often performed prior to large-scale CAR-T-cell production for patients with CLL, given their predictably poorer replicative capacity (33). Fraietta *et al.* recently examined the anecdotal improvement in T-cell expansion from cells harvested from CLL patients who were previously treated with the irreversible IL-2-inducible T-cell kinase inhibitor, Ibrutinib. They found that cells derived from such patients had improved *ex vivo* expansion and

decreased expression of immunosuppressive receptors such as PD-1 and CD200 without deleterious effects on overall T-cell function. Furthermore, continuous Ibrutinib treatment enhanced CAR-T-cell efficacy in otherwise drug-resistant ALL and CLL mouse models (64). These data suggest that the clinical differences observed between the discussed tumor subtypes may actually be related to distinct tumor biology and impaired T-cell function, rather than innate resistance to CAR-T-cell killing.

A major hurdle in modern CAR therapy is the identification of suitable target antigens. Unlike CD19, which is exclusively expressed on B lymphocytes, many antigens identified in solid tumors are also expressed at low levels on healthy tissues. Whereas B-cell aplasia is a tolerable, and potentially transient, price to pay for the eradication of R/R ALL, T-cell mediated attack of off-tumor tissues currently limits the widespread application of CAR therapy to other malignancies. Strategies are currently being devised to increase specificity to malignant targets, identify new tumor-specific markers or targeting moieties and/or limit toxicities if they do occur.

One approach currently underway involves the utilization of combinatorial antigen recognition. In this model, signal 1 (the CD3ζ chain of CARs) and signal 2 [the chimeric co-stimulatory receptor (CCR)] are spatially separated and are stimulated following recognition of two different tumor-specific antigens. The first receptor provides sub-optimal T-cell activation upon binding unless in the setting of co-stimulation provided by the co-expressed CCR (65). This approach has been demonstrated in mouse models but has not yet been tested in the clinic.

A strength of CAR-based targeting is the lack of dependence on MHC antigen presentation for antigen recognition and T-cell activation. Unfortunately, this also limits the repertoire of targets, as a majority of TAAs are intracellular neoantigens expressed solely in the context of MHC. Ikeda *et al*. recently presented their work at ASH 2015 demonstrating the ability to generate scFvs that recognize intracellular peptides loaded on endogenous MHC (66). It is unclear whether this approach will provide sufficient antigen exposure, given MHC down-regulation and saturation, but the prospect of a larger array of targetable antigens is exciting and warrants further investigation.

Despite these advances in targeting, if and when toxicity does occur, current CAR transduction techniques preclude the ability to shutdown or specifically halt the subsequent T-cell response. By design, constructs are constitutively expressed at high levels for the purpose of maximizing anti-tumor effects with varying degrees of persistence. It therefore would be useful to provide physicians the ability to shutdown an overly robust or off-target response via 'suicide' gene switches. An example of such a system is the inducible caspase 9 (iCasp9) system that allows for targeted depletion of iCasp9-transduced cells (67). Administration of a synthetic small molecule causes dimerization of the iCasp9 pro-molecules, triggering activation and apoptosis. Other approaches include transfer of the herpesvirus thymidine kinase enzyme that phosphorylates the prodrug ganciclovir to an inhibitory nucleoside or co-transduction of T cells with inert surface-expressed proteins that can be targeted *in vivo* (68).

Given that these approaches would permanently ablate a potentially curative T-cell therapy, researchers are also attempting to design an inducible CAR system. More recently, one of the first ever inducible systems was demonstrated by Wu *et al*. (69). In this model, there is constitutive expression of spatially separated signaling molecules with heterodimerizing domains that only can dimerize, and therefore signal, in the presence of a small molecule. Additional work is underway investigating the utility of co-expressed benign surface markers as a target of antibody-mediated CAR-T-cell destruction. Work by Tasian *et al*. presented at ASH 2015 demonstrated the ability to ablate CAR-T cells in a xenograft mouse model via co-expression of CD20 and rituxan therapy (68).

Unlike hematologic malignancies such as ALL and CLL, targeting solid tumors has the added hurdles of tumor infiltration and the tumor microenvironment. This environment, composed of immune cells, endothelial cells, fibroblasts, extracellular matrix and cytokines, can have varying effects on the CAR-directed T-cell response. Several pre-clinical examples have demonstrated the feasibility of coupling CAR-T cells with chemokine receptors that allow for enhanced migration and infiltration once within the tumor bed (70). Combinations of pro-inflammatory cytokines, such as IL-12, with CAR-T cells as well as combinations of CAR-T cells with immune-checkpoint inhibitors are currently being studied to diminish the inhibitory effects of the tumor microenvironment.

Conclusions

Technology based on CARs is a rapidly evolving field with significant academic and industry interest. The field is beginning to understand the basics of CAR biology and has started applying this knowledge in designing CAR constructs for optimal *in vivo* efficacy. CD19 is a phenomenal paradigm demonstrating the potential of CAR-T cells, but there are several challenges ahead. One of the largest questions in the field is whether this mode of immunotherapy will be effective in solid tumors. Questions such as optimal CAR design, mode of co-stimulation, *ex vivo* expansion, host preconditioning, antigen targeting and toxicity management are just now being addressed in the laboratory and human trials. Despite these challenges, it is apparent that this powerful platform will be moving into the forefront of oncologic care, providing new treatment options for cancer.

Funding

M. V. M. was supported by the NCI grant K08CA166039.

Conflict of interest statement: M. J. F. and M. V. M. are listed as co-inventors in patents related to CAR-T cells; patents are held by the University of Pennsylvania and Novartis.

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