In Like a Lamb; Out Like a Lion: Marching CAR T Cells Toward Enhanced Efficacy in B-ALL

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ABSTRACT

Combining synthetic biology with adoptive T-cell transfer has led to promising advances in the treatment of relapsed/refractory B-cell acute lymphoblastic leukemia (R/R B-ALL), diffuse large B-cell lymphoma (DLBCL), and mantle cell lymphoma (MCL). Chimeric antigen receptors (CARs) are synthetic receptors that redirect T-cell specificity against cancer. CARs include "built-in" signaling domains that reprogram T-cell metabolism, enhance effector function, and support long-term persistence. Despite their success in blood-based malignancies, relapse can occur in CD19-redirected CAR T-cell therapies for several reasons, including poor engraftment, impaired *in vivo* proliferation, and T-cell senescence. Herein, we explain how subtle alterations in CAR design may overcome barriers to effective adoptive immunotherapy. We also discuss how the physiochemical properties of the single-chain variable fragment (scFv) affect differentiation and persistence. Moreover, we describe innovative advances in CAR engineering and provide insight into the development of humanized scFvs whose proposed benefits include increased persistence and improved clinical outcomes. Tumor cells can evade CAR T-cell–mediated detection and elimination due to the emergence or presence of CD19-negative leukemic cell subpopulations. We also discuss the opportunities and challenges in targeting other B-ALL–associated antigens. Identifying alternate targets is fundamentally necessary to restore the success of CAR T-cell therapies in CD19-negative patients with B-ALL.

Introduction

B-cell acute lymphoblastic leukemia (B-ALL) is a hematologic malignancy characterized by the uncontrolled proliferation of progenitor B cells in the bone marrow. B-ALL is particularly insidious in children as well as young adults. Standard treatment regimens for B-ALL include chemotherapy, radiotherapy, and hematopoietic stem cell transplantation. Although the 5-year overall survival rate is 80% to 90%, disease recurrence (relapse) can occur and is characterized by a poor response to conventional treatments (refractory). This lack of durable efficacy underscores the need for alternate approaches with enhanced effectiveness against cancer (1, 2). Improved outcomes in patients with relapsed/refractory (R/R) B-ALL have been obtained through the use of adoptively transferred T cells. Using advanced principles of synthetic biology, T cells can be genetically reprogramed to recognize and lyse malignant cells in a specific manner. Chimeric antigen receptor (CAR) transgenes have been effectively used to generate tumor-reactive T cells. The unprecedented successes of CAR-expressing T cells have given rise to a succession of FDA approvals, including Kymriah (tisagenlecleucel) for pediatric patients and young adults with R/R B-ALL, Yescarta (axicabtagene ciloleucel) and Breyanzi (lisocabtagene maraleucel) for patients with diffuse large B-cell lymphoma (DLBCL), and Tecartus (brexucabtagene autoleucel) for adults with mantle cell lymphoma (MCL; refs. 2, 3).

Adoptive T-cell therapies (ACTs) involve an ordered process whereby patient T cells are isolated and expanded *ex vivo* in nutrient-rich conditions over 9 to 14 days before reinfusion (4, 5). During this process, T cells are often genetically modified with a transgene to selectively direct their cytolytic activity toward antigens expressed on the surface of tumor cells, known as tumor-associated antigens (TAAs) or tumor-specific antigens (TSA). Using viral-mediated gene delivery, transgenes such as CARs are increasingly used not just to redirect T-cell specificity but also to enhance effector function, reprogram metabolism, and improve overall persistence in B-ALL. In this review, we provide mechanistic insights into the multidimensional benefit of CARs, illuminating their important role against cancer. We will also discuss strategies explicitly relevant to (i) CAR design and (ii) the *ex vivo* expansion phase that support the generation of CAR T cells with enhanced durable efficacy.

CAR Design and Structure

CARs are modular polypeptides encoding three canonical components. The CAR ectodomain contains an extracellular antigen-binding domain that is essential for tumor reactivity (6). This antigen-recognition domain is usually derived from a mAb single-chain variable fragment (scFv; ref. 6). The scFv contains heavy and light chain moieties that are connected by a linker region and anchored via a hinge region to the plasma membrane. The scFv is fused to one (or two) intracellular costimulatory signaling domain(s) expressed in tandem with the CD3ζ chain from the T-cell receptor (TCR; **Fig. 1**; ref. 7). There is renewed interest in understanding how the physiochemical properties of these individual components, without any modification

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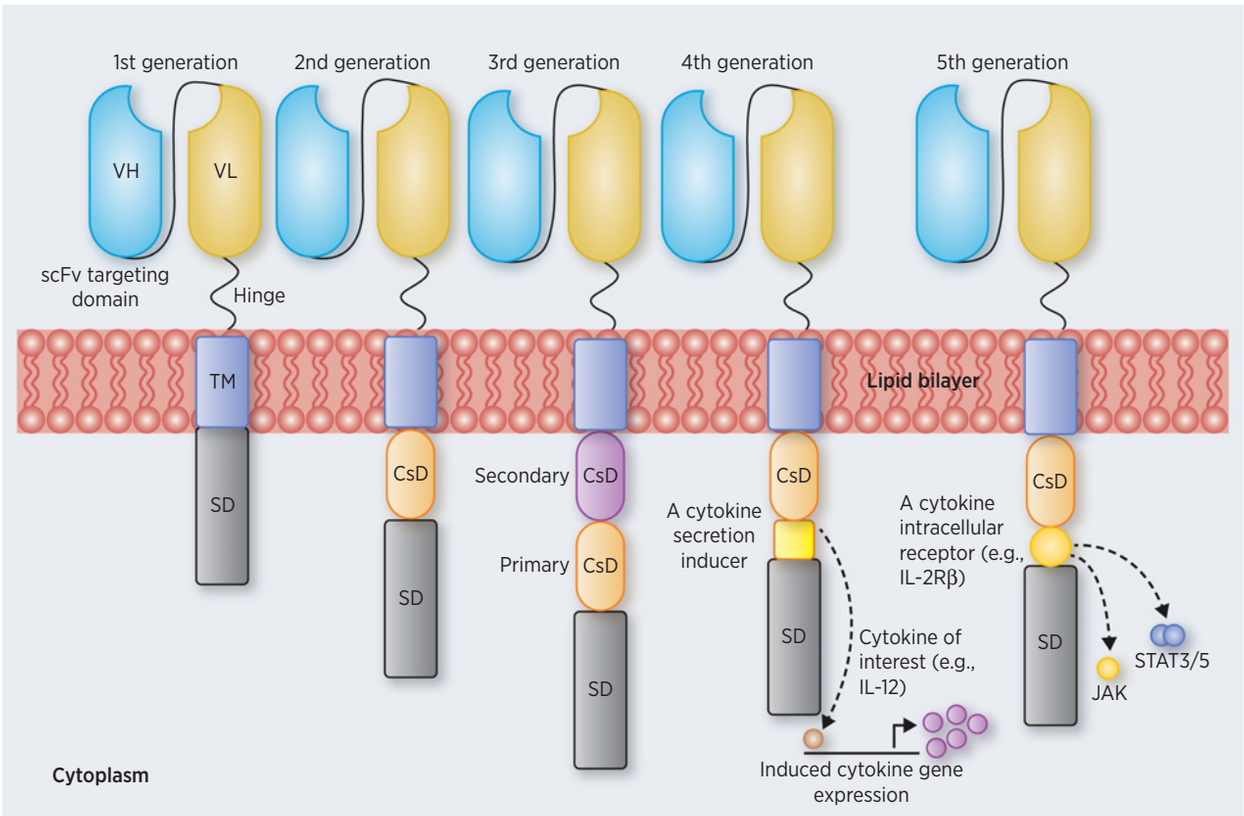

Figure 1.

A detailed anatomy of different CAR generations. First-generation CARs contain only a primary stimulation domain (e.g., CD3ζ). Second-generation CARs harbor one costimulatory domain (CsD) whereas third-generation CARs have two CsDs (a primary CsD and a secondary CsD). Fourth-generation CARs, also known as T-cell redirected for universal cytokine-mediated killing (TRUCK) or armored CARs, are based on second-generation CARs and are paired with a cytokine secretion inducer (e.g., IL-12). Fifth-generation CARs are also tailored versions of second-generation CARs as they contain an intracellular domain of a cytokine receptor (e.g., IL-2Rβ). CsD, costimulatory domain; scFv, single-chain variable fragment; SD, stimulation domain; TM, transmembrane domain.

to the antigen-binding region, can influence the efficacy of the modified T cells. For this reason, we will discuss how subtle modifications in CAR design and structure influence its overall function.

The extracellular domain

CARs combine two important aspects of an adaptive, cell-mediated immune response. In a humoral immune response, differentiated B cells recognize unique antigens through the production and secretion of antibodies. T cells also recognize unique antigens but do so through the assembly of multidimensional TCR complexes. T-cell activation occurs in a multistep process: Antigenic peptides are internalized by antigen-presenting cells, proteolytically processed, and presented to T cells in the context of MHC. CARs combine the high selectivity of antibodies with T-cell cytolytic function. By using scFvs as the antigen-binding moiety, CARs effectively redirect T-cell cytolytic activity to a specific epitope found on tumor cells, in an MHC-independent manner.

The scFv used in Kymriah and Yescarta is derived from the FMC63 murine antibody that recognizes human CD19. FMC63 targets a specific epitope found on exon 4 of the *CD19* gene (8). There are inherent limitations in the use of murine scFv-based CARs. Non-native scFvs may contain immunogenic epitopes that trigger an immune response. The potential for immunogenicity is increased when murine sequences are encoded in synthetic receptors, such as CARs, and expressed in human T cells. HLA-restricted T-cell–mediated immune responses against specific epitopes in the FMC63 scFv have been reported (9, 10). Importantly, the use of FMC63 has been increasingly recognized as an important parameter influencing CAR T-cell engraftment in the treatment of B-ALL. Similarly, the use of murine-based scFvs such as the SS1 CAR has also been associated with decreased persistence and impaired clinical efficacy in T-cell therapies against mesothelioma (11).

Antibody humanization is a useful strategy to overcome the inherent limitations of murine scFvs. To initiate this process, framework regions of the murine scFv are replaced with human coding sequences. We comprehensively describe the humanization process of a murine scFv and the proposed benefits of humanized CAR T-cell therapies against cancer (**Fig. 2**).

During antibody humanization, sequence modifications are limited to the frameworks rather than CDRs. For this reason, the binding affinities of CARs engineered with a humanized scFv should be minimally affected. In support of this premise, Qian and colleagues (12) showed that CARs designed with a humanized scFv supported T-cell proliferation and cytokine production following co-culture with $CD19^{+}$ target cell lines Nalm-6 and Daudi. In a human xenograft model of lymphoma, they showed that CAR T cells expressing a humanized scFv (hCARTs) displayed similar antitumor efficacy and survival as those of CAR T cells expressing murine scFv. These

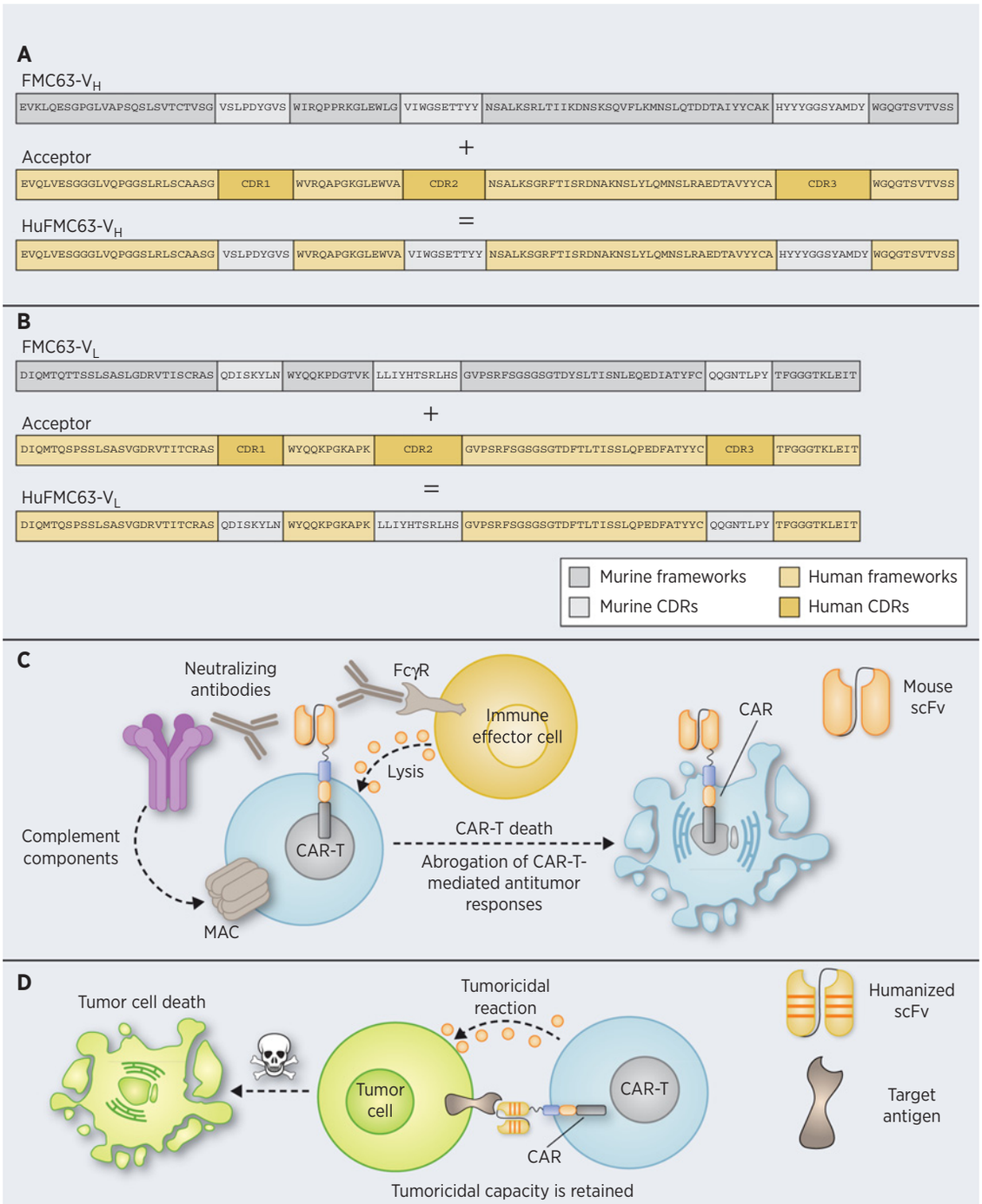

Figure 2.

The humanization process of the murine CD19-specific scFv, FMC63 (ref. 12), and the mechanism of action underlying the elimination of CAR T cells that harbor an animal-derived targeting domain. **A,** The CDRs of the FMC63-V_H are grafted onto the framework regions of an acceptor human antibody with a high-rate of amino acid sequence similarity. In some cases, a few framework residues, such as those positioned in framework 2, might get back-mutated to the native animal residues because their substitutions are considered detrimental for the antigen affinity of the antibody. **B,** The CDR grafting of the FMC63-V_L onto the acceptor frameworks of a human antibody. **C,** Neutralizing antibodies are produced by the immune system of the recipient against the murine-derived targeting domain of the CAR T cells leading to their elimination and subsequent dismantling of all CAR T-cell–related tumoricidal responses. **D,** As opposed to the previous situation, CAR T cells that harbor humanized targeting domains are much less likely to encounter neutralizing antibodies formed against them interfering with their antitumor capacity. Therefore, prolonged antitumor responses are a possible scenario. V_H, variable regions of the heavy chains; V_L, variable regions of the light chains; CDR, complementarity-determining regions; HuFMC63-V_H, humanized FMC63-V_H; HuFMC63-V_L, humanized FMC63-V_L; FcγR, Fcγ receptor; scFv, single-chain variable fragment; MAC, membrane attack complex; CAR, chimeric antigen receptor.

encouraging results advanced the use of humanized scFvs in preclinical models and clinical studies further.

Cao and colleagues (13) evaluated the therapeutic efficacy of hCARTs clinically, following infusion into patients with R/R B-ALL. Interestingly, a subset of patients in their trial had undergone prior treatment with murine scFv-based CD19-redirected CAR T cells. On the basis of tumor clearance from the bone marrow, central nervous system, and testis, it was shown that trafficking and overall cytolytic activity of hCARTs were superior to those of their murine-based derivatives (13, 14). Conclusions from this trial show that hCARTs have a decreased potential for immunogenicity, improved therapeutic effectiveness, and enhanced persistence. Moreover, hCART19s can effectively treat pediatric or adult R/R $CD19^+$ B-ALL, even following relapse from CAR T cells engineered with murine scFvs.

In a separate clinical trial (NCT02349698), Heng and colleagues (15) evaluated the efficacy and safety of CD19-redirected hCAR-Ts in patients with R/R B-ALL. They reported enhanced long-term persistence of hCAR-Ts; exemplified by a complete remission in 60% of the patients (6 out of 10 patients) at an 18-month follow-up. As persistence correlates with enhanced durable efficacy, their study provides mechanistic insights into the beneficial attributes of hCAR-Ts; ultimately culminating in lower relapse rates as well as improved clinical impact in the treatment of patients with R/R ALL. Collectively, these studies show that scFv humanization is an important optimization strategy to increase CAR T-cell bioactivity in preclinical and clinical subjects. As survival is substantially influenced by engraftment, persistence, and durable efficacy, these findings encourage the humanization of other scFvs used in T-cell–based therapies.

Generally, B-cell aplasia, as characterized by CAR T-cell–mediated elimination of healthy CD19-expressing B cells, is an established hallmark of successful CAR T-cell therapy (2). However, B-cell aplasia can result in hypogammaglobulinemia and increased risk of viral and bacterial infections (2). Such patients often require precise monitoring and healthy donor-derived plasma to complete their CAR T-cell treatment. Following antigen engagement, activated CAR T cells increase IFNγ, TNF, and GM-CSF cytokine production; macrophages respond to this inflammatory cascade by secreting IL-1 and IL-6 collectively, this process manifests as cytokine release syndrome (CRS; refs. 2, 16). Various therapeutic modalities have been developed and/or repurposed to counter the deleterious effects of CAR T-cell–induced CRS and neurotoxicity, including the administration of tocilizumab, anakinra, lenzilumab, metyrosine, or dasatinib, all of which have shown promise in a case-dependent manner (2, 16). Interestingly, CAR T cells can also be engineered to secrete neutralizing antibodies or even IL-6R- or GM-CSF–specific nanobodies to reduce the life-threatening effects of CRS and neurotoxicity (2, 16). Unsurprisingly, there is a direct relationship between the severity of toxicity and the persistence of the infused cells. Because humanized hCARTs exhibit higher persistence relative to their murine counterparts, their toxicity burden must be followed closely. To our knowledge, no clinical study has yet compared the differences between the toxicities of these two CAR T-cell platforms. In one study with 18 patients with R/R ALL, resolvable neurotoxicity was reported in one recipient whereas grade 1 to 2 and grade 3 to 5 CRS was observed in 13 and four recipients, respectively, after a single round of CD19-redirected hCART infusion (1×10^6/kg; ref. 13). In a separate study (ChiCTR1800017401) comprising two previously untreated patients with B-ALL, both recipients showed signs of grade 1–2 CRS (for which tocilizumab or corticosteroids was not advised) after a single infusion of CD19-redirected hCARTs (1×10^6/kg; ref. 17). In the phase I trials ChiCTR1800014761 and ChiCTR1800017439, five patients with B-ALL treated with CD19-redirected hCARTs experienced mild adverse events (including fever and grade 1 CRS). However, the humanized version conferred a sixfold increase in affinity to CD19 (18) that likely affected the magnitude, and rate, of the response independent of persistence. More research is necessary to fully understand how antibody humanization in the CAR format affects the overall response and toxicity profiles.

Advanced CAR design: understanding how the physiochemical properties of the ectodomain influence CAR function

Nonvariable regions confined to the CAR ectodomain can overtly contribute to the overall biophysical balance, thermodynamic stability, and tertiary structure of the scFv. The CAR ectodomain provides (i) the necessary framework to anchor the scFv to the plasma membrane and (ii) confers the necessary flexibility to adopt select conformational states upon antigen engagement. To optimize CAR efficacy, it is important to understand how subtle modifications in the framework regions of the CAR targeting domains can influence antitumor function.

The intrinsic stability of V_H and V_L chains is decreased in the scFv format. This instability is exemplified by a tendency to unfold, aggregate, and oligomerize with the scFvs contained within adjacent CARs. Several approaches have been developed to counteract the inherent thermodynamic instability of scFvs that include the inclusion of disulfide bridges to reinforce the V_H and V_L domains. In the format of a CAR, the aggregation of scFvs leads to CAR clustering, spontaneous T-cell activation and cytokine production in the absence of antigen engagement (which is recognized as tonic signaling). Sustained tonic signaling promotes progressive T-cell dysfunction, characterized by impaired engraftment, diminished proliferation, poor cytokine production, and decreased antitumor function.

In an exemplary study, Long and colleagues (19) showed how the physiochemical properties of the scFv framework affect overall CAR T-cell function. They compared the ability of T cells that were selectively engineered with either a GD2-specific CAR (containing the 14g2a scFv) or CD19-specific CAR (using the FMC63 scFv) to eliminate tumors and persist as memory cells (19). GD2 is a membrane-bound disialoganglioside overexpressed in osteosarcoma (20), melanoma (21), and neuroblastoma (22). CD19 expression is restricted to the B-cell lineage. To control for intertumor heterogeneity, they ectopically expressed CD19 into GD2-expressing osteosarcoma cells. Immunodeficient mice were engrafted with these target cells. The ability of CAR T cells with directed specificity against GD2 or CD19 to control tumor size was examined (19). As GD2- and CD19-redirected CAR T cells encountered identical tumor burdens, the observed variations in tumoricidal efficacy were attributed to intrinsic factors contained within the CAR design (19). In contrast with CD19-redirected CAR T cells, GD2-redirected CAR T cells lacked efficacy and displayed phenotypic features of exhaustion (19). Using a series of approaches, including confocal microscopy, *in vitro* killing assays, cytokine production, and xenograft models, the authors attributed this antigen-independent anergy to the aggregation of CAR molecules that lead to spontaneous cytokine production, and ultimately effector cell exhaustion (19). Furthermore, the authors concluded that CAR aggregation is strictly driven by the interactions between the framework residues of the scFvs of different GD2-specific CARs on the surface of an engineered T cell (19). These data support earlier findings showing an inherent propensity for antibody (as well as antibody-derived fragments such as scFv) oligomerization (23, 24). In general, the organization of antibodies into higher order complexes reduces their

free energy cost. Initial findings suggest that 4–1BB mitigates the phenotypic and functional features of exhaustion that result from scFv-induced CAR aggregation (19). These findings are consistent with the proposed ability of 4–1BB to counter anergy in the context of chronic viral infections (19, 25, 26), and also complement previous work demonstrating antigen-independent signaling contributes to CD28–CD3ζ CAR T-cell exhaustion *in vivo* (27). Taken together, these findings highlight the importance of meticulous CAR design and the need to understand how all components of a synthetic molecule impact therapeutic efficacy.

To overcome the intermolecular clustering of a toxin-specific scFv, Zhao and colleagues (28) introduced a disulfide bond between position 44 and 100 of the heavy and light chains, respectively. Replacing amino acids glycine and glutamine with cysteine increased the overall stability of the scFv, without any adverse effect on antigen-binding activity. Although speculative, there might be an additive benefit to such structural reconfigurations that enhance scFv stability, along with optimal costimulatory domain to enhance antitumor function following adoptive transfer.

Similarly, a recent study showed how the biophysical balance of the CAR ectodomain is an important determinant of CAR T-cell efficacy (29). Using the 763.74 scFv, Landoni and colleagues (29) developed a CAR against chondroitin sulphate proteoglycan 4 (CSPG4), which is a glycosylated transmembrane protein overexpressed in malignant melanoma. Regardless of the costimulatory endodomain (4–1BB or CD28), the inherent instability of the scFv culminated in spontaneous cytokine release, tonic signaling, and progressive dysfunction over time. Using computational modeling strategies, the authors identified critical residues within the framework regions of the CAR targeting domains (isoleucine 123 as well as glutamine 127) that regulated the scFv stability (29). Advanced structural analyses using Eris software revealed a number of amino acid substitutions that would further stabilize the underlying framework of the scFv, attenuate tonic signaling, and enhance the overall antitumor function of CSPG4-specific CAR T cells (29). As the authors found no connection between CD28 versus 4–1BB endodomains and the degree of tonic signaling, future studies are necessary to understand how CAR costimulation affects CAR desensitization in the absence of antigen engagement (29). These findings highlight the benefit of structural modeling to design CARs that can overcome the inherent instability of scFvs in the CAR format and enhance persistence following adoptive transfer (29).

Strategies for Overcoming Antigen Escape

Several parameters intrinsic to CAR T cells, including CAR expression, T-cell trafficking, *in vivo* expansion, and persistence, influence the overall outcome (including relapse) of adoptive transfer. Tumor-intrinsic mechanisms also contribute to relapse following CAR T-cell treatment. In the context of B-ALL, 60% of CAR T-cell cases exhibit relapse due to CD19 antigen loss (30). Although the expression of a chimeric entity provides specificity against a given tumor antigen, loss of the corresponding antigen renders the very same CAR T cells obsolete.

There are several established mechanisms by which tumor cells achieve antigen loss and/or antigen escape. Intuitively, tumor cells can decrease CD19 expression on their cell surface. This impedes CAR T-cell activation, resulting in a low level of antitumor function (31, 32). Mechanistically, tumor cells can achieve this through alternate splicing at the *CD19* locus. Splice variants can give rise to truncated CD19 proteins that are undetectable by flow cytometry, but present by Western blot analyses. Such a dichotomy, exemplified by the expression of variants lacking the necessary epitope to interact with the corresponding scFv used in flow cytometry reflects antigen escape rather than antigen loss.

Additional mechanisms contributing to relapse following CD19-redirected CAR T cells have been recently identified. CD123 has a low-level expression on normal hematopoietic stem cells but is upregulated in several hematologic malignancies such as acute myelogenous leukemia (AML; ref. 33), hairy cell leukemia (34), blastic plasmacytoid dendritic cell neoplasm (35, 36), and systemic mastocytosis (37). Importantly, CD123 expression distinguishes a subset of leukemia-initiating cells in AML (38). Ruella and colleagues (39) provided evidence that CD123 is ectopically expressed in B-ALL. Moreover, they showed that a subclone of B-ALL cells can undergo a transforming event giving rise to $CD19^{-}CD123^{+}$ cells with phenotypic and cytogenetic features of leukemia-initiating cells. In addition, the selective expansion of $CD19^{-}CD123^{+}$ B-ALL cells under CD19-redirected CAR T-cell immune pressure can give rise to malignant progeny. Other studies corroborated these findings showing aberrant CD123 expression in both pediatric and adult B-ALL (33, 36, 40–42).

Yu and colleagues (43) provided further insights into the mechanisms of antigen escape following CD19-redirected CAR immunotherapy in a pediatric case of primary mediastinal large B-cell lymphoma. In a detailed genomic analysis, Yu and colleagues identified a missense mutation (G210D) in one allele of the *CD19* gene. Computational modeling predicted that substitution of glycine with a negatively charged aspartic acid in codon 210 would alter the physiochemical-binding properties and subcellular distribution of CD19. Phenotypic analysis of G210D mutants revealed a cytoplasmic rather than a cell-surface localization of CD19. Of interest, Yu and colleagues attributed this mutation to a loss of DNA repair genes yielding transformants that expanded under CD19-redirected CAR T-cell–mediated selective pressure.

CAR T cells targeting the B-cell lineage marker CD22 have been investigated as an alternative approach to overcome relapse from CD19 antigen loss. Durable clinical responses have been achieved in patients with $CD19^{-}$ B-ALL treated with CD22-redirected CAR T cells (44–47). Interestingly, a recent article showed how CAR T cells could be redirected to a nucleophosmin neoepitope in AML (48). Future studies may identify similar novel targets for CAR T cells in B-ALL.

Novel antigens

B-cell–activating factor receptor

The B-cell–activating factor receptor (BAFF-R), a transmembrane protein of the TNF receptor superfamily, is essential for B-cell maturation and survival (49–51). Importantly, BAFF-R is highly expressed in B-cell malignancies. The canonical ligand for BAFF-R is the BAFF. Following activation, BAFF-R regulates tumor survival and proliferation in an NF-κB–dependent manner (52, 53). BAFF-R upregulation is associated with disease progression in patients with B-cell lymphoma and pre–B-ALL (54–56). The critical role of BAFF-R in B cells positions it as an ideal target for adoptive immunotherapies (50, 51, 57, 58).

Qin and colleagues (59) developed hCARTs against BAFF-R and provided evidence that BAFF-R–redirected CAR T cells have significant cytotoxicity against several B-ALL cell lines. Adoptively transferred BAFF-R–redirected CAR T cells successfully eradicated 10-day pre-established tumor xenografts after a single treatment and maintained efficacy against xenograft models lacking CD19

expression (59). Using patient-derived xenograft (PDX) models, tumors that evade CAR T-cell detection by CD19 antigen loss are susceptible to BAFF-R-redirected CAR T-cell cytolysis (59). Qin and colleagues (60) also developed a high-affinity humanized mAb against the natively expressed BAFF-R. By adapting the V_H and V_L chains to an scFv format and integrating into a CAR, they provided evidence that BAFF-R–redirected CAR T cells were highly effective in xenogeneic mouse models of B-cell malignancies, including those with CD19 antigen loss (61).

CSPG4

CSPG4 is a heavily glycosylated transmembrane protein overexpressed in several malignancies, including melanoma, glioma, and triple-negative breast cancer. CSPG4 is also upregulated in mixed-lineage leukemia (MLL)–rearranged leukemia blasts, a form of leukemia with the unfortunate MLL 11q23 rearrangement that occurs in 10% of all leukemias (62–71). CSPG4 is an established antitumor target. mAbs that disrupt ligand access to CSPG4 have been developed and shown therapeutic efficacy in cancer immunotherapies (72). Beyond competitive inhibition, conjugating CSPG4-specific antibodies with pro-apoptotic factors also has translational relevance (72). Fusion proteins linking the CSPG4-binding domain to soluble TRAIL (TNF-related apoptosis-inducing ligand) agonists can trigger cell death upon CSPG4 binding through the extrinsic apoptosis pathway (73). One inherent limitation in the use of CSPG4 as a target for adoptive immunotherapy is that its expression is not limited to tumor cells. Low levels of CSPG4 have been observed in activated pericytes and smooth muscle cells (74–76). This is concerning, as a recent article showed how mural cells, which are a subset of vascular smooth muscle cells surrounding capillary beds, are susceptible to CD19-redirected CAR T-cell–induced neurotoxicity (77). Gene delivery approaches permissive for transient CAR expression are safer alternatives to lentiviral-mediated CAR expression whenever toxicities are a major concern. For this reason, mRNA-based CSPG4-redirected CAR T cells might be a preferable approach to limit the potential for "on-target off-tumor" toxicities, specifically with vascular smooth muscle cells found in various organs, including the brain (78, 79). It is encouraging to assert that mRNA-transfected CAR T cells can induce tumoricidal reactions similar to those mediated by virally transduced CAR T cells (80).

Thymic stromal lymphopoietin receptor

Thymic stromal lymphopoietin receptor (TSLPR) is a heterodimeric receptor complex activated by the thymic stromal lymphopoietin (TSLP) cytokine. TSLPR is overexpressed in 5% to 15% of patients with B-ALL. *CRLF2* translocations and deletions result in alternative promoter activation and TSLPR overexpression in B-ALL (81–86). *CRLF2* gene rearrangements are associated with a poor prognosis in patients with B-ALL (87–92). TSLPR activation promotes JAK/STAT signal transduction in B-ALL blasts (93, 94). Given its restricted expression in normal tissues, its role as a B-ALL oncoprotein, and its cell-surface overexpression and association with poor clinical outcomes, TSLPR is a potential target for CAR T-cell therapies against B-ALL. Qin and colleagues (95) evaluated the efficacy of TSLPR-targeting CAR T cells against *CRLF2*-overexpressing B-ALL *in vitro* and *in vivo*. TSLPR-targeting CAR T cells demonstrated potent cytotoxicity *in vitro*. Moreover, the anti-leukemic activity of TSLPR-targeting CAR T cells was demonstrated in mice engrafted with a TSLPR-expressing B-ALL cell line. TSLPR-redirected CAR T cells also eliminated human *CRLF2*-rearranged TSLPR-overexpressing ALL in PDX models. These findings might implicate the cytokine receptor TSLPR as a promising therapeutic target for patients with *CRLF2*-rearranged B-ALL.

Other proposed targets

CD20 is a nonglycosylated transmembrane phosphoprotein unique to B cells. Although CD20 is abundant on mature B cells, it is absent on pro-B cells, plasma cells, and hematopoietic stem cells (96). CD20 regulates cell cycle progression as well as differentiation, inhibits apoptosis, and facilitates calcium entry (97). Properties unique to CD20 make it an attractive target for CAR T-cell therapies against cancer. In particular, CD20 is neither internalized nor secreted following ligand/antibody engagement. There are several mAbs against CD20, including *rituximab* (FDA approved in 1997), *ofatumumab* (FDA approved in 2009), and *obinutuzumab* (FDA approved in 2013), which have been investigated or used for the treatment of B-ALL and other hematologic malignancies (98–100).

The cell surface glycoprotein CD52 is another potential target that has not received much attention. Whereas CD52 is expressed on differentiating B and T cells, it is absent on terminally differentiated circulating plasma cells. *Alemtuzumab* is a humanized CD52-specific mAb that has shown promising results in patients with chronic lymphocytic leukemia leading to its FDA approval in 2000 (101). Although its clinical benefit in the treatment of ALL has been poor (102), repurposing the scFv in a CAR construct for CAR T-cell therapy may restore its potential as a therapeutic option for ALL.

Multitargeting CARs

Dual-targeting strategies

To overcome the adaptive response of B-ALL cells to CD19-redirected CAR T cells, and the aberrant expression of CD123, Ruella and colleagues (39) devised a dual receptor approach targeting both CD19 and CD123 simultaneously and set out to test this construct using a novel preclinical model of relapse. In this xenograft model, NSG mice were injected with primary ALL blasts ($CD19^+$ $CD123^+$) isolated from a patient before CD19-redirected CAR T-cell treatment, as well as after relapse ($CD19^-$ $CD123^+$). As CD123-redirected CAR T cells demonstrated efficacy against B-ALL cells exhibiting resistance to CD19-redirected CAR T cells, their findings highlight the therapeutic potential of dual receptor CARs in preventing relapse due to antigen loss. Complementing the findings from Ruella and colleagues, a more recent study demonstrated that CAR T cells with two discrete scFv domains, one against CD19 and the other against CD123, demonstrated cytotoxic effects against their corresponding targets both *in vitro* and *in vivo*. This further supports the idea that a dual CAR approach is effective in augmenting the response against leukemic blasts and reducing overall rates of disease relapse (103).

Simultaneous multispecific targeting can enhance the durability of B-ALL remission while minimizing the risk of antigen escape–associated relapse (44, 103). A bispecific CAR platform is one approach to effectively overcome escape variants. As surface receptor engagement is an important determinant of efficacy, simultaneously targeting two distinct antigens also raises the overall T-cell antitumor responses. Beneficial attributes of bispecific CAR platforms in advanced B-cell malignancies and glioblastoma have been established (44, 104–106). In one innovative design, CD19- and CD22-specific scFvs were combined to form a single bivalent receptor (44). These bispecific CAR T cells, targeting both CD19 and CD22, demonstrated potent cytotoxic effects against $CD19^+CD22^+$, $CD19^-CD22^+$, and $CD19^-CD22^+$ cells in B-ALL (44).

In one pilot study (including 51 patients with B-ALL), the efficacy and safety of a sequential CAR T-cell infusion strategy (targeting CD19

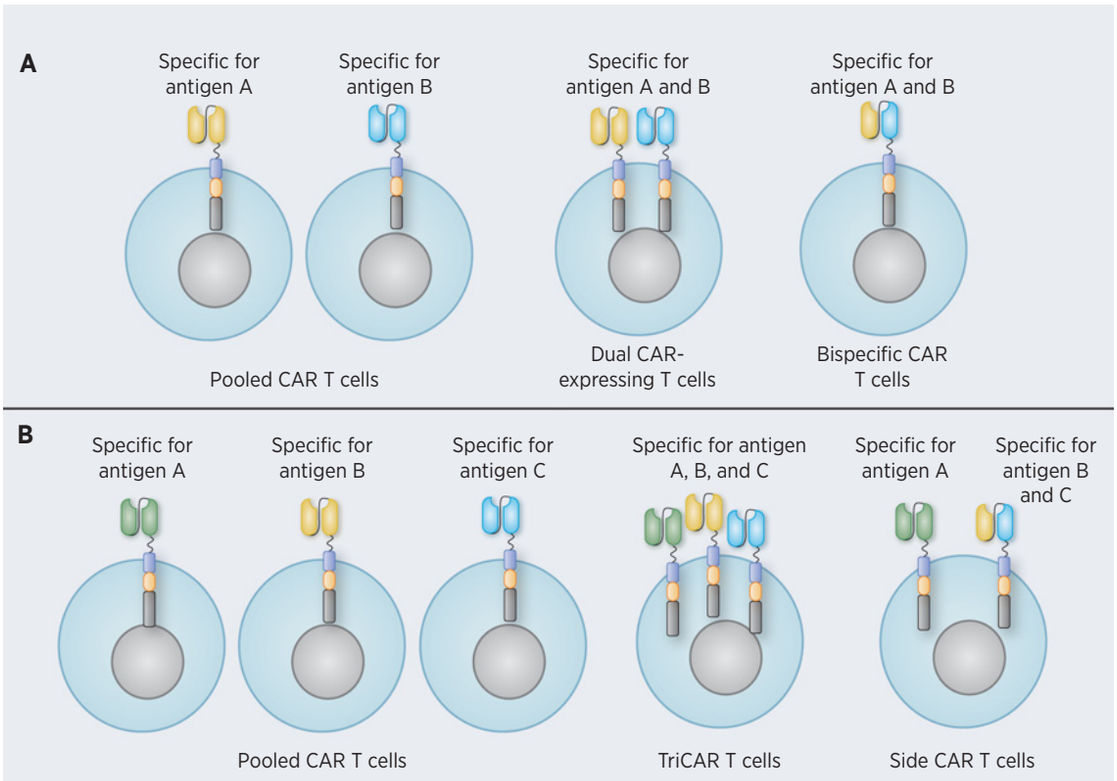

Figure 3.

Different strategies for having multitargeting CAR T cells. **A,** Dual-targeting CAR T cells. Pooled CAR T cells are a combination of two populations of distinct CAR T cells each targeting a different antigen of interest (e.g., CD19 and CD20). Dual CAR-expressing CAR T cells express two different CAR constructs on a single cell with each CAR targeting a different antigen of interest. Bispecific CAR T cells are equipped with a bispecific targeting domain thus enabling them to target two antigens of interest without the need for the expression of two distinct CARs. **B,** Trivalent CAR T cells. Pooled CAR T cells are a combination of three populations of distinct CAR T cells each capable of targeting a different antigen of interest (e.g., CD19, CD20, and CD22). TriCAR T cells express three different CAR constructs on a single cell with each CAR targeting a different antigen of interest. Side CAR T cells co-express a conventional CAR specific for antigen A alongside a bispecific CAR specific for antigen B and C.

followed by CD22) were established (107). The authors reported a minimal residual disease-negative response rate of 96.0% among the 51 patients with B-ALL and a median progression-free survival of 13.6 months (with a median follow-up of 16.7 months; ref. 107). The benefits of a sequential CAR T-cell infusion strategy, including an increased leukemia-free survival rate, were corroborated by other studies (108, 109). **Fig. 3A** illustrates different approaches for having dual-targeting CAR T cells.

Trivalent CARs

Given the incidence of $CD19^{-}$ disease relapse, approaches to broaden the specificity of CAR T cells against additional tumor antigens have been developed (110, 111). One such study targeted three distinct leukemia antigens (CD19, CD20, and CD22) to evaluate whether a trivalent CAR platform could control disease progression during $CD19^{-}$ relapse (110, 111). Two second-generation trivalent CAR T-cell products targeting (i) CD19 (using the FMC63 scFv), (ii) CD20 (using a rituximab-derived scFv), and (iii) CD22 (using the m971 scFv) were developed (110). The first CAR T-cell product expressed three individual CARs, with each CAR redirecting T-cell cytotoxicity against a unique leukemia antigen (hereafter referred to as TriCAR); the second CAR T-cell product (hereafter referred to as SideCAR) expressed a conventional CD19-targeting CAR as well as a CD20- and CD22-targeting bispecific CAR achieved through a tandem arrangement (110). *In vitro* cytolytic activity was enhanced in T cells expressing either TriCAR or SideCAR relative to CD19-redirected CAR T cells (110). Trivalent CAR T cells were also functionally effective against $CD19^{-}$ $CD20^{+}CD22^{+}$ target cells highlighting their translational relevance against CD19 escape variants (110). In **Fig. 3B**, we outline various trivalent CAR designs. Future studies will likely provide more insights into their therapeutic potential and clinical impact.

It can be an arduous task to find one TSA, let alone two or three antigens that are strictly restricted to only tumor cells. In this regard, one of the main downsides of multitargeted CAR T cells might be the increased risk of on-target off-tumor toxicities delivered to healthy tissues. In the past years, researchers have meticulously devised several counterstrategies to overcome such limitations that include inhibitory CARs; in this context, the absence of a specific antigen on tumor cells permits downstream signaling cascades inducing tumoricidal responses, or logic-gated CAR T cells that enforce antitumor reactions only toward tumor cells that simultaneously express two target antigens recognized by the CAR constructs (2, 6). Although these countertactics sound reasonable, their practicality can only be determined in clinical trials in years to come. Another potential strategy for reversing adverse events attributed to CAR T cells is to equip T cells

with safety switches that enable their rapid elimination from the circulation upon the introduction of a safety switch-activating agent (2, 6). Aside from these, transient CAR expression seems like one of the most reliable strategies and has been well tolerated by a number of patients with breast cancer, melanoma, and pancreatic cancer enrolled in clinical trials NCT03060356 and NCT01897415. According to one study with six patients with pancreatic ductal adenocarcinoma, mRNA-based mesothelin-redirected CAR T cells showed no sign of CRS, neurotoxicity, or any other serious dose-limiting toxicities (112). However, there are inherent limitations in the use of mRNA-based CAR T cells and they require frequent infusion to sustain antitumor responses.

Future Opportunities and Challenges

Rapid advances in the development of CAR T cells for B-ALL paved the way for a number of FDA approvals against a broadening range of hematologic malignancies. However, the therapeutic potential of CAR T cells can be further expanded if infallible countertactics are undertaken, including advanced CAR design and engineering to enhance T-cell persistence and extend the limited durable efficacy displayed by senescent T cells, or dual targeting approaches to eradicate tumor cells that evade immunosurveillance by antigen escape and antigen loss. Accumulating evidence suggests that optimizing CAR design by including fully human or humanized targeting domains in the CAR construct, selecting a suitable costimulatory domain, and identifying alternate target antigens will enhance the therapeutic promise of CAR T cells even further. The discovery of novel target antigens and the development of multitargeted CAR T cells can substantially diminish the risk of disease relapse. However, multitargeting strategies might result in life-threatening side effects if the targeted antigen sets are not cancer-specific. Moreover, because finding TSAs is challenging, identifying and targeting aberrantly glycosylated forms of antigens, known as Tn and Sialyl Tn glycoform, that are only expressed by tumor cells might be an alternative worth considering. Combining other treatment modalities, such as oncolytic virotherapy or bispecific T-cell engagers (BiTEs®), may also enhance the clinical efficacy of adoptively transferred CAR T cells. We are at the nexus of important breakthroughs that will propel the field of CAR T-cell therapies to even greater heights.

Authors' Disclosures

R.S. O'Connor reports a patent for CAR T-cell therapies licensed to Novartis AG. No disclosures were reported by the other authors.

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