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CART AND IMMUNOTHERAPY (M RUELLA AND P HANLEY, SECTION EDITORS)

Multi-Specific CAR Targeting to Prevent Antigen Escape

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Abstract

Purpose of Review Chimeric antigen receptor (CAR) T cell therapy has demonstrated remarkable remission induction rates for relapsed/refractory B cell malignancies. However, loss of the CAR-targeted antigen, known as antigen escape, accounts for a substantial percentage of relapses following CAR therapy and is a major barrier to durable remission. Here, we discuss mechanisms for antigen escape and strategies to prevent this pattern of relapse, including the use of multi-specific CARs, which recognize and target multiple tumor-associated antigens simultaneously.

Recent Findings Preclinical and early clinical trial data indicates that multi-specific CAR therapy for B cell malignancies is both safe and effective. Optimal combinations of target antigens, as well as different multi-specific CAR formats, are currently being evaluated.

Summary Although still in early stages of development, multi-specific CAR therapy represents a promising approach to mitigate antigen loss–related relapses and improve durability of remission in patients with refractory B cell malignancies, and may be applicable to other types of cancer.

Keywords CAR T cell · Antigen loss · Antigen escape · Multi-specific · Bivalent · Lineage switch

Introduction

Chimeric antigen receptor (CAR) T cell therapy is a rapidly evolving immunotherapeutic treatment modality for adult and pediatric patients with cancer. CAR T cells are genetically modified cells that contain a synthetic receptor that targets a specific antigen. A monovalent CAR targets one antigen and is composed of a single chain variable fragment (scFv), hinge region, transmembrane domain, one or more costimulatory domains, and an intracellular signaling domain (CD3 zeta). Monovalent CAR T cell therapy has been proven to be an effective strategy to treat multiple types of cancer, including adult and pediatric relapsed and refractory (r/r) B cell acute lymphoblastic leukemia (B-ALL) (CD19 CAR, 70–90% complete remission (CR) at 28 days), non-Hodgkin's lymphoma (NHL) (CD19 CAR, 54–74% CR at 3 months), and multiple myeloma (BCMA CAR, 94% overall remission/response rate (ORR)) [1]. CARs targeting IL-13 receptor alpha 2, HER2, and EGFRvIII have shown promise in the treatment of recurrent multifocal glioblastoma multiforme, suggesting a role for CAR T cell therapy in brain tumors [2]. Unfortunately, the use of CAR T cells in solid tumors is more challenging for multiple reasons, including difficulty with CAR T cells trafficking to the tumor, tumor microenvironment impeding CAR T function, and expression of targeted antigens on healthy tissues, leading to increased on-target/off-tumor toxicity [1].

While the majority of current CAR T cell products target a single tumor antigen, recent data shows that relapse frequently occurs due to loss of the target antigen, termed antigen escape, as is seen after treatment with CD19 and CD22 monovalent CARs [3, 4]. Up to 60% of relapses after CD19 CAR are due to loss of CD19 antigen [3]. Similarly, some patients who receive CD22 CAR relapse with diminished CD22 site density, rendering them unable to respond to further CD22 directed therapies [5••, 6]. Antigen loss has also been reported with the B cell maturation antigen (BCMA) in BCMA CAR clinical trials for the treatment of multiple myeloma [7]. Efforts are being made to overcome the

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problem of antigen escape, including the use of multi-specific antigen targeting CAR T cells, which target two or more tumor antigens simultaneously. Studies show that targeting multiple antigens can result in a synergistic effect and can help overcome variable antigen expression levels, which may diminish CAR T cell effectiveness [4, 8].

Sequential immunotherapeutic targeting of a second antigen after relapse associated with antigen loss is one approach to multi-specific targeting. However, as was seen with CD22 CAR therapy in leukemia patients with antigen-negative relapse after CD19 CAR, patients often relapse with loss of both antigens. This observation prompted the development of a bivalent CAR construct targeting both antigens simultaneously, in hopes that patients would be less likely to relapse via antigen escape [5••]. Multi-antigen targeting through CAR T cells harnesses the treatment principle that is already inherent to cancer therapy—targeting multiple oncogenic pathways simultaneously through the use of different types of chemotherapy helps prevent tumor cells from developing resistance to a single agent and thus minimizes the risk of relapse. This review outlines the various mechanisms for antigen escape and strategies to prevent antigen-negative relapse, and summarizes multi-specific CAR T cell therapies currently undergoing development and testing.

Mechanisms of Antigen Escape

A comprehensive discussion of the mechanisms of CAR T cell resistance can be found elsewhere but will be reviewed briefly here [9]. Relapse after CAR T cell therapy can occur with a preserved expression of the antigen target, due to failure of the CAR T cells to expand or function once infused, as seen after CD19 CAR. Strategies to improve CAR persistence can help prevent this [10] and will not be discussed in this review. However, a more frequent pattern of relapse is the loss of the target antigen. Antigen-negative relapse was first noted by Grupp et al. in a patient treated with CD19 CAR [11] and is now known to account for 60% of relapses post-CD19 CAR and up to 30% of relapses after treatment with blinatumomab, a bispecific antibody targeting CD19/CD3. One possible mechanism for this is through selective pressure causing the proliferation of a CD19-negative B-ALL precursor population that exists at the time of CART treatment. Often, CD19-negative populations cannot be identified at the time of diagnosis, but patients still go on to relapse with loss of the target antigen, suggesting that other mechanisms may also contribute to emergence of antigen-negative leukemia (Fig. 1a). CD123, a putative marker on leukemia stem cells, is often expressed on leukemia with CD19-negative relapse following CD19 CAR therapy, suggesting that stem cell populations may be a source for antigen escape [3].

Antigen escape can occur through loss of the target epitope, which accounts for 10–20% of relapses after CD19 CAR in pediatric B-ALL. In a study by Sotillo et al. comparing patient samples pre- and post-CD19 CAR therapy, they found that some patients harbored a deletion within the CD19 locus, while others had alterations in transcriptional regulation [12]. Patient samples were found to have variable splice sites, as well as nonsense, missense, and frameshift mutations, leading to exon skipping and expression of truncated CD19 variants with altered CD19 function and loss of recognition by CD19 CAR. The target epitope of the CAR, FMC63, is thought to be affected by skipping exon 2 specifically. Analysis of CD19-negative relapses from patients treated on the ELIANA trial demonstrated that all patients harbored different mutations that resulted in CD19 loss [13]. CD19 protein lacking exon 2 is expressed in a more stable form that is found more often in the cytosol or retained in the endoplasmic reticulum rather than on the leukemia cell surface, thus conferring a survival advantage as it is not targetable by subsequent CD19-directed therapy [14]. Based on these findings, targeting essential exons of the antigen of interest may be a way to overcome antigen escape [12].

Relapses after CD22-directed CAR therapy for patients with B-ALL can also occur due to diminished and variable expression of CD22 on the surface of leukemia cells, rather than complete loss of the targeted epitope as with CD19 [5••]. Indeed, leukemic cells with lower CD22 expression can evade CD22 CAR leading to eventual relapse, suggesting low sensitivity of the current CAR formats as a limitation [6]. Recently published data from a monovalent CD22 clinical trial (clinicaltrials.gov/NCT02315612) suggests that prior treatment with inotuzumab (anti-CD22 monoclonal antibody) or CD22 CAR causes diminished CD22 expression on the leukemia cell surface, similar to the selective pressure seen with CD19 CAR [15]. These patients have decreased response rates and faster relapses post-CD22 CAR, indicating that sequential targeting and diminished site density of the target antigen at the time of treatment can impair CAR efficacy and lead to antigen-negative relapse [15]. In a study by Fry et al., a comparison of leukemia cells pre- and post-CAR T cell therapy in two patients who relapsed with diminished CD22 site density found no genetic mutations in the CD22 locus nor qualitative changes in mRNA levels or alternative CD22 isoforms. This suggests that post-transcriptional effects cause diminished CD22 cell surface expression levels [5••]. Additional mechanisms for antigen loss have been postulated for patients with lymphoma, such as post-translational changes, loss of DNA repair proteins, or clonal evolution due to selective pressure from treatment with antigen-directed CAR [16].

In addition to the mechanisms already described for antigen loss post-CAR, recent studies in B-ALL have shown that patients may relapse with lineage switch to T cell or myeloid leukemia [17]. In xenograft models, relapse has occurred with

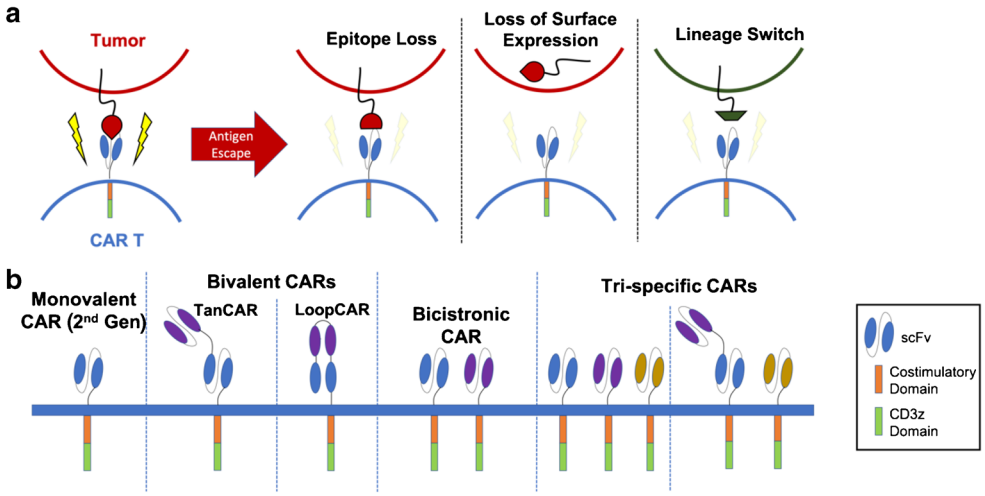

Fig. 1 Identifying and addressing mechanisms of leukemia relapse through multi-specific CAR targeting. (**a**) Mechanisms of leukemic antigen escape following monovalent CAR pressure. B-ALL cells can escape CAR pressure through loss of the CAR-targeted surface epitope, diminished surface expression, or by switching to a cell lineage which does not express the CAR-targeted protein. (**b**) Multi-specific antigen targeting to address antigen loss**.** A standard second-generation CAR recognizes a single antigen to drive T cell activation and cytotoxicity. Bivalent CARs include two scFv regions, arranged in tandem or loop structure, each targeting a different antigen and capable of generating full CAR activation. Bicistronic CARs express two full-length second-generation CAR constructs targeting different antigens. Tri-specific CARs incorporate a combination of monovalent and/or multi-specific CAR constructs to target three antigens, further broadening specificity

the loss of transcription factors *Pax5* and *Ebf1*, which are important in B cell development. Loss of these factors is seen in lineage switch in patients with pre-B-ALL, where relapse occurs with acquisition of T cell or myeloid genes. This is due to genetic reprogramming, rather than due to selection of a pre-existing myeloid or T cell leukemia population. When CD19 CAR T cells persist, they not only alter CD19 expression but also affect overall B cell identity, resulting in the emergence of leukemic resistance via lineage switch [17]. This phenomenon was also seen following CD19 CAR in one patient with chronic lymphocytic leukemia/small lymphocytic lymphoma and in two patients with MLL-rearranged ALL [18, 19]. Ultimately, further studies to understand and prevent leukemic resistance via lineage switch post-CAR are necessary.

Strategies to Overcome Antigen Loss

Despite the success of monovalent CARs in the clinic, post-CAR T relapses occur and are associated with the outgrowth of leukemia clones which have modulated their target antigen expression such that they evade CAR-targeted cytotoxicity. As described previously, monovalent CAR pressure leads to tumor immune evasion through loss or diminished surface expression of the target antigen, as seen after CD19 and CD22 CAR therapy [5••, 12, 20]. These findings highlight the need to develop strategies to improve durability of CAR response in order to achieve long-term remissions in a higher percentage of patients. Strategies which are currently being explored include inducing increased expression of the CAR-targeted antigen, broadening CAR specificity by developing constructs which are specific for multiple tumor-associated antigens, and targeting alternative antigens which are integral for tumor survival and cannot be readily downregulated.

Modulating Antigen Density to Improve CAR Response

Relapse following CAR infusion has been linked to decreased surface expression of CAR-targeted antigen [5••, 21]. This is particularly problematic for CD22-directed CAR therapy, with clinical trial results showing that eight of 20 patients treated with CD22 CAR subsequently relapsed with reduced leukemic CD22 expression [5••, 6]. Follow-up preclinical studies have shown that as CD22 antigen density decreases, CAR efficacy and persistence diminish, and similar results have been found with CD19 and anaplastic lymphoma kinase (ALK) CARs [6, 22, 23]. Thus, exogenous modulation of target antigen expression is an attractive strategy to improve durability of CAR response and potentially salvage CAR function in patients who relapse with antigen-low leukemia. Bryostatin 1, a protein kinase C modulating drug which increases CD22 expression on chronic lymphocytic leukemia, pre-B-ALL, and lymphomas, improves CD22 CAR persistence and durability of antileukemic response in a xenograft model of pre-B-ALL [6, 24]. A phase I clinical trial to evaluate the safety of Bryostatin 1 as a CD22 CAR co-therapy will open later this year at the National Cancer Institute (NCI). Similarly, in the context of acute myelogenous leukemia (AML), all-trans retinoic acid has been shown to enhance CD38 CAR cytotoxicity by increasing CD38 antigen

expression [25]. Thus, the principle of improving CAR response by increasing antigen density may be more broadly applicable and should be explored for other CAR targets.

Multi-Antigen Targeting Through Sequential or Co-infusion of CARs

A logical strategy to address antigen loss following monovalent CAR pressure is to infuse multiple CAR products which each target different antigens. While the principle of multi-CAR infusion is promising, clinical and preclinical findings have highlighted the challenges of preventing relapse and maintaining a persistent multi-specific response [4, 26]. Clinical data has shown that sequential treatment of diffuse large B cell lymphoma (DLBCL) with CD19 and CD22 CAR can drive the loss of both the CD19 and CD22 antigens, suggesting that sequential CAR infusion is not sufficient to prevent antigen escape [16]. In a clinical trial evaluating the efficacy of T cells co-transduced with both CD19 CAR and CD22 CAR, the pre-infusion CAR population was heterogeneous, with some cells expressing only one of the CARs and some expressing both [26]. However, despite encouraging results with CR achieved in five of seven patients, there was preferential expansion of T cells expressing only the CD19 CAR and a lack of persistence of both the CD22 CAR and double CAR-positive cells [26]. Xenograft experiments have similarly demonstrated that co-infusion or sequential infusion of monovalent CD19 and CD22 CAR leads to the outgrowth of a single dominant CAR population and cannot clear a heterogeneous population of CD19 or CD22 single-positive leukemia cells [4]. These findings suggest that infusing multiple populations of CAR T cells targeting different antigens may not be sufficient to generate durable multi-antigen pressure on malignant cells. While co-infusion and co-transduction have limitations, particularly in their inherent heterogeneity which can lead to immune competition, multi-antigen targeting with homogeneous multi-specific CAR products—either as bivalent or bicistronic CARs—have shown promising initial results.

Bivalent CAR Constructs

Several designs for multi-specific CARs have been explored (Fig. 1b). The bivalent "tandem" CAR construct, which consists of two scFv regions linked to a single CAR endodomain, was first introduced by Grada et al. in 2013 with simultaneous targeting of CD19 and HER2 [27]. The bivalent structure represents a conceptually simple mechanism to endow dual-antigen specificity, allowing for binding of either scFv to trigger a full second-generation CAR signal and drive T cell activation and cytotoxicity. This format has been evaluated against numerous leukemic antigens in preclinical models [4, 28, 29]. Given the clinical efficacy of both CD19 and CD22 CAR T cells, bivalent CARs specific for CD19 and CD22 have been developed by Qin et al. to extend durability of response against these antigens. The bivalent CAR is cytotoxic against both single-antigen-positive and double-antigen-positive leukemia cells, demonstrating equivalent in vivo and in vitro functionality to its monovalent CAR predecessors [4]. Development of the construct incorporating the CD19- and CD22-binding domains presented challenges, and maximum cytokine production and in vivo leukemia clearance was achieved by incorporating a loop structure in the extracellular domain and situating the CD22 scFv more distal to the membrane than the CD19 scFv [4]. The influence of extracellular domain conformation and scFv positioning on bivalent CAR functionality is thus an important parameter to evaluate in the design of future constructs.

The safety and efficacy of this bivalent CAR targeting CD19 and CD22 are currently being evaluated in phase I clinical trials for both pediatric and adult B cell malignancies (Table 1) [30, 31•]. Notably, the pediatric trial for r/r B-ALL has achieved CR in four of four patients 28 days post-CAR infusion, with 3 of 4 patients in MRD-negative remission [30]. The adult trial for r/r B-ALL and DLBCL has achieved CR in two of seven patients, including one B-ALL patient at 60 days and one DLBCL patient at three months [31•]. Both trials show that the bispecific CAR is safe, with minimal toxicities reported thus far. A bivalent CAR targeting CD19 and CD20 for the treatment of r/r B cell NHL is also being evaluated in a phase I clinical trial for adult patients. Early data from the trial has shown CR achieved in two of six patients at three and nine months, with no CD19 or CD20 antigen loss reported in any treated patient [32•]. Long-term follow-up data from these trials will be critical to determine whether bispecific CAR targeting is sufficient to decrease likelihood of antigen escape and improve durability of remission.

There are several other functional bivalent CAR constructs currently in preclinical development. Targeting CD133 in conjunction with CD19 in a third-generation bivalent CAR format generates T cell responses against CD133+ and CD19+ cells and extends the duration of leukemic clearance in a murine xenograft model of mixed lineage leukemia (MLL), when compared with CARs targeting only CD19 [33]. Bispecific "nanobody" CARs containing single-antibody domains specific for both CD20 and HER2 are also capable of broadening T cell specificity and cytotoxicity to each of these antigens in vitro [34].

While linking two scFvs is a logical way to generate dual-antigen pressure with a single CAR construct, multi-specific antigen targeting has also been achieved by developing a CAR with a single receptor that can recognize multiple target antigens. Lee et al. generated a CAR with a targeting domain consisting of a truncated form of APRIL, a ligand of BCMA and transmembrane activator and calcium-modulator and cyclophilin (TACI), which are co-expressed on tumor cells in 78% of patients with multiple myeloma (MM). This

Table 1 Notable multi-specific CAR trials for hematologic malignancies

Antigen targets & CAR format	Clinicaltrials.gov identifier:	Name/phase	Location	Patients and diseases treated	Preliminary data (if applicable)
CD19/CD22 bicistronic	NCT03289455	CD19/22 CAR T cells (AUTO3) for the treatment of B cell ALL (AMELIA); phase I/II [37]	London, UK	Age 1–24 years with high-risk relapse or relapse post HSCT B-ALL	- No deaths or DLTs - No grade 2 or higher CRS - 6 of 8 patients achieved MRD-negative CR at 1 month (75% ORR) - No loss of CD19 or CD22 in 3 patients that relapsed
CD19/CD22 bicistronic; anti-PD1 (pembro)	NCT03287817	CD19/22 CAR T cells (AUTO3) for the treatment of diffuse large B cell lymphoma (Alexander); phase I/II [38]	London, UK	Age 18 years or older with r/r DLBCL	- No deaths or DLTs - 1 patient developed grade 1 CRS; no grade 2 or higher CRS - 2 of 5 patients achieved CR at 3 months - 4 of 5 patients responded (80% ORR)
CD19/CD22 bivalent	NCT03241940	CD19/CD22 chimeric antigen receptor T cells and chemotherapy in treating children or young adults with recurrent or refractory CD19-positive B acute lymphoblastic leukemia; phase I [30]	Palo Alto, CA	Age 2–17 years with r/r pre-B-ALL or lymphoma	- 3 of 4 patients had reversible CRS - 2 patients had grade 1 neurotoxicity - 4 of 4 patients achieved CR at 28 days (3 of 4 MRD negative)
CD19/CD22 bivalent	NCT03233854	CD19/CD22 chimeric antigen receptor T cells and chemotherapy in treating patients with recurrent or refractory CD19-positive diffuse large B cell lymphoma or B acute lymphoblastic leukemia; phase I [31•]	Palo Alto, CA	Age 18 years or older with r/r B-ALL or DLBCL	- 6 of 6 developed reversible CRS - 3 developed neurotoxicity (grade 2 or lower) - 3 patients have CR, 2 have PR, 1 died of PD
CD19/CD22 bivalent	NCT03330691	Pediatric and young adult leukemia adoptive therapy (PLAT)-05: a phase 1 feasibility and safety study of dual specificity CD19 and CD22 CAR T cell immunotherapy for CD19+ CD22+ leukemia	Seattle, WA	Age 1–26 years with CD19+ CD22+ r/r leukemia	Not applicable
CD19/CD22 bivalent	NCT03448393	Phase 1 dose escalation study of CD19/CD22 chimeric antigen receptor (CAR) T cells in children and young adults with recurrent or refractory CD19/CD22-expressing B cell malignancies	Bethesda, MD	Age 3–30 years with r/r B-ALL or lymphoma	Not applicable
CD19/CD20 bivalent	NCT03019055	Phase 1/1b study of redirected autologous T cells engineered to contain an anti CD19 and anti CD20 scFv coupled to CD3ζ and 4-1BB signaling domains in patients with relapsed and/or refractory CD19 or CD20 positive B cell malignancies	Milwaukee, WI	Age 18–70 years with r/r B-NHL or CLL/SLL; must have either CD19+ or CD20+ disease	-No DLTs - 2 of 6 achieved CR, 2 of 6 achieved PR, 2 of 6 have PD at day 28 - 1 patient with PR eventually progressed at day 90 - Patients with PD remained either CD19+ or CD20+
CD19/CD22 sequential infusion	NCT03620058	Phase 1 study of autologous anti-CD22 chimeric antigen receptor redirected T cells alone and when co-administered with humanized anti-CD19 chimeric antigen receptor redirected T cells in patients with chemotherapy-resistant or refractory ALL	Philadelphia, PA	Age 18 years or older with r/r B-ALL	Not applicable

MRD, minimal residual disease; *DLT*, dose-limiting toxicity; *CRS*, cytokine release syndrome; *ORR*, overall response rate; *PR*, partial response; *CLL/SLL*, chronic lymphocytic leukemia/small lymphocytic lymphoma; *pembro*, pembrolizumab; *PD*, progressive disease

APRIL-based CAR caused cytolysis of MM cells in vitro when either BCMA or TACI was detected. More importantly, it demonstrated tumor clearance in models of antigen escape (BCMA+/TACI−, BCMA−/TACI+) in vivo when compared with scFv CAR targeting BCMA, which allowed for proliferation of the BMCA-negative tumor population. This multi-specific CAR model offers yet another strategy for prevention of post-CAR relapse via antigen loss [35].

Bicistronic CAR Constructs

In contrast to bivalent CAR constructs containing two scFv domains in one receptor, an alternative is to generate an expression of two separate CAR constructs on the surface of the same cell using a single vector. A bicistronic CAR is transduced as two full-length CARs on a single vector, with a cleavable linker that allows each CAR to be expressed separately on the cell surface. This construct functions in the presence of either target antigen to trigger T cell activation, while avoiding issues with low transduction efficiency and preferential activation of one CAR when two separate vectors are utilized [8].

Bicistronic CAR T cells engineered by Qin et al. to target CD19 and CD22 outperformed both bivalent and monovalent CARs targeting the same antigens and improved durability of remission in a xenograft model [8]. These CD19/CD22 bicistronic CAR T cells were shown to eradicate leukemia in xenograft models of antigen loss, which could not be controlled by either CD19 or CD22 monovalent CAR T cells individually [8]. Similarly, targeting of CD19 and CD123 with a bicistronic CAR also successfully prevents antigen loss–mediated relapse, which occurred after treatment with the CD19 CAR alone, in a murine xenograft model of B-ALL [3]. Superiority of multi-specific targeting has also been demonstrated with CD19/CD20 bicistronic CARs, which more effectively kill leukemia at decreased effector-to-target ratios in vivo when compared with CD19 or CD20 CARs [36]. This finding suggests that in the context of high leukemia burden, multi-specific antigen targeting may improve CAR T cell performance and future studies should determine whether this can be generalized to other bicistronic CARs.

Bicistronic CARs are currently being tested in clinical trials (Table 1). The Amelia trial, a phase I/II clinical trial evaluating a bicistronic CAR (AUTO3) targeting CD19 and CD22, has shown promising initial results for pediatric B-ALL with six of eight patients achieving CR at one month post-CAR [37]. Thus far, no relapse with loss of the CD19 or CD22 has been observed. The Alexander trial, a phase I/II clinical trial evaluating AUTO3 followed by PD1 blockade with pembrolizumab for the treatment of adult r/r DLBCL, has also demonstrated some success with two of five patients in CR at three months post-CAR [38]. However, similar to the ongoing bivalent CAR trials, long-term follow-up data will be crucial to determine the durability of response.

Alternative Targets

Though targeting multiple antigens is a promising strategy to overcome antigen escape, it may also be important to consider alternative targets that are more functionally important, such that tumor cells are less likely to survive without them. For example, CAR T cells targeting the cytokine receptor thymic stromal lymphopoietin receptor (TSLPR) are effective at eradicating leukemia in vivo, and late relapses occur with a preserved expression of TSLPR [39]. This suggests that the leukemia cells need this cytokine receptor to survive, unlike CD19 and CD22, as leukemia cells have found a way to proliferate in their absence [39]. Our lab is currently exploring multi-antigen targeting incorporating TSLPR and either CD19 or CD22 as a strategy to treat TSLPR overexpressing leukemia, mediated by CRLF2 rearrangement, which makes up about half of Philadelphia-chromosome like B-ALL. This type of leukemia is more aggressive, with higher relapse rates, indicating a need for more targeted effective therapy [39]. A clinical trial using monovalent TSLPR CART in adults with TSLPR-overexpressing B-ALL is anticipated to open by the end of 2019, and knowledge from this trial can guide further explorations into multi-antigen targeting incorporating this cytokine receptor.

Though CAR T cells have been more widely used in treatment of B-ALL, they have applicability across multiple hematologic malignancies, including AML and multiple myeloma. These malignancies have different tumor-associated antigens with more complex on-target/off-tumor toxicities beyond B cell aplasia, as is seen with CD19 CAR therapy. Broadly speaking, CAR T cell therapy for AML has not been as successful as it has been for B-ALL, in part because target antigens (i.e., CD33, CD123) exist on hematopoietic progenitor cells, leading to myeloablation. Recently, studies have looked at targeting Fms-like tyrosine kinase-3 (FLT3), which is mutated in about 30% of patients with AML. Patients harboring this mutation have worse outcomes and require hematopoietic stem cell transplant as their only option for curative therapy. The initial FLT3 CAR construct targeting the scFv was unable to distinguish wild-type and mutant FLT3, suggesting a need for an alternative target. Recent data shows that targeting the ligand of FLT3 may be a more effective strategy to eradicate leukemia, while minimizing on-target/off-tumor toxicity. This suggests that it may be beneficial to explore various structural components of the target antigen as potential CAR targets going forward [40]. FLT3 CAR has also been utilized in combination with the FLT3 inhibitor crenolanib, which causes upregulation of FLT3 expression and thus improved CAR targeting and cytotoxicity in vivo. Additionally, treatment with both crenolanib and FLT3 CAR in conjunction leads to less FLT3-negative or FLT3 low escape variants, suggesting combination therapy as a potential strategy to prevent antigen loss [41].

Conclusion and Future Directions

As CAR T cell therapy continues to evolve and expand to treat various types of malignancies, prevention of relapse post-CAR will be a driving factor for scientists and clinicians alike. Antigen loss remains a significant barrier to overcome, though modulating target density with complementary therapies, targeting multiple antigens simultaneously, and targeting more functionally important antigens to the tumor cell are promising prevention strategies. Data from ongoing and future clinical trials with multi-specific CARs will be essential to determine whether these are sufficient to mitigate antigen loss.

With preclinical data demonstrating improved durability of remission using bispecific CAR targeting [3, 4, 8], a natural progression is to expand CAR targeting to more than two tumor-associated antigens as a potential strategy to overcome antigen escape. Tri-specific CAR T cells are under development to treat hematologic malignancies and glioblastoma multiforme [42, 43]. Fousek et al. developed two variants of a tri-specific CAR targeting the B-ALL-associated antigens CD19, CD20, and CD22. The first variant (TriCAR) expresses three monovalent second-generation CARs targeting each antigen individually. The second variant (SideCAR) expresses a monovalent second-generation CD19 CAR and a bivalent CAR containing scFvs targeting both CD20 and CD22 (Fig. 1b). Both variants demonstrate improved in vitro cytotoxicity against triple-positive ALL cells at low effector-to-target ratios when compared with monovalent CD19 CAR, suggesting that multi-antigen targeting may improve CAR functionality in the setting of a high leukemia burden [42].

A trivalent CAR targeting the glioblastoma-associated antigens HER2, IL13Ra2, and EphA2 has also been developed [43]. However, while this trivalent CAR demonstrated cytotoxicity against each target antigen comparable to that of monovalent CARs, patient data suggests that up to 68% of glioblastomas may be triple-negative for these antigens, indicating the need to target a combination of antigens that encompasses the greatest percentage of glioblastoma variants [44]. Though preliminary data suggests that tri-specific CAR targeting may be effective, it is not yet known if this will translate into less relapse via antigen loss.

Beyond broadening CAR functionality to mitigate antigen escape, multi-specific targeting is also being harnessed to improve the safety of CAR therapy by minimizing on-target/off-tumor toxicities. The bivalent and bicistronic constructs previously described are activated by the presence of either target antigen, termed an "OR-CAR." By linking one scFv to CD3 zeta and the other scFv to a costimulatory signaling domain, a format known as an "AND-CAR", a full second-generation CAR signal can be generated only through ligation of both CARs, limiting CAR efficacy to double-antigen-positive target cells. Selecting a combination of antigens which are only overexpressed on tumor cells can thus limit CAR cytotoxicity against single-positive non-malignant cells. Proof of concept of the AND-CAR format has been demonstrated in a number of preclinical studies [45–47]. Narrowing of CAR specificity can also be achieved with a format known as a "NOT-CAR", in which standard second-generation CAR T cells co-express an scFv conjugated to an inhibitory T cell endodomain (such as PD1 or CTLA4) [48]. This format can prevent CAR activation against cells expressing the antigen recognized by the inhibitory-conjugated scFv and represents another compelling strategy to restrict CAR toxicity to malignant cells.

In summary, multi-specific CARs have broad applicability in treating multiple types of malignancy and show promise as a strategy to prevent antigen escape. As more clinical data becomes available, lessons learned from patients will help to further optimize CAR design and function to minimize risk of relapse.

Compliance with Ethical Standards

Conflict of Interest Zachary Walsh, Savannah Ross, and Terry J. Fry declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national institutional guidelines).

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