Engineering-enhanced CAR T cells for improved cancer therapy

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Chimeric antigen receptor (CAR) T cell therapies have evolved from a research tool to a concept-shifting therapy with impressive responses in B cell malignancies. This Review summarizes the current state of the CAR T cell field, focusing on CD19- and B cell maturation antigen-directed CAR T cells—the most developed of the CAR T cell therapies. We discuss the many challenges to CAR T cell therapeutic success and innovations in CAR design and T cell engineering aimed at extending this therapeutic platform beyond hematologic malignancies.

Although antitumor immunity by T lymphocytes has been known for decades, translating it into anticancer therapies has been challenging. However, biological advances, such as the generation of single-chain antibody fragments (scFv)1, the elucidation of pathways mediating the activation of functional memory T cells2,3, and molecular cloning4 have led to the engineering of chimeric antigen receptor (CAR) T cells, introducing a new era of cancer immunotherapy5,6 and permitting the treatment of large groups of patients with genetically augmented patient-derived T cells.

The first generation of CAR T cells fused the scFv antibody fragment to T cell signaling domains comprising the immunoreceptor tyrosine-based activation motif, offering a relatively simple method of endowing T cells with major histocompatibility complex-independent recognition of antigens7. Over the following two decades, the CAR platform evolved into second- (two-domain) and third-generation (three-domain) CARs that incorporated additional signal transduction domains, including cytoplasmic domains from important T cell costimulatory receptors such as CD28, CD137 (4-1BB) and CD134 (OX-40) (reviewed in refs. 8,9). These additional signaling domains promote both the persistence and antigen activity of CAR T cells following adoptive transfer10,11,12, and were essential to avoid the anergy observed with first-generation CARs13,14.

The remarkable ability of a CAR to reprogram T cell specificity led to attempts at clinical translation. The earliest clinical application used a simple CAR design comprising a CD4 ectodomain fused to a CD3ζ cytoplasmic domain to treat human immunodeficiency virus-infected patients15, and established both the safety and antigen activity of CAR T cells following adoptive transfer16,17, and were essential to avoid the anergy observed with first-generation CARs18,19.

As preclinical models of adoptive T cell therapy are limited during their clinical development to determine the kinetics and quality of the infused CAR T cells, measure tumor cell dynamics and assess cytokine levels and repertoires during therapy have proven pivotal in improving our understanding of these complex therapies and enhancing their clinical application. These correlative studies have highlighted many factors that are essential to safely achieving both deep and durable clinical responses in otherwise treatment-refractory cancers. Here, we discuss the important role of correlative science in developing CAR T cell therapies, and highlight the challenges still faced during clinical application and the new technologies promising to address these complications to help extend this therapeutic modality beyond B cell malignancies.

Efficacy and toxicity of CD19-specific CAR T cell therapies

Normal and malignant B cells uniformly and exclusively express CD19 (ref. 18)—the dominant signaling moiety of a tetramolecular complex consisting of CD21, CD81 and CD225, which modulates B cell receptor signaling and mediates immunoglobulin-induced B cell activation19. Given CD19’s broad expression within the B cell lineage from early pro-B cells to subsets of plasma cells (Fig. 1), as well as its generally uniform expression on B cell malignancies20, this molecule became a prime target of CAR T cell approaches. The initial encouraging results in relatively small studies in non-Hodgkin’s lymphoma (NHL)21,22, chronic lymphocytic leukemia (CLL)23–25 and acute lymphoblastic leukemia (ALL)26–28 have since been confirmed in larger cohorts29–31. So far, the first patients with CLL treated with anti-CD19 CAR T cells have sustained remission beyond 9 years32, and the first ALL patient to be treated with the same engineered product has been in remission for more than 7 years33.

Generally, the overall response rate has been highest in B cell ALL (>80%), variable in lymphomas (~63–100%) and lower in CLL (50–70%)34,35,36,37. Patients with CLL, who achieved remission with anti-CD19 CAR T cell treatment sustained their disease-free state38,39,40. In ALL, however, only 20–40% of patients sustained remission on this therapy38,41,42,43,44,45. Loss of CD19 expression is a major mechanism of resistance in ALL, accounting for around two-thirds of relapse cases and is a well-recognized phenomenon in lymphoma as well46,47. Loss of CAR T cell engraftment may account for most of the remaining cases of relapse48. Initial small trials49,50 followed by larger ones51,52,53 also confirmed the immense potential

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Described recently, the adoption of which should greatly facilitate systems. Fortunately, a new consensus grading system for CRS was treated with anti-CD19 CAR T cells33,36,39,50. Additionally, during the has also been observed in patients with lymphoma and leukemia but was observed in every CAR T cell trial for ALL 26,29,33,38,39, more addition to CRS, a somewhat unique and unexpected neurotoxic-drome are commonly observed in patients with lymphoma treated- signage CAR 24,28,35,49, ζ disease and CAR design. Severe neurotoxicity was seldom reported in patients. This toxicity can range from mild delirium to severe encephalopathy. The incidence of neurotoxicity may depend on the reasons for these differences are not understood, and remain an important subject of study in the post-marketing phase. Although CD19-specific CAR T cell therapies have shown remarkable clinical activity against B cell malignancies, these deep and durable responses do come at the cost of some unique adverse effects. Cytokine release syndrome (CRS) is the most frequently observed adverse event in CART19-treated patients. Most cases of CRS are mild or moderate in severity and manageable. However, the frequency of severe CRS across studies, reported in 19.8–38.8% of patients, most of whom remained disease free. Although clinical responses were generally sustained in NHL, most disease-free patients would display normal B cell recurrence and loss of detectable CAR T cells, suggesting that other mechanisms were responsible for long-term tumor control in NHL. Interestingly, although patients with ALL and CLL generally achieved their best overall response within the first month following CAR T cell infusion, patients with lymphoma often continued to improve beyond the first month, with some patients not achieving their maximum response until 6 months post-CAR T cell treatment23,33,44,46. The reasons for these differences are not understood, and remain an important subject of study in the post-marketing phase.

for this therapy in NHL. Both the CD2831 and 4-1BB cosignaling anti-CD19 CAR T cells34 induced complete remission in 40–50% of patients, most of whom remained disease free. Although clinical responses were generally sustained in NHL, most disease-free patients would display normal B cell recurrence and loss of detectable CAR T cells, suggesting that other mechanisms were responsible for long-term tumor control in NHL.

Generating hypotheses with correlative studies
Defining the kinetics, homing and bioactivity of the cell therapy product and tumor response to treatment in each patient requires diligent monitoring, as these are critical components in the continued translational cycle from the bench to the bed and back again. Furthermore, the US Food and Drug Administration (FDA) mandates that sponsors observe study participants for delayed adverse events for as long as 15 years following the infusion of modified cells62. To this end, it is desirable to include a correlative studies laboratory in an organization that operates according to good clinical laboratory practice39 (Fig. 2), to ensure that biospecimens from patients on cell therapy are handled by qualified personnel following experimental processes specified by standard operating procedure (SOP). Sample analytics and biobanking are two critical activities in such a laboratory, both of which should be carried out using rigorously validated, SOP-defined procedures. As most phase I trials are run in academic centers, some of the analytical methods would have to be developed and validated for novel, innovative therapies such as the CRISPR–Cas9-mediated disruption of endogenous genes in mature T cells, combined with lentiviral delivery of a tumor-targeting T cell receptor41. An example is the frequent monitoring of CAR T cell bioactivity in terms of changes in cytokine and soluble cytokine receptor levels23,26,30,33,42–45 in serum early after infusion, given that high-grade toxicities may develop rapidly upon treatment.

The value of correlative studies is underscored by the identification of a rise in interleukin-6 (IL-6) levels in association with the onset of CRS in patients, which played a central role in prompting the evaluation of IL-6/IL-6 receptor blockade in severe CRS31. This insight proved life saving for many patients, and formed the foundation for co-developing anti-IL-6 and CD19 CAR T cell therapy, leading to their concurrent FDA approval for severe CRS and B cell ALL, respectively. More extensive analyses of serum from patients in multiple trials have led to the discovery and validation of biomarkers of CRS and neurotoxicity, providing insight into the mechanisms that drive them59 and potential paths to predicting these complications of CART1 cell therapy. Although not all studies agree on the precise cytokines48 or biomarkers7 to interrogate, they all focus on identifying predictive markers and developing algorithms to distinguish patients at increased risk of developing life-threatening toxicities.

Biobanked cells from patients have played a critical role in identifying mechanisms of resistance to CD19-specific CAR therapy. One of the earliest reports of CART19 in ALL revealed evidence of relapse in the context of loss of CD19 expression, which has been demonstrated to be the dominant resistance mechanism in ALL, occurring through various genetic mechanisms and rare intergenic causes40–42. Early loss of CAR T cell preceded by normal B cell recovery is another commonly observed event associated with relapse46. Analyses of the T cells used for manufacturing the CART19 cells, as well as the product itself, have revealed a number of associations that link CAR T cell quality to outcome. In particular, the presence of naive-like CD27+CD45RO+ cells in the apheresis product used for CART19 generation was shown to predict engraftment and clinical response in CLL40. The reinfusion of relapsing patients with leukemia with a murine scFv-based CAR has been associated with reduced expansion compared with first infusion33,35,46, suggesting that an immune-mediated mechanism may underlie resistance to retreatment. Humanizing or developing a fully human scFv fragment might therefore enhance therapeutic success41. Recently, defects in death receptor signaling have been identified in a subset of ALL that is resistant to CD19-specific

Fig. 1 | B cell malignancies at the different stages of B cell development. Normal B cell developmental lymphocytes (top) often share immunophenotypic characteristics with their malignant counterparts (bottom), reflecting the expansion of a dominant clone leading to the development of leukemia or lymphoma.
CAR T cell therapy, providing additional resistance mechanisms beyond CD19 loss.65–67

Correlative studies have also revealed differential kinetics of CAR T cells in responding and non-responding patients with CLL and ALL, which led to the development of an in vitro, proliferation-based potency assay.40,67 This correlation between clinical response and in vivo CART19 cell proliferation was not evident in trials of the same product when used for NHL, in contrast with their effector progeny that have lost that ability and instead directly lyse the tumor. Two studies recently confirmed that this therapy depends on a functional, self-renewing T cell pool, demonstrating that in CLL the advanced age of the patient population in combination with effector-memory skewing limited CART19 cell functionality (Fig. 3)40,67. Furthermore, response to therapy in CLL can be predicted based on the presence of a pool of more functional early memory cells.40,67 CAR T cells and other therapies that rely on immune system activation may therefore have limited effect in malignancies that terminally skew T cell differentiation or occur in aged populations where T cells are less functional at baseline. That baseline functionality of the T cell pool plays a substantial role in dictating response rates was confirmed in a separate study, which revealed that CD8 T cell dysfunction at apheresis and the rapid expression of immune checkpoint molecules after infusion marked CAR T cells from non-responding and partially responding patients with ALL.67 Therefore, CAR T cells are subject to inhibition via endogenous immune checkpoint pathways such as programmed cell death protein 1 (ref. 73). Inhibitory receptor–ligand interactions normally dampen T cell functions to prevent an overactive immune response and sustain a memory T cell pool. In CAR T cells, this can result in failure to eliminate the tumor and loss of T cell persistence. Whereas checkpoint blockade can improve responses, other immune-suppressive factors in the microenvironment can impair CAR T cell function. Immune-suppressive cytokines, metabolic competition and high inhibitory ligand expression levels all serve to modulate the function of cell-based therapies.59,68–71

Enhancing CAR T cell potency by genome engineering

Although the natural basis of CAR T cell efficacy, as laid out in the previous sections, presents the foundation of immunogenic therapies with CAR T cells (and probably other systems that depend on sustained tumor control), CAR T cell engineering may also impact cell function, as recently reported.57,58. CAR T cells produced with lentivirus display quasi-random integration of the vector throughout the genome, introducing the potential for genomic activation or disruption events.75,80. Although the majority of CAR T cells

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**Fig. 2 | Operational pipeline for integrating correlative studies in translational science laboratories.** Novel therapies that have been developed and preclinically validated in research laboratories are handed off to the process development (PD) team for scale-up and the development of a current good manufacturing practice (GMP) process. In collaboration with the GMP teams, SOPs and documentation forms are developed and GMP staff are trained in the new procedures. In parallel, the correlative studies laboratory ensures that all supportive assays, protocols and forms are in place, staff are trained, routine, qualified assays are developed and biobanking is ensured. This same team is also involved in protocol development, which is led by the clinical operations team with feedback from the study clinicians and the research laboratory that developed the new process. When a new clinical trial begins, the correlative studies laboratory starts receiving biospecimens from the clinic, manufacturing facility or collaborating laboratories, and logs these samples into the laboratory information management system (LIMS) to be processed as specified by standard operating procedures and examined using validated assays by qualified personnel. Aliquots are retained from each specimen for future translational studies. The data are reviewed by subject matter experts (SMEs) before being reviewed by the quality control (QC) manager and entered into a database. A staff statistician cleans and analyzes the data for reporting purposes (for example, to the FDA) or for scientific meetings and manuscript preparation.

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**Clinical impact of T cell biology and CAR engineering**

Although CART19 therapy has been efficacious in ALL and NHL, many factors contributing to patient response remain poorly understood. As patient-derived T cells are used to target a tumor-associated cell-surface protein, the immune system is repurposed to treat the malignancy. Thus, the therapeutic efficacy still depends on T cell memory and effector functions. This also includes T cell fitness, which is affected by the malignancy and previous therapies, and, most importantly, the ability of the CAR-redirected T cells to sustain the antitumor response, because most tumors exist in actively growing and dormant phases, which can last from several years to decades.65–67. By harnessing T cells, this form of immunotherapy abides by similar target cell quiescence–reactivation principles to induce a cure. Naive and memory T cells retain the ability to proliferate vigorously in response to cognate antigen recognition, in contrast with their effector progeny that have lost that ability and instead directly lyse the tumor. Two studies recently confirmed that this therapy depends on a functional, self-renewing T cell pool, demonstrating that in CLL the advanced age of the patient population in combination with effector-memory skewing limited CART19 cell functionality (Fig. 3)40,67. Furthermore, response to therapy in CLL can be predicted based on the presence of a pool of more functional early memory cells.40,67 CAR T cells and other therapies that rely on immune system activation may therefore have limited effect in malignancies that terminally skew T cell differentiation or occur in aged populations where T cells are less functional at baseline. That baseline functionality of the T cell pool plays a substantial role in dictating response rates was confirmed in a separate study, which revealed that CD8 T cell dysfunction at apheresis and the rapid expression of immune checkpoint molecules after infusion marked CAR T cells from non-responding and partially responding patients with ALL. Therefore, CAR T cells are subject to inhibition via endogenous immune checkpoint pathways such as programmed cell death protein 1 (ref. 73). Inhibitory receptor–ligand interactions normally dampen T cell functions to prevent an overactive immune response and sustain a memory T cell pool. In CAR T cells, this can result in failure to eliminate the tumor and loss of T cell persistence. Whereas checkpoint blockade can improve responses, other immune-suppressive factors in the microenvironment can impair CAR T cell function. Immune-suppressive cytokines, metabolic competition and high inhibitory ligand expression levels all serve to modulate the function of cell-based therapies.59,68–71

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generated in this process are polyclonal, the CAR T cell population undergoes rapid changes after infusion due to, among other factors, selective expansion of CAR T cell clones for reasons that are currently poorly understood. In most patients, a multitude of clones contribute to the antitumor response. Two recently published reports concern clonal CD8+ CAR T cell expansion in two patients, in whom the CAR was shown by sequencing vector integration sites to have integrated into the CBL and TET2 gene loci. In the case of the TET2 integration, the patient’s CAR T cell population underwent delayed expansion accompanied by tumor clearance, complete remission status and contraction of the clonal population. The CBL-integrated clone underwent a similar, albeit less dramatic, expansion process. CBL knockdown had previously been associated with decreased T cell activation thresholds, reduced reliance on costimulation and decreased sensitivity to programmed cell death protein 1 inhibition, which could represent mechanisms for the therapeutic effect. These cases highlight how lentiviral integrations have revealed locus-specific regulation and protective effects. For instance, CAR expression from the T cell receptor-α constant (TRAC) locus optimized CAR expression and protected cells from exhaustion compared with integration in other sites. The genomic landscape of the CAR transgene cassette can therefore play an important role in how individual CAR T cells function. Unique cases such as the TET2 and CBL loci integration events are informative not only on how genome regulation can influence CAR expression and function, but also in terms of novel regulators of these functions. The identification of TET2 disruption as an enhancer of T cell persistence has sparked a wide array of research focused on knocking out TET2 to improve CAR function and determine the mechanisms underlying this selective advantage.

Natural killer cells have also been engineered to express a B cell targeting CAR combined with constitutive secretion of IL-15 (ref. 87). Preclinical studies have similarly demonstrated a beneficial effect of CAR T cells co-expressing IL-15 (ref. 88). Based on these findings, several clinical trials have been launched to evaluate T cells engineered to express this cytokine in conjunction with a tumor-targeting CAR. However, IL-15 was separately demonstrated to drive antigen-independent growth of T cells, resulting in a pre-leukemic disorder in mice. Therefore, the addition of a safety switch to the CAR and IL-15 construct should allow for control of the infused cells, as indicated in the design of one of these trials (NCT03721068), which targets GD2 in brain cancers using anti-GD2 CAR with IL-15 and the iCaspase 9 safety switch. Moreover, preclinical studies have shown augmented antitumor efficacy of IL-18 co-expressing T cells in a CD19-redirected T cell model. Similar combination therapies have been shown to jointly blunt tumor function and boost T cell potency. Next-generation CAR T cell therapies incorporating such engineering approaches are expected to further raise the therapeutic index.

Tumor-directed T cells encounter numerous inhibitory signals in the tumor bed, the most notorious of which is transforming growth factor-β. CAR T cells engineered to express a dominant-negative transforming growth factor-β receptor showed augmented potency against a solid tumor model, leading to the development of an ongoing clinical trial to target prostate cancers with a prostate-specific maturation antigen-specific CAR T cell (NCT03089203).
Current limitations
- Immunogenicity of the scFv
- Undesired interactions within T cells and other cell types (for example, macrophages)
- CAR T becomes dysfunctional or loses functional persistence in vivo (CD28)

Solutions
- Human/humanized scFv
- Engineer CAR with natural ligand of targeted protein
- Use spacer domains (for example, from CD4, CD8 or CD28)
- Engineer out the interaction domains
- Use cosignaling domain designed to boost persistence
- Mutate ITAM to reduce CAR T cell dysfunction
- Pharmacological control of the CAR activity (for example, using dasatinib)

Tumor resistance to CAR T cell therapy
Extensive clinical data have revealed mechanisms by which tumor cells escape CAR T cell targeting, and have informed engineering advances to overcome this. The anti-CD19 CAR was shown to require minute quantities of target antigen to display full effector function; therefore, the tumor cells could only escape this pressure via antigen loss25,60,61,99 or antigen masking50. Routine analyses revealed that a pediatric patient with ALL treated with murine anti-CD19 CAR relapsed with the original disease two months post-treatment51. In the ensuing months and years, similar patterns of relapse were observed from routine correlative studies, which were later confirmed by clinical pathology52,53,54,55. The molecular basis of these and similar cases of antigen loss-related anti-CD19 CAR T cell therapy were shown to be related to the acquisition of open reading frame-disrupting mutations in the target antigen, compounded by altered messenger RNA splicing in tumor cells66,67. Others similarly observed antigen-negative relapse in CD19-directed CAR T cell therapies50,68,69. Additionally, mixed-lineage leukemia-rearranged leukemias displayed lineage-switch-related relapse with loss of CD19 protein expression50,68, further illustrating how the immense immune pressure exerted by CD19-specific CAR T cells mediates Darwinian selection of the malignant cell pool. These examples demonstrate the impact of T cell and tumor cell physiology on clinical responses to single antigen-directed CAR T cell therapies. This knowledge has been used to prevent relapse through a bispecific CAR T cell that recognizes two antigens present on the tumor surface. This anti-CD19/anti-CD22 bispecific CAR T cell has been used successfully to treat an adult patient with ALL who has remained disease free for more than 1 year post-therapy70. Antigen loss has now also been observed in a patient on CD22-targeting CAR treatment71, whereas others have shown that downregulation was sufficient to evade CART22 treatment72.

CAR design
As described above, many factors independent of the CAR itself impact therapeutic efficacy. Correlative studies by various groups targeting the same tumor-associated antigen (TAA) (for example, CD19) with an scFv derived from the same monoclonal antibody (for example, FMC63) but different spacer domains, cosignaling domains and so on have allowed the identification of several pain points and success stories of chimeric receptors (Fig. 4). First, it has become obvious that cosignaling has to be engineered into the CAR, as even the transduction of memory T cells could not rescue a first-generation CAR73. Hence, around the time that CD28 cosimulation was discovered as an essential component to memory T cell formation and effector differentiation74, second-generation CARs were developed that included this domain75. However, comparative clinical studies to demonstrate the differential impact of CD28 and other cosignaling domains on effector and memory function in vivo are lacking and would be useful, because despite the CAR-contained CD28 driving a profound effector differentiation, it can also render the T cells dysfunctional with loss of persistence76.

Early data also revealed the profound impact of the spacer domain on CAR T cell function (reviewed in ref. 100). Most early-generation CARs, including those in first-generation CAR designs77, used scFv derived from mouse antibodies. T cells discern minute differences between cancerous and normal cells, and a single difference in amino acid residues can induce a robust immune response against this non-self entity100–103. This same selective threat elimination machinery deletes recombinant proteins containing minimal sequence divergence from the native protein just as efficiently as foreign threats104,105. It should therefore come as no surprise that suicide genes105 and CARs incorporating non-human sequences are readily targeted by the immune system106,107. Moreover, the poor expansion of re-infused CAR T cells108,109 was correlated with the detection of patient-derived T cell epitopes in the CAR105. The field is therefore moving away from incorporating non-human tumor-targeting moieties into the CAR107. That being said, the remarkable response rates with a non-human CAR in multiple myeloma recently suggest that deep molecular remissions are possible (see below).

Extending CD19 CAR T cell therapy beyond CD19+ malignancies
Although multiple myeloma derives from plasma cells (the terminal stage of B cell differentiation), myeloma precursor cells may express CD19. Pilot studies suggested a potential benefit to targeting CD19 in myeloma110, yet little or no activity was apparent in the vast majority of treated patients, indicating that an alternative target antigen is needed to address this disease with CAR T cell therapy112. Myeloma cells uniformly express B cell maturation antigen (BCMA) (Fig. 1), leading to the development of BCMA-specific CAR T cells. Currently, 90 relevant clinical trials are listed on ClinicalTrials.gov, with a few moving forward towards their commercial roll-out (see Tables 1 and 2 for the constructs and trials furthest along in their development).

BCMA has been targeted by various groups using a diverse array of chimeric receptors (reviewed in refs. 126,127). Although human anti-BCMA CARs gained traction in myeloma128–131, non-human-derived BCMA CARs with an anti-BCMA murine132–135 (or alpaca) immunoglobulin136–138 chain are further along in clinical trials (Table 2).

Lymphodepletion using cyclophosphamide and fludarabine before adoptive T cell transfer further boosts CAR T cell expansion139 by depleting cytokine sinks140 and immune-suppressive cells141,142. Although response rates vary widely among different BCMA-specific CAR T cell products, the biggest challenge remains the durability of response, with patients appearing to ultimately progress regardless of the product140.

The mechanisms that underlie myeloma resistance to BCMA CAR T therapy are coming to light through correlative analysis of biobanked specimens from early-phase clinical trials. Comparisons are difficult to make across trials, institutions and therapies, even though they all target the same myeloma-associated antigen, as differences in the cell manufacturing process, vector used, CAR design and trial participant selection criteria, among other factors, are
likely to affect outcomes. Modulation of BCMA expression may also play a role. Early studies preselected patients based on the expression of BCMA. Although to date no notable association between baseline BCMA expression and clinical response to BCMA CAR T therapy has been reported in the published literature, several studies have observed a reduction in BCMA expression following therapy, which may be contributing to resistance\(^\text{122}\). The mechanism of BCMA downregulation in myeloma is not entirely understood, but this protein is shed naturally from the cell surface by the γ-secretase complex\(^\text{143–145}\). The resulting increased concentrations of this protein is shed naturally from the cell surface by the β-secretase, which may be contributing to resistance\(^\text{122}\). The mechanism of BCMA downregulation in myeloma is not entirely understood, but this protein is shed naturally from the cell surface by the γ-secretase complex\(^\text{143–145}\). The resulting increased concentrations of this protein is shed naturally from the cell surface by the β-secretase, which may be contributing to resistance\(^\text{122}\).

**CAR T cell therapy for solid tumors**

Although CAR T cells can mediate deep and durable cancer remission in B cell malignancies, achieving comparable clinical responses in non-hematopoietic solid cancers remains a daunting task. Nevertheless, a complete response to CAR T cell therapy of recurrent multifocal glioblastoma was achieved using multiple intracavitary and intraventricular infusions of autologous T cells genetically redirected to IL-13 receptor α2 (ref. \(^\text{148}\)), laying the foundation for additional investigations into how to apply effective CAR T cell therapy in this and other non-hematopoietic solid cancers\(^\text{5,6,149–151}\).

CAR T cell therapy trials have established that deep, durable remissions with CAR-engineered cells correlate with a minimal proportion of early memory T cells within the apheresis product used to generated the CAR T cells showed a correlation with early engraftment. Although prospective studies using selected subsets of T cells are necessary to confirm the role of these T cells in outcome, these data suggest that some resistance to therapy may be intrinsic to the T cell product.

### Table 1 | Summary of BCMA-targeted CAR structures

| Manufacturer | CAR name | Gene delivery system | Species of antigen-binding domain | Structure of antigen-binding domain | Hinge and transmembrane domain | Signaling domain | Safety switch |
|--------------|----------|----------------------|----------------------------------|-------------------------------------|--------------------------------|-----------------|--------------|
| National Cancer Institute | CAR-BCMA | Retroviral vector | Mouse scFv | CD8η | CD28-CD3ζ | No |
| bluebird bio/ Celgene | Idecabtagene vilcucel/ bb2121 | Lentiviral vector | Mouse scFv | CD8η | 4-1BB-CD3ζ | No |
| Hrain Biotechnology | BCMA CAR T | Retroviral vector | Mouse scFv | CD8η | 4-1BB-CD3ζ | EGFRt |
| Nanjing Legend/ Janssen | Ciltacabtagene autoleucel/ LCAR-B38M | Lentiviral vector | Alpaca VHII | CD8η | 4-1BB-CD3ζ | No |
| University of Pennsylvania | CART-BCMA | Lentiviral vector | Human scFv | CD8η | 4-1BB-CD3ζ | No |
| Memorial Sloan Kettering Cancer Center | MCARH171 | Retroviral vector | Human scFv | CD8η | 4-1BB-CD3ζ | EGFRt |
| Memorial Sloan Kettering Cancer Center | JCARH25 | Lentiviral vector | Human scFv | CD28 | 4-1BB-CD3ζ | No |
| Fred Hutchinson Cancer Research Center | FCARH143 | Lentiviral vector | Human scFv | NA | 4-1BB-CD3ζ | EGFRt |
| CARsgen Biotherapeutics | CT053 | Lentiviral vector | Human scFv | NA | 4-1BB-CD3ζ | No |
| IASO Biotherapeutics | CT103A | Lentiviral vector | Human scFv | CD8α | 4-1BB-CD3ζ | No |
| Poseida Therapeutics | P-BCMA-101 | piggyBac DNA modification system | Human Centyrin | NA | 4-1BB-CD3ζ | Yes (activated by rimiducid) |

\(^{\text{EgFRt, truncated epidermal growth factor receptor; VHH, variable heavy-chain domain; NA, not applicable.}}\)
| Manufacturer | Name of product | Clinical trial registered number | Year data updated | Number of patients evaluated | Enrollment based on BCMA expression | Number of lines of previous therapies | Disease burden at time of infusion | Conditioning therapy | Infusion dose | Efficacy | Reference(s) |
|--------------|----------------|----------------------------------|------------------|-------------------------------|------------------------------------|------------------------------------|-------------------------------|-------------------|----------------|----------|----------------|
| National Cancer Institute | CAR-BCMA | NCT02215967 | 2018 | 16 | Yes | Average=9.5 (range =3-19) | Relapsed/refractory cases with BCMA uniformly expressed on tumor cells, including extramedullary diseases, and >40% of patients carried high-risk cytogenetics | Cyclophosphamide and fludarabine | 9×10^6 CAR+ T cells per kg | Overall response rate (%) | 81 | Ref. 131 |
| bluebird/celgene/celgene | idecabtagene vicleucel/bb2121 | NCT02658929 | 2020 | 62 | Yes | Above 3 | 44% of relapsed/refractory cases had ≥50% bone marrow CD138⁺ plasma cells | Cyclophosphamide and fludarabine | 50×10^6, 150×10^6, 450×10^6 or 800×10^6 CAR+ T cells in total | 76 | 39 | 26 | 34.2 | 8.8 | Ref. 132 |
| bluebird/celgene | bb21217 | NCT03274219 | 2020 | 46 | Yes | Average=6 (range =3-17) | 57% of relapsed/refractory cases were triple refractory | Cyclophosphamide and fludarabine | 150×10^6, 300×10^6 or 450×10^6 CAR+ T cells in total | 55 | 18 | 30 | NA | NA | Ref. 133 |
| hrain biotechnology | BCMA CAR T | NCT03093168 | 2019 | 44 | No | Above 2 | 19.6% had extramedullary plasmacytoma | Cyclophosphamide and fludarabine | 9×10^6 CAR+ T cells per kg | Overall response rate (%) | 80 | 41 | 18 | Not reached | 15 | Ref. 134 |
| Nanjing legend/janssen | Ciltacabtagene autoleucel/ LCAR-B38M | NCT03090659 | 2018 | 57 | Yes | Average=3 (range =1-9) | 51% of relapsed/refractory cases had ≥40% tumor BCMA expression (patients with extramedullary involvement were included) and 37% of patients had stage III disease | Cyclophosphamide | 0.07×10^6–2.1×10^6 CAR+ T cells per kg | 88 | 68 | 5 | Not reached | 15 | Ref. 135 |
| Nanjing legend/janssen | Ciltacabtagene autoleucel/ LCAR-B38M | NCT03548207 | 2020 | 97 | No | Average=6 (range =3-18) | 87.6% of relapsed/refractory cases were triple refractory | Cyclophosphamide with or without fludarabine | 0.21×10^6–1.52×10^6 CAR+ T cells per kg | Overall response rate (%) | 88 | 76 | 12 | Not reached | 12 | Ref. 136 |
| Manufacturer | Name of product | Clinical trial registered number | Year data updated | Number of patients evaluated | Enrollment based on BCMA expression | Number of lines of previous therapies | Disease burden at time of infusion | Conditioning therapy | Infusion dose | Efficacy | Reference(s) |
|--------------|----------------|----------------------------------|-------------------|-----------------------------|------------------------------------|-------------------------------------|----------------------------------|-------------------|-------------|----------|--------------|
| University of Pennsylvania | CART-BCMA | NCT02546167 | 2019 | 25 | No | Average = 7 (range = 3-13) | A median of 65% had myeloma cells on bone marrow biopsy, 28% had extramedullary disease and 96% carried high-risk cytogenetics | Cyclophosphamide or no conditioning therapy | 10×10^6– 500×10^6 CAR-T cells in total | 48 | 25 | 20 | 17 | 2, 2 and 4 in three cohorts respectively | Ref. 11 |
| Memorial Sloan Kettering Cancer Center | M CARH171 | NCT03070327 | 2018 | 11 | Yes | Average = 6 (range = 4-14) | 82% had high-risk cytogenetics | Cyclophosphamide with or without fludarabine | 72×10^6, 137×10^6, 475×10^6 or 818×10^6 CAR-T cells in total | 64 | NA | NA | NA | Ref. 12 |
| Memorial Sloan Kettering Cancer Center | Orvactagene autoleucel/JCARH25 | NCT03430011 | 2018 | 8 | No | Average = 10 (range = 4-15) | 50% had high-risk cytogenetics | Cyclophosphamide and fludarabine | 50×10^6 or 150×10^6 CAR-T cells in total | 100 | 38 | 25 | NA | NA | Ref. 13 |
| Fred Hutchinson Cancer Research Center | F CARH43 | NCT03338972 | 2018 | 11 | Yes | Average = 8 (range = 6-10) | The median percentage of bone marrow plasma cells was 58% (range = 20% to >80%) and 100% had high-risk cytogenetics | Cyclophosphamide and fludarabine | 50×10^6 or 150×10^6 CAR-T cells in total | 100 | 36 | 46 | NA | NA | Ref. 14 |
| CARsgen Therapeutics | CT053 | NCT03716856, NCT03302403 and NCT03380039 | 2020 | 24 | Yes | Average = 4.5 (range = 2-11) | 41.7% had extramedullary involvement | Cyclophosphamide and fludarabine | 50×10^6, 100×10^6, 150×10^6 or 180×10^6 CAR-T cells in total | 88 | 79 | NA | NA | 18.8 | Ref. 15 |
| | | NCT03975907 | 2020 | 12 | No | Average = 6 (range = 3-7) | 14.2% had extramedullary disease and 35.7% had high-risk cytogenetics | Cyclophosphamide and fludarabine | 100×10^6 or 150×10^6 CAR-T cells in total | 100 | 42 | 25 | NA | NA | Ref. 15 |
| | | NCT03915184 | 2020 | 10 | No | Average = 6 (range = 3-10) | 93% were triple refractory, 36% had extramedullary disease and 64% had high-risk cytogenetics | Cyclophosphamide and fludarabine | 150×10^6– 300×10^6 CAR-T cells in total | 100 | 40 | 10 | NA | NA | Ref. 15 |
appropriate target antigens, these requirements include the need for CAR T cells to: (1) traffic to sites of disease; (2) migrate through tumor endothelial and stromal barriers before infiltrating into tumors; (3) broadly attack cancer cells in the face of heterogeneous antigen expression; and (4) thrive in a harsh tumor microenvironment (TME) characterized by hypoxia, oxidative stress, nutrient deprivation and acidic pH, as well as many immunosuppressive soluble cytokines and factors, overexpression of inhibitory molecules with coordinated expression of inhibitory receptors on T cells, and the presence of an array of immune cells with immunosuppressive function, including regulatory T cells, tumor-associated macrophages, myeloid-derived suppressor cells and tumor-associated neutrophils (Fig. 3). Ultimately, CAR T cell therapy may achieve greater efficacy in patients harboring solid tumors once approaches are developed that address each of these barriers together.

An expanding cadre of tumor-specific antigens and TAAs that could be targeted using CAR T cell therapy in non-hematopoietic solid cancers have been identified, including mesothelin, folate receptor alpha, human epidermal growth factor receptor 2 (HER2), IL-13 receptor α2, epidermal growth factor receptor variant III (EGFRvIII), claudin 18.2, mucin 1, cell-surface associated (MUC1), glypican-2, carbonic anhydrase IX and others. Nevertheless, identification of an antigen with restricted expression on solid cancer cells has been challenging. Ideally, CAR T cells should be highly specific for a tumor-restricted antigen, expressed uniformly and at high levels on cancer cells, but not on vital healthy tissue. The importance of antigen exclusivity was demonstrated in CAR T cell trials targeting TAAs such as HER2 and carbonic anhydrase IX, which are expressed by both cancer cells and normal tissues, and which resulted in severe toxicity. The need for consistent antigen expression was illustrated in a clinical trial targeting mutant EGFRvIII, a CAR target antigen with highly restricted but heterogeneous expression in glioblastoma. Although intravenous T cell infusion resulted in CAR T cell trafficking to the brain with accompanied antigen-directed activity against EGFRvIII+ cancer cells, the heterogeneous EGFRvIII expression and potential antigen loss resulted in outgrowth of antigen-negative disease. In some cases, targeting antigens with more restricted and uniform expression in tumors, or those preferentially expressed on organs that are not essential for patient survival, such as follicle-stimulating hormone receptor, may pave the way toward broader and safer antitumor activity. Nevertheless, heterogeneous TAA expression is common in solid tumors, highlighting the need to develop multi-antigen targeting approaches or strategies that improve epitope spreading and engagement of endogenous antitumor immunity. Evidence already exists for epitope spreading and bolstering of endogenous immunity in clinical trials and in preclinical models of CAR T cells in solid tumors, suggesting that antigen spreading may be necessary to improve activity. As an alternative approach to address both antigen heterogeneity and the threat of antigen loss, so-called universal immune receptors (UIRs) were created (reviewed in ref. 156). These CARs do not directly recognize the tumor antigen, but rather recognize a tag, such as biotin, on an antigen-targeted ligand (for example, an antibody or scFv fragment) that serves as an immunologic tag, such as biotin, on an antigen-targeted ligand (for example, an antibody or scFv fragment) that serves as an immunologic bridge between the CAR and TAA. UIRs allow the modified T cells to recognize multiple distinct TAAs simultaneously or sequentially, thus addressing both the heterogeneity and TAA loss observed with monospecific CARs, with the added benefit of dose-dependent control of T cell activity. Clinical trials of UIR T cells are ongoing (for example, NCT03680560, NCT03266692 and NCT03189836). Another approach, referred to as dual or tandem CARs, allows CAR T cells to recognize two or more distinct antigens rather than one. Proof of this principle has been established in solid tumor models using a HER2/MUC1 bispecific CAR for breast cancer cells in vitro, a HER2/IL-13 receptor α2 bispecific CAR for the treatment of a glioma xenograft in vivo, and an EGFR/epithelial cell
adhesion molecule/HER2 tri-specific against Raji lymphoma cells engineered to express these TAAs\(^\text{160}\). Alternatively, diversification of TAAs recognized by single CAR T cell products for solid tumor treatment may be achieved using SynNotch systems for the conditional expression of a second CAR following engagement of a primary CAR with a cognate TAA, thereby allowing for potential localized expression of a CAR specific for a distinct antigen at the site of primary target encounter\(^\text{161}\). An alternative approach would be through bicistronic vectors for the engineered co-expression of a CAR specific for one antigen and a soluble bispecific T cell engager specific for a second antigen\(^\text{162}\).

Although for hematopoietic cancers intravenous infusion of CAR T cells may target cancer cells in natural immune cell environments such as the blood, lymph nodes and bone marrow, it remains challenging to deliver CART T cells targeting solid tumors to distant tumor deposits. In some cases, direct intratumoral or regional delivery of T cells may facilitate and improve T cell infiltration and antitumor activity, particularly for compartmentalized cancers\(^\text{163,164}\). Lymphodepleting chemotherapy as a preconditioning regimen may also augment CAR T cell accumulation in solid tumors after intravenous infusion. Following intravenous administration of indium-111-labeled tumor-infiltrating lymphocytes to patients with metastatic melanoma, the cells rapidly accumulated in the lungs, liver and spleen before progressively localizing in tumor deposits\(^\text{165}\). In these trials, tumor-infiltrating lymphocyte accumulation was enhanced with previous lymphodepletion and associated with an improved clinical response to treatment\(^\text{166,167}\). Still, the natural trafficking of T cells to tumors requires that they respond to chemokines produced in the TME\(^\text{168}\), and that tumor-derived chemokines be matched to the expression of the appropriate chemokine receptors on the infused T cells to permit trafficking\(^\text{169}\). Although most CAR T cells do not naturally express cognate receptors for the chemokines produced by tumors, it is possible to engineer matched chemokine receptor expression to achieve enhanced infiltration and killing of solid tumors\(^\text{169–171}\). CAR T cells may also be outfitted to produce chemokine ligands, such as CCL19 and other factors, to foster chemokine receptor-dependent recruitment of endogenous T cells and dendritic cells to tumor sites when infused without previous lymphodepletion\(^\text{172}\).

CAR T cells trafficking to solid tumor sites also encounter formidable physical barriers that can both block T cell infiltration and disable T cell function. Major barriers include the fibrotic tumor stroma, comprised of extracellular matrix (ECM) and tumor-associated fibroblasts (CAFs), and the abnormal vasculature at the tumor site. Solid malignancies, such as pancreatic, ovarian and breast cancers, often contain fibrotic tumor stroma that may impede entry into solid tumors\(^\text{172–174}\). Thus, engineering CARs that specifically bind to vascular endothelial growth factor receptor 2 using CARs can augment T cell infiltration and enable selective targeting of tumor endothelium\(^\text{174}\), and CARs targeting the angiogenic integrin α\(_\text{v}\)β\(_\text{3}\) on the vascular endothelium can disrupt tumor vessels and suppress tumor outgrowth\(^\text{175}\). CAR T cells may also be combined with antivasculature agents, including antivascular endothelial growth factor or prostaglandin E\(_\text{2}\) antibodies\(^\text{176}\), antitumor endothelial marker 1/endoxin immunotoxin\(^\text{177}\) or agents targeting molecules on the tumor endothelium, such as Fas ligand, which establishes a tumor endothelial death barrier and kills incoming effector CD8 T cells\(^\text{178}\). Together, these findings provide rationale for further investigation and the use of stroma-disrupting strategies as both preparative and combinatorial regimens to augment T cell entry into solid tumors in TAA-targeted CAR T cell trials.

In the stroma and tumor bed, CAR T cells contend with overexpression of inhibitory checkpoint ligands with coordinated expression of inhibitory receptors on T cells, immunosuppressive soluble cytokines and factors, various immunosuppressive cell types and a hypoxic and nutrient-deprived environment. Both tumor cells and immune cells in the TME can regulate CAR T cell activation through the expression of inhibitory signals that block T lymphocyte activation and function, thereby circumventing otherwise effective immune control of tumor progression.

### Future prospects

Over the past decade, an astounding series of proof-of-concept trials have taken place, with validation of early results in phase II trials\(^\text{179–181}\) leading to the approval of CD19-specific CAR T cell therapies for select B cell malignancies. Separately, insight into the biology of CRS has led to biomarker-driven trials (NCT02906371) and the discovery and validation of a novel biomarker profile of this potentially lethal toxicity\(^\text{181}\). Additional observations from routine and translational studies have revealed mechanisms of resistance and response, as well as identification of the natural basis of successful and failed CAR T cell therapy\(^\text{182,183}\). Novel therapies started to incorporate small molecules, which proved to augment T cell function and simultaneously inhibit the malignant population\(^\text{184–186}\). Combination trials also targeted more than one surface protein, either on the same target cell (as with CD19 and CD22) or on precursors and progeny of the tumor (as with CD19 and CD20, CD22 or BCMA)\(^\text{187,188}\). In the next few years, we are likely to witness increased efficacy of CAR T cells for solid tumors—a major current focus in this field. However, a better understanding and monitoring of the tumor will be essential for CAR T cell therapy to be offered to patients in the early stages of their disease, before genomic instability and evolution of the tumor complicate treatment.

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Author contributions
J.J.M. developed the concept of the paper. All authors contributed to the first draft of the manuscript and approved the final version.

Competing interests
J.J.M., D.J.P. Jr and M.C.M. are inventors on several patents issued and pending in the field of CAR T cell therapy for cancer, which are assigned to the University of Pennsylvania. Under the University of Pennsylvania’s policies, J.J.M., D.J.P. Jr and M.C.M. either currently, or may in the future, receive royalties from the licensing of these patent rights. M.C.M. is an inventor on issued and pending patents related to CAR technology, and receives royalties from the licensing of this IP. He is also a founder and equity holder in Calabatta Bio, J.X., S.-J.C. and M.A.C. have no conflicts of interest. J.Z. receives research funding from and is chairman of the Medical and Scientific Advisory Board of IASO Biotherapeutics. D.J.P. Jr is or has provided consultation for Iovance Biotherapeutics, Bellicum Pharmaceuticals, Neon Therapeutics, and Tmunity Therapeutics, and holds patents in the areas of tumor-infiltrating lymphocytes and gene-engineered T cells. J.J.M. receives research funding from IASO Biotherapeutics, consult for Sincere of America, Shanghai Unicar Therapy, Johnson & Johnson, Poseida, and IASO Biotherapeutics, and is on the Medical and Scientific Advisory Board of IASO Biotherapeutics and Poseida Therapeutics. J.J.M. further holds patents related to CAR T cell manufacturing and biomarkers.

Additional information
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