Published in final edited form as:

Curr Opin Oncol. 2015 November ; 27(6): 466–474. doi:10.1097/CCO.0000000000000232.

Smart CARs Engineered for Cancer Immunotherapy

Saul J. Priceman¹, Stephen J. Forman¹, and Christine E. Brown^{1,2}

¹Departments of Cancer Immunotherapy & Tumor Immunology and Hematology & Hematopoietic Cell Transplantation, Beckman Research Institute at City of Hope National Medical Center, Duarte, CA 91010.

Abstract

Purpose—Chimeric antigen receptors (CARs) are synthetic immunoreceptors that can redirect T cells to selectively kill tumor cells, and as “living-drugs” have the potential to generate long-term anti-tumor immunity. Given their recent clinical successes for the treatment of refractory B-cell malignancies, there is a strong push toward advancing this immunotherapy to other hematological diseases and solid cancers. Here, we summarize the current state of the field, highlighting key variables for the optimal application of CAR T cells for cancer immunotherapy.

Recent Findings—Advances in CAR T cell therapy have highlighted intrinsic CAR design and T cell manufacturing methods as critical components for maximal therapeutic success. Similarly, addressing the unique extrinsic challenges of each tumor type, including overcoming the immunosuppressive tumor microenvironment and tumor heterogeneity, as well as mitigating potential toxicity, will dominate the next wave of CAR T cell development.

Summary—CAR T cell therapeutic optimization, including intrinsic and extrinsic factors, is critical to developing effective CAR T cell therapies for cancer. The excitement of CAR T cell immunotherapy has just begun, and will continue with new insights revealed in laboratory research and in ongoing clinical investigations.

Keywords

Chimeric antigen receptor (CAR); T cell; immunotherapy

Introduction

Adoptive T cell immunotherapy has risen to the forefront of treatment approaches for cancer. In particular, T cells engineered to express chimeric antigen receptors (CARs) have demonstrated impressive clinical efficacy with significant improvements in patient outcomes for a number of hematological cancers (1-5). Expanding the use of CAR T cell therapy to solid cancers is proving less tractable and is an area of intense research. Enhancements in current CAR T cell design are under active investigation, with preclinical studies and retrospective analyses of clinical trials aimed at broadening their utility for multiple cancer

²**Correspondence:** C.E.B., Departments of CITI and Hem/HCT, City of Hope, 1500 E. Duarte Rd., Duarte, California 91010; cbrown@coh.org.; phone: (626) 256-4673.

Conflicts of interest: None

types. CAR T cell optimization can be broken down into several components: 1) the CAR design, 2) the T cell population and *ex vivo* expansion methods, 3) the tumor microenvironment, and 4) safety considerations (**Figure 1**). Addressing each of these components will be critical to unleash the full potential of CAR T cells. This review covers the status of CAR T cell therapy, discussing both preclinical and clinical studies that shape our up-to-date knowledge and future prospects for this exciting immunotherapy approach.

Mechanics of CAR T-Cell Engineering

The early embodiments of CAR T cell therapy evaluated highly differentiated *ex vivo*-expanded T cell products engineered to express ‘first-generation’ CARs that incorporated the intracellular activating CD3 ζ domain, but lacked co-stimulatory signaling. These first-generation products demonstrated that CARs could exert potent redirected HLA-independent cytotoxic activity, and thereby confer novel T cell-mediated anti-tumor immunity. Patient therapeutic responses, however, were limited, primarily due to suboptimal persistence of the adoptively transferred cells (6-8). Next-generation CARs have focused on refining T cells for improved persistence, expansion and optimal anti-tumor activity. Below, we will summarize recent advancements in optimizing the therapeutic product through modified CAR designs and T cell expansion protocols.

Advances in CAR Design

CARs are modular synthetic immunoreceptors consisting of three major functional components—the antigen-binding domain, the extracellular linker/spacer, and the intracellular signaling domain (**Figure 1**). Together these building blocks recapitulate native T cell function, including antigen-dependent cytokine production, proliferation and serial tumor cell killing. While many of the refinements in CAR design have been determined empirically, general principles are emerging as greater panels of CARs are generated and compared side-by-side in preclinical studies and in patients.

The antigen-binding domain is most commonly composed of a single-chain variable fragment (scFv) derived from a monoclonal antibody, or in certain instances a receptor ligand. CARs directly recognize cell surface antigens in an MHC-independent fashion, rendering them universal for all patients and insensitive to tumor escape by MHC down-regulation. While the antigen-binding domain determines the overall specificity of the CAR and is important for predicting the potential for off-tumor toxicities, questions remain regarding optimal CAR binding affinity. Several groups report that, similar to the T cell receptor (TCR) (9), CAR antigen binding requires a minimum affinity for T cell activation, beyond which T cell function is not significantly enhanced (10-12). Modulating CAR affinity, however, may be a strategy to fine-tune the level of antigen expression required for T cell activation (10, 12, 13), enhancing tumor selectivity for over-expressed self-antigens. In addition to antigen-binding affinity, epitope location may also be an important parameter for an optimized antigen-binding domain. For instance, studies evaluating CD22-specific CARs find that scFv binding to membrane-proximal epitopes improves CAR T cell effector function and *in vivo* antitumor activity as compared to scFvs that recognize membrane-distal epitopes (14, 15). While the majority of CARs developed to date target extracellular antigens, with recent successes in generating high-affinity antibodies to specific peptide-

MHC complexes, targeting intracellular antigens with CAR T cells is now feasible, thus broadening the pool of potential tumor-associated antigen targets (16).

The intracellular signaling domain has been extensively evaluated both preclinically and clinically and can greatly impact the functional activity of CARs. A major advancement in 'first-generation' CAR design was achieved by addition of a co-stimulatory signal engineered in series with the CD3 ζ activation domain [reviewed in (17)]. These 'second-generation' CARs typically incorporate the intracellular costimulatory domain of CD28 or 4-1BB, enhancing CAR T cell function via increased cytokine production, T cell proliferation and killing in the setting of recursive exposure to antigen (18-21). This translates to more durable tumor regression in xenograft models with significantly improved T cell survival. While CD19-CARs incorporating either CD28 or 4-1BB costimulation mediate remarkable clinical responses against hematological malignancies (1-5), 4-1BB-CARs persist longer in patients compared to CD28-containing CARs (3-5). Current thinking postulates that while CD28 costimulation appears to yield greater potency, higher cytokine secretion, and earlier killing activity, the slower activation of 4-1BB yields longer persistence and therefore more durable tumor control (17). Multiple alternative costimulatory domains have been investigated preclinically, [OX40 (22), ICOS (23), NKG2D (24) and CD27 (25)], and each is unique with respect to activation and persistence, for both CD4 and CD8 T cells. 'Third-generation' CARs encompass two or more co-stimulatory domains in *cis*, and in certain preclinical settings have shown benefit compared to second-generation CARs (19, 26, 27). However, overstimulation of T cells through strong CAR signaling may result in deleterious effects on overall CAR T cell function and/or promote activation-induced cell death (27, 28). Therefore, fine-tuning CAR signaling is a critical design component, and outstanding questions remain regarding the optimal co-stimulatory domain and how it impacts overall CAR T cell function.

Often under-appreciated components of CAR design are the extracellular linker/spacer and the transmembrane domain, the segments that connect the antigen-binding and the intracellular signaling domains. This region determines the proximity and flexibility for CAR-antigen immunological synapse formation, the propensity for CAR dimerization, as well as overall CAR stability. Importantly, several studies comparing CAR spacers suggest that one-size does not fit all, and that optimal spacer length for each antigen-CAR combination requires empirical determination (28-30). Commonly incorporated CAR spacers range from the relatively long IgG1- or IgG4-Fc domains, to shorter hinge regions of CD8 α or CD28. Recent reports have identified unintended interactions of the commonly used IgG-Fc spacer domains with Fc gamma receptors (Fc γ Rs) expressed by innate immune cells, which results in rapid elimination of the adoptively transferred CAR-expressing cells in NSG mouse models (31-33). To overcome this issue, deletions or mutations have been engineered into the IgG-Fc spacer domain to block interactions with Fc-receptors (31-33), and these CAR designs are currently being clinically evaluated. Although few studies have addressed the contribution of the transmembrane domain, it likely plays an important role in expression stability and function of the CAR (34). Further studies are warranted to elucidate the full impact of these structural components in CAR design.

The integration of these CAR structural components and their critical effects on T cell function are an activate area of investigation. Antigen-binding, extracellular spacer and intracellular signaling domains cooperatively modulate the effectiveness and durability of CAR T cell-mediated anti-tumor immunity. Examples of this interplay include recent reports that constitutive CAR signaling, mediated through scFv fragment aggregation in the absence of antigen, results in T cell exhaustion and inferior *in vivo* antitumor efficacy (35, 36). These exhaustion-related adverse effects are enhanced by CD28 costimulatory signaling, but reduced with 4-1BB (35). Additionally, scFvs targeting membrane-proximal epitopes may benefit from longer spacer regions, which would not be necessary for membrane-distal epitopes (29), again suggesting interdependence of CAR domains. It has also been suggested that the ability of the CAR to engage associated molecules within the endogenous TCR complex is required for optimal CAR function (37). Thus, a detailed understanding of the integration of these structural components is crucial for optimal CAR design, and is an area of intense investigation.

T cell Subsets and Ex Vivo Expansion Methods

An equally critical design component impacting therapeutic efficacy is the final T cell phenotype of the manufactured product, which is influenced by both the starting population for genetic engineering and the *ex vivo* expansion methods [reviewed in (38)]. Preclinical studies evaluating the optimal T cell subtype for adoptive therapy – differentiated terminal effectors versus less-differentiated naïve/memory subsets – have converged on the paradoxical finding that the most effective T cell product *in vivo* inversely correlates with T cell effector phenotype and *in vitro* cytotoxicity potential. Instead, less-differentiated naïve (T_N), stem memory (T_{SCM}) and central memory (T_{CM}) T cells, defined by expression of lymphoid homing receptors CCR7 and CD62L, mediate superior *in vivo* persistence and anti-tumor activity compared to more differentiated effector memory (T_{EM}) and short-lived effector (T_{EFF}) T cells [reviewed in (39)]. This has been established in syngeneic mouse models, with human T cells in xenograft mouse models, and in non-human primate studies (40-44). Indeed, retrospective analyses from adoptive T cell clinical trials find that the frequency of less-differentiated memory T cells in the infused product, either T_{CM} (1, 45) or T_{SCM} (45, 46), positively correlates with T cell persistence and clinical outcome.

Strategies for manufacturing less-differentiated T cells to enhance the efficacy of CAR therapy continue to evolve. Since *ex vivo* T cell expansion inevitably drives T cell differentiation, limiting culture time yields younger and more potent T cell products (47). In addition, manufacturing platform variations related to T cell activation strategies, cytokine conditions, and CAR design (35, 36) critically define the immunophenotype and overall functional potency of the final CAR T cell product. An integral step in *ex vivo* T cell expansion is TCR activation, with several platforms currently employed for production of clinical-grade T cell products, including soluble anti-CD3 antibody (OKT3) in the presence of IL-2 cytokine (2), OKT3 plus CD28 antibody co-stimulation (1, 3-5), and co-culture with antigen-presenting cells (48) (**Figure 1**). While the use of OKT3 in the presence of IL-2 has demonstrated utility in expanding CD8⁺ T cells over other activation methods (49), a recent study demonstrates that OKT3/CD28 magnetic bead stimulation produces T cells with a younger phenotype that outperforms OKT3/IL-2 expanded cells in pre-clinical tumor

models (50). Clinical validation for the therapeutic potential of CAR T cell products manufactured using OKT3/CD28 bead stimulation has been extensively established for CD19-CARs (1, 3-5). Inclusion of γ c cytokines during *ex vivo* manufacturing, including IL-2, IL-7, IL-15 and/or IL-21, promotes extended expansion of the engineered T cells [reviewed in (51)]. Historically, IL-2 has been utilized during the expansion process, however, it is well-established that IL-2 promotes T cell differentiation. Several recent studies demonstrate that cell products manufactured using alternative cytokine cocktails, including IL-7 and/or IL-15, maintain a more stem/central memory-like phenotype and display improved persistence and anti-tumor activity (44, 46). Support with IL-21 also limits differentiation and improves memory CD8⁺ T cell yield (52, 53), showing promising clinical responses (54). Additionally, the use of pharmacological immune modulators to perturb differentiation pathways is under investigation, including inhibition of glycolysis (55), induction of Wnt/ β -catenin signaling (56), blockade of AKT (57), and combinations thereof (58) for improved adoptive T cell therapy.

Another approach to produce less-differentiated T cell products is the selective enrichment of specific T cell subsets with high self-renewal potential for CAR engineering. The majority of T cell products manufactured for clinical application utilize unselected peripheral blood mononuclear cells, where the frequency of memory T cell subsets, as well as the CD4:CD8 ratios can vary greatly from patient to patient. This variability could explain some differences in clinical responses between patients. Good manufacturing practices (GMP) platforms using magnetic bead isolation have been established to enrich defined central memory T cell subsets (41, 59), or to standardize CD4:CD8 ratios (60), with the intent of overcoming this inherent variability. Other less frequent T cell subsets, such as IL-17-producing T cells (Th17 and Tc17), are also being explored based on their stem-like memory characteristics and show promising therapeutic potential (23, 61, 62). Further, the use of virus-specific memory T cells with known antigen specificity for CAR-engineering may provide protection against viral reactivation, and can be combined with a vaccination approach for specific expansion of CAR T cells either *in vitro* or in patients (63, 64). As the field explores these rarer T cell populations for CAR therapy, including T_{SCM} (40, 44), Th17 (61), and virus-specific T cells (59), or attempts to generate large banks of allogeneic T cells for an ‘off-the-shelf’ product (65, 66), developing long-term expansion conditions that maintain a ‘younger’ T cell phenotype becomes critical. Furthermore, as CAR T cell therapy moves towards commercialization, these platforms need to become more streamlined, cost-effective, reproducible, and amenable to clinical translation under GMP.

Challenges for Clinical Translation of CAR Therapy

Thus far, the most responsive clinical setting for CAR T cell therapy has been acute lymphoblastic leukemia (ALL), with complete response rates reported for greater than 70% of patients treated with CD19-CARs, independent of patient age, disease burden or prior treatments, and importantly, across multiple institutions and CAR platforms (3-5). While the overall successes of CD19-CARs for other B-cell malignancies, including chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma (NHL), are promising, they have not quite reached the level of clinical responses seen for ALL (1, 2, 67). Additionally, while clinical trials using HER2-CAR T cells for sarcoma (68), GD2-CAR T cells for

neuroblastoma (45), and IL13R α 2-CAR T cells for glioblastoma (69) hint at the therapeutic opportunities of CAR T cells for non-hematological solid cancers, these responses do not approach those of CD19-CAR T cells. Variations in overall response rates have highlighted critical aspects of the tumor microenvironment that exist in solid cancers, and even under the umbrella of B-cell malignancies. Therefore, beyond the aforementioned CAR T cell intrinsic factors, addressing the extrinsic factors, including effective tumor penetration of T cells, the immunosuppressive tumor microenvironment, and tumor heterogeneity will be required to move beyond the current successes with CD19-CARs for ALL.

Effective tumor trafficking and penetration of T cells is critical for the success of adoptive T cell therapy, and is influenced by T cell delivery route and the tumor microenvironment. The most common route of administration is intravenous (i.v.), with established efficacy against B-cell malignancies. An outstanding question remains as to whether non-systemic routes of delivery may elicit more direct and durable therapy for solid tumors. For instance, intracranial delivery of CAR T cells has shown safety and bioactivity in patients with glioblastoma (69), and intraperitoneal delivery of CAR T cells for direct targeting of ovarian cancers and mesotheliomas demonstrates effectiveness in preclinical models (19, 70). Recently, Adusumilli *et al.* (71) show that intrapleural CAR T cell administration outperforms i.v. delivery in preclinical mesothelioma models, yielding impressive CAR potency and persistence, and providing systemic anti-tumor immunity in a surprising CD4-dependent manner. The frequency of CAR T cell administration may also contribute to overall efficacy, where one may envision that a staggered accumulation of T cells in tumors by repeat infusion provides more durable therapy; a strategy that has been clinically evaluated by several groups (68, 69, 72). It is well established that in solid cancers, T cell accumulation and migration is hampered by interstitial pressure and by the hostile tumor microenvironment. Heparanase has recently been implicated in modifying the extracellular matrix in tumors to allow for optimal T cell infiltration and anti-tumor activity, which is transcriptionally down-regulated in *ex vivo* cultured T cells (73). T cells engineered to express heparanase effectively infiltrate tumors and demonstrate potent anti-tumor activity in preclinical solid cancer models (73). Migration and invasion of adoptively transferred T cells is also regulated by various chemokines. For instance, tumor-derived C-C motif ligand-2 (CCL2) (74) and forced T-cell expression of its receptor, CCR2 (75), promote tumor infiltration and increased functionality of CAR T cells. The continued development of strategies to augment CAR T cell trafficking and infiltration of tumors will advance adoptive T cell therapy application, particularly for solid cancers.

The immunosuppressive tumor microenvironment is also an area of intense focus, which can prevent effective infiltration of T cells, as well as suppress their survival and function once inside the tumor. CAR design modifications, including co-stimulatory signaling, may provide some protection against the immunosuppressive microenvironment, as CD28-containing CARs demonstrate resistance to CTLA-4-mediated inhibition in preclinical models of B-cell malignancies (76). Several groups have generated CAR T cells engineered to secrete cytokines, such as IL-12 (77-79), which can stimulate T cell-mediated immune responses in the local tumor microenvironment. Likewise, Curran *et al.* (80) show that constitutive expression of CD40L in T cells supports endogenous immune responses and

improves functionality of CD19-CAR T cells in lymphoma models. Combinatorial immunotherapy strategies are also emerging as promising approaches to overcome tumor-mediated immunosuppression and improve adoptive T cell therapy, including the potential for PD-1 checkpoint inhibitors to augment CAR T cell therapy (81), or inhibition of molecular pathways that drive immunosuppression in solid cancers [reviewed in (82)]. For example, tumor expression of IDO, an enzyme that depletes tryptophan, severely impedes survival and expansion of CD19-CAR T cells (83). Likewise, the transcription factor STAT3 (84, 85), along with one of its target genes, Arginase (86), can greatly hamper adoptive T cell therapy, suggesting that specific inhibitors of these suppressive pathways may be important strategies to boost CAR T cell therapy. Lessons learned from other cellular immunotherapies have spurred new approaches towards releasing the immunosuppressive brakes to enhance adoptive CAR T cell-mediated anti-tumor immunity.

Another major challenge for CAR immunotherapy is the heterogeneous expression of tumor antigens. In the CD19-CAR clinical trials to date, disease recurrence of CD19-negative or epitope-mutant tumors has diminished remission duration (4, 5, 87). For solid cancers, tumor heterogeneity and antigen escape are major drivers in tumor evasion of immunotherapy (88, 89), and indeed outgrowth of antigen negative/low tumors has been demonstrated in early clinical studies with IL-13R α 2-specific CAR T cells for advanced glioma (69). Several strategies have been incorporated into CAR design to improve durability of therapy in the presence of tumor heterogeneity. Dual-targeting CARs enhance the activation of T cells against tumors that express multiple tumor antigens, which has been demonstrated with IL-13R α 2 and HER2 for gliomas (90), HER2 and MUC1 for breast cancers (91), and PSMA and PSMA for prostate cancers (92). Additionally, unleashing an endogenous immune response during adoptive T cell therapy could promote antigen spreading to other tumor-specific neo-antigens, and thus reduce the potential for antigen escape (93, 94). Multi-targeting CARs hold promise for a more personalized medicine approach, as new targets are identified, new CARs are validated for safety and efficacy in the clinic, and novel patient screening procedures come online.

While enhancing potency and durability of CAR T cell therapy has dominated recent investigations, safety considerations in the clinic are equally important. Of the serious toxicities associated with CD19-CAR T cell therapy, the most common is cytokine release syndrome, which in its most severe form is commonly managed by using the anti-IL6 receptor antibody tocilizumab with or without corticosteroids (95). Off-tumor toxicity has not been a significant issue for CD19-CARs that also target normal B cells, as B cell aplasia is clinically manageable, and often used a surrogate marker for CD19-CAR T cell functional duration (1-5). One of the biggest challenges for translating this approach to other cancers has been the identification of amenable antigens for immunological targeting (96), as many cancer antigens have normal tissue expression that may cause on-target off-tumor toxicity (97). The immunogenicity of non-physiological CAR components is also an issue for safety and CAR persistence (72). Humanizing scFvs is an important optimization step in CAR development to minimize potential anti-CAR immunogenicity (12, 13). Other CAR-intrinsic strategies have been evaluated to address safety concerns with CAR T cells. One approach has been to transiently express CARs in T cells using non-viral methods to ensure limited persistence of the CAR-expressing T cell product (98-100). While this approach requires

repeat infusions of CAR T cells for clinical efficacy, the short lifespan of the engineered product may mitigate unmanageable toxicities.

A more direct approach to blunting undesirable side effects is incorporation of suicide genes in CAR T cells to provide a safety net when targeting tumor antigens that may also have low, but detectable, normal tissue expression. Examples include addition of iCaspase 9 for dimerization-induced apoptosis that has been shown to rapidly eliminate modified T cells in patients (101), or engineering T cells to express specific cell surface antigens for immunologic targeting such as the truncated epidermal growth factor (EGF) receptor that has been shown to render modified T cells susceptible to anti-EGFR antibody (i.e. Erbitux) elimination in mice (102). Other strategies are being evaluated in the preclinical setting, including dissociating CAR components for improved selectivity of tumors (91, 92, 103), incorporating inhibitory CARs (iCARs) to reduce on-target off-tumor toxicity (104), and potentially driving expression of CAR with regulated promoters to improve tumor selectivity. The incorporation of safety switches will be important additions to CAR designs as optimizations aim to improve the potency and durability of this therapy.

Conclusions

While the excitement of CAR T cells has spread across the immunotherapy world, there are considerable unknowns in translating this therapy beyond CD19, to treat other hematological malignancies and solid cancers. Enthusiasm for adoptive T cell therapy in the treatment of advanced cancers is producing tremendous efforts to address the challenges of CAR T cell potency, persistence, patient safety, the immunosuppressive tumor microenvironment, and tumor heterogeneity. Effectively translating this technology beyond CD19-CARs will likely require sophisticated optimization and engineering innovations of CAR T cell products to tackle each of these abovementioned hurdles. Preclinical studies and clinical investigations are currently underway to address many of these challenges in the field, and will likely continue to invigorate the development of CAR-based immunotherapies for a wide spectrum of malignancies.

Acknowledgements

We greatly thank Dr. Sandra Thomas for illustration generation, manuscript editing and scientific feedback; Dr. Elizabeth Budde and Dr. Monique Dao for scientific feedback and manuscript review. This work was supported by the California Institute for Regenerative Medicine (CIRM) grant TR3-05641, NCI Lymphoma SPORE grant P50 CA107399-08, and Prostate Cancer Foundation Challenge Award grant. The authors of this publication have a license agreement with and receive research support from Mustang Therapeutics Inc in the area of CAR-engineered T cell therapy. The terms of this arrangement have been reviewed and approved by City of Hope in accordance with its conflict of interest and commitment policy.

Disclosure of Funding: This work was supported by the California Institute for Regenerative Medicine (CIRM) grant TR3-05641, NCI Lymphoma SPORE grant P50 CA107399-08, and Prostate Cancer Foundation Challenge Award grant.

References

1. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med.* 2011; 3(95):95ra73.

2. Kochenderfer JN, Dudley ME, Kassim SH, Somerville RP, Carpenter RO, Stetler-Stevenson M, et al. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2015; 33(6):540–9. [PubMed: 25154820]
- 3**. Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K, et al. Efficacy and Toxicity Management of 19-28z CAR T Cell Therapy in B Cell Acute Lymphoblastic Leukemia. *Sci Transl Med*. 2014; 6(224):224ra25. [Clinical study showing an overall complete response rate of 88% in refractory ALL patients using CD19-28z CAR T cells, with clarification on sCRS toxicity management with IL-6 receptor blockade.]
- 4**. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *The New England journal of medicine*. 2014; 371(16):1507–17. [PubMed: 25317870] [Clinical study in pediatric ALL reporting a 90% complete response rate for refractory disease following autologous CD19-CAR T cell administration with 4-1BB co-stimulation, with sustained remissions associated with CAR T cell persistence.]
- 5**. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet*. 2015; 385:517–28. [PubMed: 25319501] [Clinical study in pediatric ALL reporting 70% complete response rates for refractory disease following administration of T cells engineered with a CD19-CAR containing a CD28-costimulatory domain.]
6. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, et al. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2006; 24(13):e20–2. [PubMed: 16648493]
7. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res*. 2006; 12(20 Pt 1):6106–15. [PubMed: 17062687]
8. Jensen MC, Popplewell L, Cooper LJ, DiGiusto D, Kalos M, Ostberg JR, et al. Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans. *Biol Blood Marrow Transplant*. 2010; 16(9):1245–56. [PubMed: 20304086]
9. Schmid DA, Irving MB, Posevitz V, Hebeisen M, Posevitz-Fejfar A, Sarria JC, et al. Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. *J Immunol*. 2010; 184(9):4936–46. [PubMed: 20351194]
10. Chmielewski M, Hombach AA, Abken H. CD28 cosignalling does not affect the activation threshold in a chimeric antigen receptor-redirected T-cell attack. *Gene therapy*. 2011; 18(1):62–72. [PubMed: 20944680]
- 11*. Textor A, Listopad JJ, Wuhrmann LL, Perez C, Kruschinski A, Chmielewski M, et al. Efficacy of CAR T-cell therapy in large tumors relies upon stromal targeting by IFN γ . *Cancer research*. 2014; 74(23):6796–805. [PubMed: 25297631] [This study evaluated the capacity of HER2-CAR T cells to reject large established tumors, demonstrating IFN γ mediated destruction of tumor stroma is required for full potential of CAR T cell therapy.]
12. Song DG, Ye Q, Poussin M, Liu L, Figini M, Powell DJ Jr. A fully human chimeric antigen receptor with potent activity against cancer cells but reduced risk for off-tumor toxicity. *Oncotarget*. 2015 Epub 2015/06/19.
13. Johnson LA, Scholler J, Ohkuri T, Kosaka A, Patel PR, McGettigan SE, et al. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. *Sci Transl Med*. 2015; 7(275):275ra22.
14. Haso W, Lee DW, Shah NN, Stetler-Stevenson M, Yuan CM, Pastan IH, et al. Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. *Blood*. 2013; 121(7):1165–74. [PubMed: 23243285]

15. James SE, Greenberg PD, Jensen MC, Lin Y, Wang J, Till BG, et al. Antigen sensitivity of CD22-specific chimeric TCR is modulated by target epitope distance from the cell membrane. *J Immunol.* 2008; 180(10):7028–38. [PubMed: 18453625]
16. Oren R, Hod-Marco M, Haus-Cohen M, Thomas S, Blat D, Duvshani N, et al. Functional comparison of engineered T cells carrying a native TCR versus TCR-like antibody-based chimeric antigen receptors indicates affinity/avidity thresholds. *J Immunol.* 2014; 193(11):5733–43. [PubMed: 25362181]
17. van der Stegen SJ, Hamieh M, Sadelain M. The pharmacology of second-generation chimeric antigen receptors. *Nature reviews Drug discovery.* 2015; 14(7):499–509. [PubMed: 26129802]
18. Brentjens RJ, Latouche JB, Santos E, Marti F, Gong MC, Lyddane C, et al. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat Med.* 2003; 9(3):279–86. [PubMed: 12579196]
19. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhali M, Suhoski MM, et al. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *PNAS.* 2009; 106(9):3360–5. [PubMed: 19211796]
20. Milone MC, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Mol Ther.* 2009; 17(8):1453–64. [PubMed: 19384291]
21. Finney HM, Akbar AN, Lawson AD. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. *J Immunol.* 2004; 172(1):104–13. [PubMed: 14688315]
22. Hombach AA, Heiders J, Foppe M, Chmielewski M, Abken H. OX40 costimulation by a chimeric antigen receptor abrogates CD28 and IL-2 induced IL-10 secretion by redirected CD4(+) T cells. *Oncoimmunology.* 2012; 1(4):458–66. [PubMed: 22754764]
- 23*. Guedan S, Chen X, Madar A, Carpenito C, McGettigan SE, Frigault MJ, et al. ICOS-based chimeric antigen receptors program bipolar TH17/TH1 cells. *Blood.* 2014; 124(7):1070–80. [PubMed: 24986688] [This elegant study demonstrates ICOS co-stimulation in CAR T cells promoting IL-17-producing Th17 subsets with improved persistence and anti-tumor efficacy compared with CD28 and 4-1BB.]
24. Song DG, Ye Q, Santoro S, Fang C, Best A, Powell DJ Jr. Chimeric NKG2D CAR-expressing T cell-mediated attack of human ovarian cancer is enhanced by histone deacetylase inhibition. *Human gene therapy.* 2013; 24(3):295–305. [PubMed: 23297870]
25. Song DG, Ye Q, Poussin M, Harms GM, Figini M, Powell DJ Jr. CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo. *Blood.* 2012; 119(3):696–706. [PubMed: 22117050]
26. Zhong XS, Matsushita M, Plotkin J, Riviere I, Sadelain M. Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI3kinase/AKT/Bcl-XL activation and CD8+ T cell-mediated tumor eradication. *Mol Ther.* 2010; 18(2):413–20. [PubMed: 19773745]
27. Hombach AA, Rappl G, Abken H. Arming cytokine-induced killer cells with chimeric antigen receptors: CD28 outperforms combined CD28-OX40 “super-stimulation”. *Mol Ther.* 2013; 21(12):2268–77. [PubMed: 23985696]
- 28*. Kunkle A, Johnson AJ, Rolczynski LS, Chang CA, Hoglund V, Kelly-Spratt KS, et al. Functional Tuning of CARs Reveals Signaling Threshold above Which CD8+ CTL Antitumor Potency Is Attenuated due to Cell Fas-FasL-Dependent AICD. *Cancer immunology research.* 2015; 3(4):368–79. [PubMed: 25576337] [This study provides detailed analysis of CAR structural design, evaluating the interplay between extracellular linker length and cytoplasmic signaling for overall CAR functional potency.]
29. Guest RD, Hawkins RE, Kirillova N, Cheadle EJ, Arnold J, O'Neill A, et al. The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens. *Journal of immunotherapy (Hagerstown, Md : 1997).* 2005; 28(3):203–11.
30. Hudecek M, Lupo-Stanghellini MT, Kosasih PL, Sommermeyer D, Jensen MC, Rader C, et al. Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. *Clin Cancer Res.* 2013; 19(12):3153–64. [PubMed: 23620405]

31. Jonnalagadda M, Mardiros A, Urak R, Wang X, Hoffman LJ, Bernanke A, et al. Chimeric Antigen Receptors With Mutated IgG4 Fc Spacer Avoid Fc Receptor Binding and Improve T Cell Persistence and Antitumor Efficacy. *Mol Ther*. 2015; 23(4):757–68. [PubMed: 25366031]
32. Hombach A, Hombach AA, Abken H. Adoptive immunotherapy with genetically engineered T cells: modification of the IgG1 Fc ‘spacer’ domain in the extracellular moiety of chimeric antigen receptors avoids ‘off-target’ activation and unintended initiation of an innate immune response. *Gene therapy*. 2010; 17(10):1206–13. [PubMed: 20555360]
33. Hudecek M, Sommermeyer D, Kosasih PL, Silva-Benedict A, Liu L, Rader C, et al. The non-signaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. *Cancer immunology research*. 2014; 3(2):125–35. [PubMed: 25212991]
34. Bridgeman JS, Hawkins RE, Bagley S, Blaylock M, Holland M, Gilham DE. The optimal antigen response of chimeric antigen receptors harboring the CD3zeta transmembrane domain is dependent upon incorporation of the receptor into the endogenous TCR/CD3 complex. *J Immunol*. 2010; 184(12):6938–49. [PubMed: 20483753]
- 35*. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat Med*. 2015; 21(6):581–90. [PubMed: 25939063] [This study provides an important example of antigen-independent signaling due to scFv aggregation, which results in T cell exhaustion and suboptimal functional activity with CD28, but not 4-1BB, co-stimulation.]
36. Frigault MJ, Lee J, Basil MC, Carpenito C, Motohashi S, Scholler J, et al. Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells. *Cancer immunology research*. 2015; 3(4):356–67. [PubMed: 25600436]
37. Bridgeman JS, Ladell K, Sheard VE, Miners K, Hawkins RE, Price DA, et al. CD3zeta-based chimeric antigen receptors mediate T cell activation via cis- and trans-signalling mechanisms: implications for optimization of receptor structure for adoptive cell therapy. *Clinical and experimental immunology*. 2014; 175(2):258–67. [PubMed: 24116999]
38. Klebanoff CA, Gattinoni L, Restifo NP. Sorting through subsets: which T-cell populations mediate highly effective adoptive immunotherapy? *Journal of immunotherapy (Hagerstown, Md : 1997)*. 2012; 35(9):651–60.
39. Gattinoni L, Klebanoff CA, Restifo NP. Paths to stemness: building the ultimate antitumour T cell. *Nature reviews Cancer*. 2012; 12(10):671–84. [PubMed: 22996603]
40. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell subset with stem cell-like properties. *Nat Med*. 2011; 17(10):1290–7. [PubMed: 21926977]
41. Wang X, Naranjo A, Brown CE, Bautista C, Wong CW, Chang WC, et al. Phenotypic and functional attributes of lentivirus-modified CD19-specific human CD8+ central memory T cells manufactured at clinical scale. *Journal of immunotherapy*. 2012; 35(9):689–701. [PubMed: 23090078]
42. Graef P, Buchholz VR, Stemberger C, Flossdorf M, Henkel L, Schiemann M, et al. Serial transfer of single-cell-derived immunocompetence reveals stemness of CD8(+) central memory T cells. *Immunity*. 2014; 41(1):116–26. [PubMed: 25035956]
43. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8 T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest*. 2008; 118(1):294–305. [PubMed: 18060041]
44. Cieri N, Camisa B, Cocchiarella F, Forcato M, Oliveira G, Provasi E, et al. IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. *Blood*. 2013; 121(4):573–84. [PubMed: 23160470]
45. Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood*. 2011; 118(23):6050–6. [PubMed: 21984804]
- 46*. Xu Y, Zhang M, Ramos CA, Durett A, Liu E, Dakhova O, et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR-CD19-T cells and are preserved by IL-7 and IL-15. *Blood*. 2014; 123(24):3750–9. [PubMed: 24782509] [Retrospective analysis of CD19-CAR T cell clinical data, correlating CAR T cell in vivo persistence and expansion with a younger CD8+ CD45RA+ CCR7+ T cell phenotype, with inclusion of IL-7 and IL-15 in lieu of IL-2.]

47. Itzhaki O, Hovav E, Ziporen Y, Levy D, Kubi A, Zikich D, et al. Establishment and large-scale expansion of minimally cultured “young” tumor infiltrating lymphocytes for adoptive transfer therapy. *Journal of immunotherapy*. 2011; 34(2):212–20. [PubMed: 21304398]
48. Rushworth D, Jena B, Olivares S, Maiti S, Briggs N, Somanchi S, et al. Universal artificial antigen presenting cells to selectively propagate T cells expressing chimeric antigen receptor independent of specificity. *Journal of immunotherapy*. 2014; 37(4):204–13. [PubMed: 24714354]
49. Yang S, Dudley ME, Rosenberg SA, Morgan RA. A simplified method for the clinical-scale generation of central memory-like CD8+ T cells after transduction with lentiviral vectors encoding antitumor antigen T-cell receptors. *Journal of immunotherapy*. 2010; 33(6):648–58. [PubMed: 20551831]
50. Barrett DM, Singh N, Liu X, Jiang S, June CH, Grupp SA, et al. Relation of clinical culture method to T-cell memory status and efficacy in xenograft models of adoptive immunotherapy. *Cytotherapy*. 2014; 16(5):619–30. [PubMed: 24439255]
51. Petrozziello E, Sturmheit T, Mondino A. Exploiting cytokines in adoptive T-cell therapy of cancer. *Immunotherapy*. 2015; 7(5):573–84. [PubMed: 26065481]
52. Santeoets SJ, Turksma AW, Suhoski MM, Stam AG, Albelda SM, Hooijberg E, et al. IL-21 promotes the expansion of CD27+ CD28+ tumor infiltrating lymphocytes with high cytotoxic potential and low collateral expansion of regulatory T cells. *Journal of translational medicine*. 2013; 11:37. [PubMed: 23402380]
53. Singh H, Figliola MJ, Dawson MJ, Huls H, Olivares S, Switzer K, et al. Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies. *Cancer research*. 2011; 71(10):3516–27. [PubMed: 21558388]
54. Chapuis AG, Ragnarsson GB, Nguyen HN, Chaney CN, Pufnock JS, Schmitt TM, et al. Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. *Sci Transl Med*. 2013; 5(174):174ra27.
55. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. *The Journal of clinical investigation*. 2013; 123(10):4479–88. [PubMed: 24091329]
56. Gattinoni L, Zhong XS, Palmer DC, Ji Y, Hinrichs CS, Yu Z, et al. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nat Med*. 2009; 15(7):808–13. [PubMed: 19525962]
57. Crompton JG, Sukumar M, Roychoudhuri R, Clever D, Gros A, Eil RL, et al. Akt inhibition enhances expansion of potent tumor-specific lymphocytes with memory cell characteristics. *Cancer research*. 2015; 75(2):296–305. [PubMed: 25432172]
58. Litterman AJ, Zellmer DM, LaRue RS, Jameson SC, Largaespada DA. Antigen-specific culture of memory-like CD8 T cells for adoptive immunotherapy. *Cancer immunology research*. 2014; 2(9): 839–45. [PubMed: 24852944]
59. Terakura S, Yamamoto TN, Gardner RA, Turtle CJ, Jensen MC, Riddell SR. Generation of CD19-chimeric antigen receptor modified CD8+ T cells derived from virus-specific central memory T cells. *Blood*. 2012; 119(1):72–82. [PubMed: 22031866]
60. Riddell SR, Sommermeyer D, Berger C, Liu LS, Balakrishnan A, Salter A, et al. Adoptive therapy with chimeric antigen receptor-modified T cells of defined subset composition. *Cancer journal*. 2014; 20(2):141–4.
61. Muranski P, Borman ZA, Kerkar SP, Klebanoff CA, Ji Y, Sanchez-Perez L, et al. Th17 cells are long lived and retain a stem cell-like molecular signature. *Immunity*. 2011; 35(6):972–85. [PubMed: 22177921]
62. Hinrichs CS, Kaiser A, Paulos CM, Cassard L, Sanchez-Perez L, Heemskerk B, et al. Type 17 CD8+ T cells display enhanced antitumor immunity. *Blood*. 2009; 114(3):596–9. [PubMed: 19471017]
63. Wang X, Wong CW, Urak R, Mardiros A, Budde LE, Chang WC, et al. CMVpp65 Vaccine Enhances the Antitumor Efficacy of Adoptively Transferred CD19-Redirected CMV-Specific T Cells. *Clin Cancer Res*. 2015; 21(13):2993–3002. [PubMed: 25838392]

64. Cruz CR, Micklethwaite KP, Savoldo B, Ramos CA, Lam S, Ku S, et al. Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. *Blood*. 2013; 122(17):2965–73. [PubMed: 24030379]
65. Torikai H, Reik A, Soldner F, Warren EH, Yuen C, Zhou Y, et al. Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood*. 2013; 122(8):1341–9. [PubMed: 23741009]
66. Poirot L, Philip B, Schiffer-Mannioui C, Le Clerre D, Chion-Sotinel I, Derniame S, et al. Multiplex genome edited T-cell manufacturing platform for “off-the-shelf” adoptive T-cell immunotherapies. *Cancer research*. 2015 Epub 2015/07/16.
67. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood*. 2011; 118(18):4817–28. [PubMed: 21849486]
- 68*. Ahmed N, Brawley VS, Hegde M, Robertson C, Ghazi A, Gerken C, et al. Human Epidermal Growth Factor Receptor 2 (HER2) -Specific Chimeric Antigen Receptor-Modified T Cells for the Immunotherapy of HER2-Positive Sarcoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2015; 33(15):1688–96. [PubMed: 25800760]
[Clinical study of HER2-specific CAR T cells evaluated in sarcoma patients in a dose escalation Phase I/II trial, demonstrating safety of systemic delivery of HER2-CAR T cells and potential anti-tumor responses.]
- 69*. Brown CE, Badie B, Barish ME, Weng L, Ostberg JR, Chang WC, et al. Bioactivity and Safety of IL13Ralpha2-Redirected Chimeric Antigen Receptor CD8+ T cells in Patients with Recurrent Glioblastoma. *Clin Cancer Res*. 2015 Epub 2015/06/11. [Clinical study of a Phase I trial highlighting safety and transient anti-tumor activity in advanced glioma patients with locally delivered IL13Ra2-specific CAR T cells in the brain.]
70. Song DG, Ye Q, Carpenito C, Poussin M, Wang LP, Ji C, et al. In vivo persistence, tumor localization, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB). *Cancer research*. 2011; 71(13):4617–27. [PubMed: 21546571]
- 71**. Adusumilli PS, Cherkassky L, Villena-Vargas J, Colovos C, Servais E, Plotkin J, et al. Regional delivery of mesothelin-targeted CAR T cell therapy generates potent and long-lasting CD4-dependent tumor immunity. *Sci Transl Med*. 2014; 6(261):261ra151. [The first report of intrapleural delivery of CAR T cells, demonstrating more potent and durable therapy compared with intravenous delivery. This paper also demonstrated more robust systemic anti-tumor immunity in a CD4-dependent manner.]
72. Maus MV, Haas AR, Beatty GL, Albelda SM, Levine BL, Liu X, et al. T cells expressing chimeric antigen receptors can cause anaphylaxis in humans. *Cancer immunology research*. 2013; 1(1):26–31.
- 73*. Caruana I, Savoldo B, Hoyos V, Weber G, Liu H, Kim ES, et al. Heparanase promotes tumor infiltration and antitumor activity of CAR-redirected T lymphocytes. *Nat Med*. 2015; 21(5):524–9. [PubMed: 25849134] [This study elegantly demonstrates loss of the enzyme heparanase expression in ex vivo cultured T cells compared with freshly isolated T cells, which normally disrupts the extracellular matrix in solid cancers to improve penetration of adoptively-transferred T cells.]
74. Brown CE, Vishwanath RP, Aguilar B, Starr R, Najbauer J, Aboody KS, et al. Tumor-Derived Chemokine MCP-1/CCL2 Is Sufficient for Mediating Tumor Tropism of Adoptively Transferred T Cells. *J Immunol*. 2007; 179(5):3332–41. [PubMed: 17709550]
75. Moon EK, Carpenito C, Sun J, Wang LC, Kapoor V, Predina J, et al. Expression of a functional CCR2 receptor enhances tumor localization and tumor eradication by retargeted human T cells expressing a mesothelin-specific chimeric antibody receptor. *Clin Cancer Res*. 2011; 17(14):4719–30. [PubMed: 21610146]
76. Condomines M, Arnason J, Benjamin R, Gunset G, Plotkin J, Sadelain M. Tumor-Targeted Human T Cells Expressing CD28-Based Chimeric Antigen Receptors Circumvent CTLA-4 Inhibition. *PloS one*. 2015; 10(6):e0130518. [PubMed: 26110267]
77. Chinnasamy D, Yu Z, Kerkar SP, Zhang L, Morgan RA, Restifo NP, et al. Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. *Clin Cancer Res*. 2012; 18(6):1672–83. [PubMed: 22291136]

78. Pegram HJ, Lee JC, Hayman EG, Imperato GH, Tedder TF, Sadelain M, et al. Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. *Blood*. 2012; 119(18):4133–41. [PubMed: 22354001]
79. Koneru M, Purdon TJ, Spriggs D, Koneru S, Brentjens RJ. IL-12 secreting tumor-targeted chimeric antigen receptor T cells eradicate ovarian tumors. *Oncoimmunology*. 2015; 4(3):e994446. [PubMed: 25949921]
80. Curran KJ, Seinstra BA, Nikhamin Y, Yeh R, Usachenko Y, van Leeuwen DG, et al. Enhancing antitumor efficacy of chimeric antigen receptor T cells through constitutive CD40L expression. *Mol Ther*. 2015; 23(4):769–78. [PubMed: 25582824]
81. John LB, Devaud C, Duong CP, Yong CS, Beavis PA, Haynes NM, et al. Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2013; 19(20):5636–46. [PubMed: 23873688]
82. Vanneman M, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. *Nature reviews Cancer*. 2012; 12(4):237–51. [PubMed: 22437869]
- 83*. Ninomiya S, Narala N, Huye L, Yagyu S, Savoldo B, Dotti G, et al. Tumor indoleamine 2,3-dioxygenase (IDO) inhibits CD19-CAR T cells and is downregulated by lymphodepleting drugs. *Blood*. 2015; 125(25):3905–16. [PubMed: 25940712] [This study provides an example of the effects of IDO-mediated immunosuppression on the function of CD19-CAR T cells for lymphoma eradication, and importantly that lymphodepleting chemotherapy down-regulated tumor IDO expression.]
84. Herrmann A, Kortylewski M, Kujawski M, Zhang C, Reckamp K, Armstrong B, et al. Targeting Stat3 in the myeloid compartment drastically improves the in vivo antitumor functions of adoptively transferred T cells. *Cancer research*. 2010; 70(19):7455–64. [PubMed: 20841481]
85. Kujawski M, Zhang C, Herrmann A, Reckamp K, Scuto A, Jensen M, et al. Targeting STAT3 in adoptively transferred T cells promotes their in vivo expansion and antitumor effects. *Cancer research*. 2010; 70(23):9599–610. Epub 2010/12/02. [PubMed: 21118964]
86. Mussai F, Egan S, Hunter S, Webber H, Fisher J, Wheat R, et al. Neuroblastoma Arginase Activity Creates an Immunosuppressive Microenvironment That Impairs Autologous and Engineered Immunity. *Cancer research*. 2015
87. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric Antigen Receptor-Modified T Cells for Acute Lymphoid Leukemia. *The New England journal of medicine*. 2013; 368(16):1509–18. Epub 2013/03/27. [PubMed: 23527958]
88. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, Friedman HS, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *Journal of clinical oncology*. 2010; 28(31):4722–9. [PubMed: 20921459]
89. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med*. 2015 Epub 2015/07/21. *Nat Med*. 2015 Jul 20. doi: 10.1038/nm.3910.
90. Hegde M, Corder A, Chow KK, Mukherjee M, Ashoori A, Kew Y, et al. Combinational targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma. *Mol Ther*. 2013; 21(11):2087–101. [PubMed: 23939024]
91. Wilkie S, van Schalkwyk MC, Hobbs S, Davies DM, van der Stegen SJ, Pereira AC, et al. Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. *Journal of clinical immunology*. 2012; 32(5):1059–70. [PubMed: 22526592]
92. Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nature biotechnology*. 2013; 31(1):71–5.
93. Sampson JH, Choi BD, Sanchez-Perez L, Suryadevara CM, Snyder DJ, Flores CT, et al. EGFRvIII mCAR-modified T-cell therapy cures mice with established intracerebral glioma and generates host immunity against tumor-antigen loss. *Clin Cancer Res*. 2014; 20(4):972–84. [PubMed: 24352643]

94. Ma C, Cheung AF, Chodon T, Koya RC, Wu Z, Ng C, et al. Multifunctional T-cell analyses to study response and progression in adoptive cell transfer immunotherapy. *Cancer discovery*. 2013; 3(4):418–29. [PubMed: 23519018]
95. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood*. 2014; 124(2):188–95. [PubMed: 24876563]
96. Hinrichs CS, Restifo NP. Reassessing target antigens for adoptive T-cell therapy. *Nat Biotechnol*. 2013; 31(11):999–1008. [PubMed: 24142051]
97. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther*. 2010; 18(4):843–51. [PubMed: 20179677]
98. Kenderian SS, Ruella M, Shestova O, Klichinsky M, Aikawa V, Morrissette JJ, et al. CD33-specific chimeric antigen receptor T cells exhibit potent preclinical activity against human acute myeloid leukemia. *Leukemia*. 2015 Epub 2015/02/28.
99. Beatty GL, Haas AR, Maus MV, Torigian DA, Soulen MC, Plesa G, et al. Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies. *Cancer immunology research*. 2014; 2(2):112–20. [PubMed: 24579088]
100. Deniger DC, Yu J, Huls MH, Figliola MJ, Mi T, Maiti SN, et al. Sleeping Beauty Transposition of Chimeric Antigen Receptors Targeting Receptor Tyrosine Kinase-Like Orphan Receptor-1 (ROR1) into Diverse Memory T-Cell Populations. *PloS one*. 2015; 10(6):e0128151. [PubMed: 26030772]
101. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *The New England journal of medicine*. 2011; 365(18):1673–83. [PubMed: 22047558]
102. Wang X, Chang WC, Wong CW, Colcher D, Sherman M, Ostberg JR, et al. A transgene-encoded cell surface polypeptide for selection, in vivo tracking, and ablation of engineered cells. *Blood*. 2011; 118(5):1255–63. [PubMed: 21653320]
103. Lanitis E, Poussin M, Klattenhoff AW, Song D, Sandaltzopoulos R, June CH, et al. Chimeric antigen receptor T Cells with dissociated signaling domains exhibit focused antitumor activity with reduced potential for toxicity in vivo. *Cancer immunology research*. 2013; 1(1):43–53. [PubMed: 24409448]
104. Fedorov VD, Themeli M, Sadelain M. PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses. *Sci Transl Med*. 2013; 5(215):215ra172.

Key Points

- Major challenges exist to successfully extend CAR T cell therapy beyond CD19-CARs for B-cell malignancies and effectively translate to other hematological malignancies and solid cancers.
- Chimeric antigen receptor (CAR) design and T cell manufacturing are intrinsic variables that define the CAR T cell product, and are critical determinants of overall therapy, likely to be empirically determined for each tumor antigen and cancer type.
- Extrinsic factors related to the tumor microenvironment that impact the ability of CAR T cells to penetrate the tumor, retain potency within the hostile tumor microenvironment and overcome antigen heterogeneity of the tumor, have spurred more complex CAR T cell engineering strategies and combination therapies.

Figure 1. Key Variables in CAR T Cell Therapy

The diagram depicts the processes (**Steps 1-5**) of CAR T cell therapy moving in a counter-clockwise direction, starting with patient leukapheresis and ending with infusion of engineered CAR T cells. **1. Leukapheresis and T cell enrichment:** The first key variables include T cell isolation, in particular whether the starting population is unselected peripheral blood mononuclear cells or enriched T cell subsets (i.e. memory) with potentially defined CD4:CD8 ratios. **2. Activate T cells:** The next key variables in T cell manufacturing are T cell activation, including CD3 antibody stimulation along with CD28 co-stimulation or antigen presenting cells. **3. Engineer CAR T cells:** Key variables in CAR design dictate an optimized CAR, and include the antigen-binding domain, the extracellular linker/spacer, and the intracellular signaling domain. **4. Expand CAR T cells:** Optimized CARs are then introduced into T cells using various engineering strategies (viral or non-viral delivery). Key variables in T cell expansion include cytokines, immune-modulators, and *ex vivo* culture time and expansion conditions. **5. Infuse CAR T cells:** Finally, key variables in adoptive transfer include patient pre-conditioning regimens, route of T cell administration, as well as T cell dose and frequency of infusions. The tumor microenvironment, antigen expression heterogeneity in tumors, and safety considerations, are also important factors in optimizing CAR T cell therapy (discussed in the body of this review). Cumulative consideration of each key variable is critical in developing the most effective CAR T cell product.