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CAR T Cells: New Approaches to Improve Their Efficacy and Reduce Toxicity

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Abstract

The durable remission of B-cell leukemia and lymphoma following chimeric antigen receptor T (CAR) cell therapy has brought this new form of adoptive immunotherapy to center stage with the expectation that CART cell therapy may provide similar efficacy in other hematological and solid cancers. Herein, we review recent advances in the areas of CAR design that improve CART cell proliferation, engraftment and efficacy as well as clinical application strategies that are designed to improve clinical efficacy while reducing the risk for toxicity and broaden patient access to this promising form of cancer immunotherapy.

Keywords

Chimeric antigen receptor; adoptive transfer; genetic engineering; T cell; toxicity; immunotherapy

Introduction

The advent of advanced gene transfer and cell cultivation technologies now makes it possible to redirect the effector functions of a patient's T cells against an antigen on the cancer cell surface via a chimeric antigen receptor (CAR), commonly composed of an extracellular antigen binding moiety (e.g. antibody scFv) fused to intracellular signaling domains for T cell activation. Adoptive transfer of CART cells redirected against CD19 can result in durable, complete remission of B cell lymphomas and leukemias, including a nearly 90% response rate in acute lymphoid leukemia[1]. Still, toxicities such as cytokine release syndrome (CRS) and occasional neuro-toxicities do occur, and similar success in solid tumors still remains elusive, given the immunosuppressive tumor microenvironment (TME) and difficulties in identifying sound target antigens that are not also expressed on normal tissues. Indeed, on-target, off-tumor toxicities have been noted in some CART therapy

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trials[2, 3], however, advances in gene transfer platforms, CAR design and clinical strategies that potentiate and focus antitumor activity while sensitizing the tumor cells and its associated microenvironment to CART cell attack now provide an opportunity to safely and effectively treat these cancers.

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Several outstanding questions in the field of CAR T cell therapy exist; among these are what defines a potent cell product, what factors are most important to achieve engraftment and expansion of a cell product, and what factors are most responsible for achieving a durable anti-tumor response. It is becoming increasingly clear that T cell engraftment and persistence are the keys to anti-tumor efficacy [4]. The field has therefore concentrated on three complementary approaches to improve efficacy: (1) facilitating in vivo expansion and engraftment by either increasing cell quality or dose, or providing host conditioning, (2) genetic manipulation of T cells to increase their proliferative capacity or become resistant to the inhibitory TME and (3) selecting a starter T cell population that would be predicted to have a favorable proliferative potential.

Host and tumor conditioning

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One way to increase the efficacy of CART cell therapy is to “condition” the host with chemotherapy and/or radiation, resulting in lymphodepletion prior to administering CAR T cells [5]. Lymphodepletion may exert its effects in three different ways: (1) reduction in regulatory T cells (Treg) and immunosuppressive macrophages [6]; (2) reduction in disease burden, and/or (3) facilitating homeostatic proliferation by increased availability of endogenous cytokines, including IL-7 [7]. Although chemotherapy with or without radiation is a common conditioning strategy, it is relatively non-specific. Alternative, mechanism-based conditioning regimens such as Treg-reducing strategies with IL-2 immunotoxin (Ontak) or daclizumab (anti-CD25) could be considered. Recently, targeted small molecule drugs, such as CSF-1R and IDO inhibitors, have also been shown to affect the immunosuppressive myeloid-derived suppressor cells (MDSC) [8, 9]. Combinations of T cells with a variety of other targeted drugs may have beneficial effects by sensitizing tumors to T cell-mediated killing and may also have independent effects on T cell biology[10–12]. Although it is fairly well established that lymphodepleting chemotherapy aids in infused T cell engraftment, it is not clear that lymphodepletion is absolutely required for effective therapy, particularly when the T cell product is optimally cultured and has adequate potency. Finally, the importance of lymphodepletion and the regimen used may be disease-specific and therefore bears consideration.

Engineering potency

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Beyond host conditioning, productive CART cell proliferation, effector function and survival rely upon coordinate delivery of an activation signal, often via a TCR CD3 ζ or an Fc receptor subunit, in parallel with a secondary costimulatory signal. The modular design of a CAR permits the inclusion of CD3 ζ and costimulatory signaling domains in tandem for *cis* signaling that attempts to mimic natural costimulation *in trans* during TCR activation for full T cell activation. In a competitive repopulation study by Savoldo et al., first generation CART cells lacking a costimulatory signaling domain did not persist, whilst second

generation CART containing a CD28 signaling domain proliferated and persisted after co-administration[13], emphasizing the need for costimulation. The concept now emerges that costimulatory domain selection can influence the nature of CART cell persistence and function. CD28 and 4-1BB have been most widely applied in CART therapy, with a growing body of patient and preclinical data suggesting that CD28 signaling supports robust effector function but with coordinate exhaustion and reduced capacity to persist in vivo, compared to 4-1BB signaling that promotes T cell survival[14–17]. Accordingly, CART may be tuned for short-term or long-lasting effects in vivo, through provision of CD28 or 4-1BB signaling, respectively. Still, CARs that include alternative costimulatory molecules from the TNFR superfamily such as CD27 [16], OX40 (CD134) or inducible costimulatory (ICOS) [18] can confer CART cells with qualities that rationalize their clinical investigation. Like 4-1BB, CD27 promotes CART resistance to antigen-induced cell death and anti-apoptotic Bcl-xL upregulation associated with enhanced survival and antitumor activity in vivo, compared to CD28[16]. In contrast, OX40 confers CART with a capacity for antigen-stimulated proliferation and cytokine production that is superior to first generation CARs but reduced relative to CD28[19]. Evidence that ICOS signaling promotes the development of human CD4+ T helper 17 (Th17) cells [20] has restored interest in the use of ICOS in second generation CARs, and led to the generation of Th17-like tumor-specific CART cells capable of producing both IL-17 and IFN- γ with an enhanced capacity to persist, relative to CD28 or 4-1BB containing CART cells [21]. Whether and which combination of two or more costimulatory domains in the same CAR best enhances net CART activity requires further investigation as both additive [19, 22] and diminished benefit have been reported [23]. However, what is growing clear is that CD4+ and CD8+ CART cells require distinct costimulation signals for optimal persistence and activity. Compared to CD28 and 4-1BB, ICOS-based CARs significantly increase CD4+ T cell in vivo persistence, an effect heightened by ex vivo culturing under Th17 conditions [24]. CD4+ ICOS-CART cells also bolstered CD8+ CD28- and 4-1BB-based CART cell persistence and antitumor activity when co-administered in preclinical models, lending credence to tailored CART design and culture conditioning for various T cell subsets prior to infusion for maximized efficacy.

Conventional CARs are designed to possess costimulatory domains in tandem with a TCR signaling domain for *cis* signaling, however costimulatory signals can be disentangled from CD3 ζ signaling by the creation of dual CART cells that transmit signal 1 (TCR) through a first generation CD3 ζ -based CAR and signal 2 (costimulation) through a secondary CAR of distinct antigen specificity containing only a costimulatory endodomain for ligand dependent costimulatory signaling *in trans* [25–27]. These dual CART cells receive TCR and costimulatory signals *in trans* akin to natural T cell activation and are similar in potency to standard *cis*-signaling CARs, but have the added benefit of achieving full activation only upon simultaneous encounter and stimulation with two discrete antigens on cancer cells, improving their ability to sense tumor and dampening their response to healthy tissues bearing single antigen. *Trans* costimulatory signaling in CART cells can also be achieved through the engineered surface expression of a costimulatory ligand, such as 4-1BBL, for presumed autocrine crosslinking with its costimulatory receptor. Using this approach, CD28-based CD19-specific CART cells outfitted with 4-1BBL showed the greatest persistence and mediated complete tumor eradication in a preclinical NALM-6 model, while

standard CD28-based CD19 CART cells showed reduced persistence and partial responses[28]. Using a different multi-chain CAR signaling approach, Wang et al. engineered a scFv based CAR containing the transmembrane and cytoplasmic domains of a stimulatory killer immunoglobulin-like receptor (KIR), KIR2DS2, into human T cells along with DAP12, an ITAM-containing adaptor, for multi-chain CAR signaling [29]. T cells expressing the KIR-CAR and DAP12 exhibited antitumor activity that was superior to second generation CD3 ζ -based CARs carrying either CD28 or 4-1BB domains in a xenograft model, which reflected a heightened potency on the per cell basis, and not increased proliferation and persistence in vivo. Accordingly, this multi-chain approach may provide utility in solid tumors that resist conventional CART therapy.

Enhanced CART potency may also be conferred by engineering cells to resist immunosuppression in the TME through the use of a dominant negative TGF β , or immune potentiating “switch receptors” comprised of an extracellular inhibitory receptor domains (e.g. PD-1, CTLA-4) to intracellular costimulatory domains, including CD28, which invigorate antitumor activity in response to inhibitor ligands[30]. CART cells can also be engineered for inducible or constitutive activity using activating transcription factors, cytokines or anti-apoptotic molecules. CART potency may also be limited by accessibility issues in solid tumors that may be overcome by regional cell delivery [31] or engineered chemokine receptors e.g. CCR2b[32] for specific trafficking. The use of CART cells engineered to disrupt solid tumor masses via Heparinase[33], and its stroma via combination with FAP-[34], PSMA-[35] or VEGFR-[36] CARTs also hold promise.

Optimized T cell subsets for CART therapy

Basic immunologists have uncovered different subsets of T cells with varying degrees of proliferative capacity, effector function, and memory differentiation, many of which are characterized by the expression of specific cell-surface markers. For example, T central memory (T_{cm}) cells, characterized as CD45RO⁺ and CCR7⁺, have the capacity for long-lived persistence, and retain their ability to proliferate upon antigen encounter[37]. In a non-human primate model, low doses of T_{cm} can engraft and establish memory[38], and remains an area of clinical investigation. Stem cell-like memory cells are also attractive candidates for use in CART cell therapy given their less differentiated phenotype, proliferative capacity and ability to differentiate into full effectors[39]. In addition, although the ratio of CD4:CD8 T cells in the peripheral blood of normal donors is typically 2:1, it is possible that an immunotherapeutic drug has a different optimal ratio for tissue penetration and tumor eradication. Although some investigators use bulk populations of unselected T cells as the cell product, others use T cell products of defined composition in terms of ratio of CD8⁺ central memory T cells and CD4 T cells[40]. Finally, resident memory T cells that are chronically exposed to antigen, such as EBV-specific or CMV-specific T cells, may offer some benefits, as these are naturally-occurring persisting and effective T cells in normal donors [41], and would be amenable to post-infusion antigen-specific boosting strategies.

Improving CART cell safety

CART cells could potentially cause toxicity by any one of three mechanisms: (1) on-target, off-tumor effects, (2) off-target effects, or (3) activation-related cytokine production and release.

On-target, off-tumor effects are dependent on the choice of target antigen. Indeed, the most challenging roadblock to broadening the application of CART cell therapies remains the selection of the target antigen [2, 3]. High-affinity CART cells detect and can lyse targets expressing approximately 100 copies of the target antigen[42]; however, affinity-tuning may decrease the threshold for CART cell activation, which may broaden the therapeutic window of CART cells to tissues that only express high levels of antigen[43]. The ideal target antigen for a CART cell is one that is expressed homogeneously on the tumor cells and has a role in maintaining tumorigenicity (to prevent antigen escape variant recurrences of tumor), and is not expressed in any essential normal tissues (to prevent toxicity). The B cell antigen CD19 is the prototypical CAR antigen: it is expressed homogeneously in multiple tumors of B cell origin, its expression is typically homogeneous, and it is not expressed in a life-sustaining tissue. On the contrary, its expression on normal B cells can enable isolated B cell aplasia to serve as a biomarker of continuing CART cell function. Other antigens that could fall into this category of non-essential tissue antigens include hormone receptors (thyroid-stimulating, follicle-stimulating, estrogen, etc.) and tissue antigens such as prostate-specific membrane or stem cell antigen (PSMA and PSCA, respectively). Other potential target antigens include mutated oncogenic antigens such as EGFR variant III (though its expression is heterogeneous), viral antigens expressed on tumor cells such as EBV or polyoma virus for lymphoma or Merkel cell cancer, tumor-specific neo-antigens, and cancer testis antigens.

Off-target effects are the result of imperfect characterization of the specificity of the antigen receptor or unforeseen molecular mimicry of antigens. In this case, the engineered receptor recognizes an antigen that is similar to but was not predicted to bind to the receptor. In the case of transduced T cells, there are limited reports of this effect, e.g. where an engineered T cell receptor specific for a cancer-testis antigen cross-reacted with a peptide from the unrelated protein Titin[44]. Off-target effects are particularly difficult to predict from pre-clinical studies, given that animal models by definition do not express the same antigenic repertoire as humans. Toxicity from off-target effects may be controlled by ablating or silencing the CART cells.

Activation-related cytokine production or release is an expected event resulting from effective antigen encounter by CART cells. Activated T cells produce and release IFN γ , IL-2, and various other cytokines, which may in turn activate other immune cells, including macrophages. Cytokine release syndrome can be severe[45], and can be managed clinically with anti-cytokine therapy and/or corticosteroids. One of the advantages of anti-cytokine therapy is that it directly targets the secondary mediator of toxicity without necessarily impacting the T cell engraftment[14], and it generally has a rapid onset of action. However, abrogating the cytokine release syndrome may not have an impact on on-target or off-target

toxicity, and it may be difficult to clinically distinguish these forms of toxicity in a first-in-human study of a novel CART cell product.

To manage potential toxicity, or to eliminate CAR T cells after a sustained engraftment and eradication of disease, many investigators are developing ‘suicide genes’ to incorporate into CAR T cells. Candidate genes such as nucleoside kinases, including viral thymidine kinase or a mutated version of human thymidylate kinase, can be inactivated with nucleoside analog drugs, thereby inhibiting DNA synthesis and proliferation of the transduced cells. The main advantage of Herpes Simplex Virus – thymidine kinase (HSV-TK) as a safety switch is the ready availability and specificity of the inhibitor drug, ganciclovir[46]; however, introduction of a viral transgene also increases the risk for immunogenicity of the CART cells. Furthermore, elimination of the transduced T cells may take several days, which may not be acceptable in the face of immediately life-threatening toxicity. Human inducible caspase 9 is a fusion protein of caspase 9 and a drug-sensitive dimerizing domain of FK506. Upon binding to the drug, the fusion protein dimerizes and initiates apoptosis in the iCaspase9-transduced T cell. The advantages of this system are that (1) it is based on human sequences, (2) the transduced cells are eliminated rapidly because no DNA synthesis is required, and (3) it has been shown to be safe and rapidly effective in an allogeneic stem cell transplant study[47]. The main disadvantage of this system is the availability of the dimerizing drug. Alternative safety switches are based on expression of a well-characterized, targetable surface antigen on the transduced T cells, such as CD20 or variants of CD20[48], or truncated EGFR[49]; in case of severe uncontrolled toxicity, an infusion of rituximab or cetuximab, respectively, would theoretically target the transduced T cells for elimination. The advantages of this system are its lack of immunogenicity, the ready availability and safety of the antibodies that could be used to trigger CART cell elimination, and the dual-purpose nature of the additional transgene (which can also be used to measure the persistence of the transduced T cells). To our knowledge, these systems have not yet been tested clinically to determine their efficacy and kinetics as a CART cell elimination system in the setting of clinical toxicity.

Directing CART cell attack to tumor sites anatomically may also limit toxicity and improve efficacy. This may be achieved via intratumoral or local intralymphatic delivery or by engineering CART cells to express specific chemokine receptors for trafficking to tumor[32]. Cloaking the target on normal tissue with antigen-specific antibody may effectively reduce toxicity but limits response to therapy[2]. Newer strategies now make it possible to genetically engineer CART cells for conditional attack of cancer and relative sparing of normal tissue. Some of these approaches include dual *trans*-signaling CART cells [25–27], as well as inhibitor CARs containing for restricted functionality upon encounter of specific antigen on the normal cells, but not tumor[50]. Alternatively, one might engineer T cells for conditional expression of the CAR using conditionally inducible promoters that are active only in the TME.

Broadening patient access through universal approaches

Strategies are being devised to make CART cell therapy universal in application through the generation of (1) so-called universal T cells, and (2) universal immune receptors. Generation

of universal effector cells could provide a route for therapy in patients with autologous T cells of poor quality or quantity, and streamline commercialization of a standardized CART cell product. Some approaches include the use of immortalized NK cell lines (e.g. NK92), or allogeneic T cell products such as allo-depleted T cells, allogeneic T cells with restricted specificity (e.g. virus-specific T-cells), NKT cells, or gamma-delta T cells. The advent of genome-editing technologies now makes it possible to ablate endogenous TCR and MHC genes from allogeneic donor T cells to create universal CART cell with reduced risk for graft versus host disease and host-mediated response against the transferred product[51].

Universal immune receptors contain an extracellular moiety that serves as a scaffold for the binding of intermediate tumor-targeting molecules (e.g. tagged antibodies) so that the universal receptor can be “armed” for antigen specificity or directed against a targeting molecule “painted” on the tumor cell surface. Accordingly, universal immune receptors serve as a standardized platform for adaptable redirected T cell specificity for the targeting of multiple and diverse antigens either simultaneously or sequentially[52]. Various approaches have now been described including those that rely upon biotin-avidin, antibody-Fc receptor, antibody-antigen and metabolite-receptor interactions, and are at the early phase of clinical investigation.

Conclusion

CAR T cells are a nascent form of immunotherapy with tremendous potential for inducing durable anti-tumor effects. Numerous investigative avenues to explore range from clinical manipulations, such as combinations of drugs administered to the patient host, to genetic engineering strategies to manipulate the T cell product, all of which have the potential to increase the safety, potency, and broad applicability of CAR T cell technology to better the lives of patients with cancer.

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Table 1

Summary of approaches to increase CAR T cell safety and efficacy

Strategies to increase efficacy	
Increase dose	
Increase host conditioning	
Genetic engineering of CAR signaling domains to increase engraftment	
Additional transgenes to increase potency/engraftment/tissue penetration	
Selection of optimized T cell populations	
Multiple antigen targeting to address tumor heterogeneity	
Strategies to increase safety	
Selection of tumor-specific antigens	
Affinity tuning of CAR receptors	
Dual-CAR systems to recognize antigen combinations	
Suicide systems	
Anatomic localization	
Refined pre-clinical testing models	