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Adoptive Immunotherapy for Cancer or Viruses

Marcela V. Maus[1], **Joseph A. Fraietta**, **Bruce L. Levine**[2], **Michael Kalos**[2], **Yangbing Zhao**[2], and **Carl H. June**[2]

[1]Translational Research Program, Abramson Cancer Center and Departments of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

[2]Departments of Medicine and Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

Abstract

Adoptive immunotherapy, or the infusion of lymphocytes, is a promising approach for the treatment of cancer and certain chronic viral infections. The application of the principles of synthetic biology to enhance T cell function has resulted in substantial increases in clinical efficacy. The primary challenge to the field is to identify tumor-specific targets to avoid off-tissue, on-target toxicity. Given recent advances in efficacy in numerous pilot trials, the next steps in clinical development will require multicenter trials in order to establish adoptive immunotherapy as a mainstream technology.

Keywords

adaptive immunity; adoptive immunotherapy; gene transfer; chimeric antigen receptor

Introduction

Adoptive immunotherapy, or cell therapies, is undergoing a period of growth and enthusiasm following encouraging data of clinical efficacy. Virus- directed cell therapies are under investigation for the treatment of chronic viral infections like HIV and for viruses that cause morbidity and mortality in the immunosuppressed setting of bone marrow transplantation. In addition, cell therapies are poised to take a prominent role in both hematologic malignancies and solid tumors. Here we will review the history and rationale of immunotherapy and advances in understanding the principles of T cell transfer that are thought to impact clinical results. We will also discuss strategies and methods that are important in developing appropriate, effective, reliable, and scalable culture systems. Our current understanding of methodologies of engineering cells to re-direct them to specific targets, endowing immune cells with additional functions and safety features, and combining cells with other immune and targeted therapies is discussed in this review (Figure 1). Finally, we will illustrate how immune monitoring and biomarkers can determine the effects

Address correspondence to: Marcela Maus or Carl June, Smilow Center for Translational Research, 3400 Civic Center Blvd, Bldg 421, Philadelphia, PA 19104-5156, Phone: (215) 573-3269; Fax (610) 646-8455;, Marcela.Maus@uphs.upenn.edu or cjune@exchange.upenn.edu.

and fate of cell therapies in the clinical setting, and conclude with a brief discussion on what elements will be required to establish a new pillar of medical treatments built around personalized cell therapies.

History and rationale for adoptive immunotherapy

Given the abilities of T cells to recognize and kill target cells, it is not surprising that most investigations of adoptive T cell therapy have targeted chronic viruses and cancer.

Viruses

Cell and gene therapy strategies have been proposed from the earliest days of the HIV epidemic (1) (2). The first clinical use of chimeric-antigen receptor (CAR) modified T cells was in HIV infection. In this setting, the CAR was composed of the receptor for the HIV envelope protein, namely the extracellular and transmembrane portions of the CD4 protein, fused to the TCR zeta signaling molecule (CD4z CAR). The proposed mechanism of action was for transduced T cells to lyse HIV-envelope expressing T cells. Between 1998 and 2005, three clinical studies evaluated the CD4z CAR expressed in autologous CD4+ and CD8+ T cells via a retroviral vector in subjects with active viremia (3) or in T cell–reconstituted patients with chronic HIV-1 infection (4). These studies showed that infusion of re-directed T cells was feasible and safe; in addition, T cells trafficked to reservoirs of infection (mucosa) and had modest effects on viremia. A decade later, analysis of the data collected from these protocols in a long-term follow-up study demonstrated the safety of retroviral modification of human T cells and the long-term persistence of CAR-modified T cells, with an estimated half-life of at least 17 years (5). This study added to the literature indicating that T cells were not as susceptible to retrovirus-mediated insertional mutagenesis as hematopoietic stem cells. In 2009, the remarkable story of the “Berlin patient” was published (6); this was the first report of a patient being functionally cured of HIV infection following an allogeneic hematopoietic stem cell transplant for acute myelogenous leukemia. The donor was homozygous for the CCR5 **Δ**32 mutation, which confers genetic resistance to HIV infection. This has challenged the field to develop cell-therapy based approaches that do not require myeloablative chemotherapy or allogeneic donors. One approach has been to develop gene therapy strategies to reduce CCR5 expression, either through shRNA encoded by lentiviral vectors (7) or through gene-editing strategies using zinc-finger nucleases (ZFN) to disrupt the CCR5 gene in T cells (8). In these cases, autologous gene-modified T cells are reinfused with the goal of reconstituting the T cell repertoire in HIV-infected patients. Interpretation of T cell effects on viremia and control of HIV may be affected by ongoing treatment with highly active anti-retroviral therapy (HAART), and carefully designed trials with scheduled, carefully monitored, treatment interruptions are underway.

Patients with hematologic malignancy undergoing allogeneic bone marrow transplantation are also at high risk for viral illness, particularly from reactivation of chronic viruses such as CMV, EBV, and HHV6; primary adenovirus infection can also cause acute and severe illness in this immunocompromised population. Although pharmacologic treatments for these viruses are available, they often have limited efficacy, must be administered recursively, and have significant side effects. For these reasons, several transplant centers have focused on developing donor-derived virus-specific T cells that can be administered as

a donor lymphocyte infusion, either prophylactically or as treatment (9) (10). Because of the limitations in approaching healthy donors and single-patient manufacturing lots of virus-specific T cells, some centers have developed 'third-party' T cell banks derived from a panel of donors selected to span the most common HLA alleles (11) (12) (13). The Baylor group has pioneered the use of T cell lines that are specific for three to five viruses simultaneously, and have administered these to patients either as donor-derived or as third-party derived lymphocyte infusions (11, 14-16). Importantly, the incidence and severity of graft vs host disease has been limited or tolerable in all of these studies. These forms of adoptive immunotherapy are the most clinically advanced, with publication of Phase II, multicenter trials [11].

Cancer

Immunotherapy for cancer has a long and somewhat checkered history; the first observations of immune system engagement having anti-tumor effects are often attributed to William Coley, who observed regression of sarcoma following severe bacterial infections in the 1890s (17). However, it was the seminal finding that allogeneic immune reconstitution after bone marrow transplant had anti-leukemic effects (18) that definitively identified the anti-cancer effects of immune cells. Allogeneic bone marrow transplant remains the most potent, widely available form of cellular immune therapy and offers curative potential for hematologic malignancies. Researchers soon noted that the major mediators of the graft-vs-leukemia effect were T cells (19), while a contribution by NK cells was noted later (20) but can also be quite potent (21). There is a strong rationale to combine T cell therapy with NK cell therapy because NK cells do not cause graft vs host disease while they may limit resistance to T cell therapy through the emergence of MHC class I deficient tumor cells.

In the case of relapse after allogeneic transplantation, withdrawal of immunosuppressive therapy and/or donor lymphocyte infusions (DLI) are considered standard therapies but do have the potential to cause or worsen graft vs host disease. Ex vivo activation and culture of donor lymphocytes has also been clinically evaluated and appears to have modest benefit over standard DLI (22), particularly in hematologic malignancies aside from chronic myeloid leukemia (CML). Although CML was formerly one of the most common indications for transplant, it tends to be the most response to immune manipulations such as DLI; in the modern era, it is most often treated with tyrosine kinase inhibitors such as imatinib, dasatinib, and nilotinib. The limitation of these inhibitors is that they are expensive and while they result in long term remissions in most cases, they are not curative. The major opportunity for research in CML is to combine targeted agents such as kinase inhibitors with adoptive transfer therapy, with the goal of developing a curative regimen.

In solid tumors, investigators have hypothesized that tumor-infiltrating lymphocytes are the result of a naturally-occurring, yet ineffective, T cell response to the tumor. The observation of tumor-infiltrating lymphocytes (TILs) has spawned three forms of immune-based clinical interventions designed to convert TILs into effective cells: First, systemic administration of cytokines and immunologically active proteins such as IL-2 and interferon, which are currently approved for melanoma; second, systemic administration of antibody therapies aimed to modify T cell activation and relief of checkpoint blockade, such as ipilimumab

(anti-CTLA-4), anti-PD-1 and anti-PD-L1, anti-4-1BB and anti-CD40, to name a few. Checkpoint blockade therapy has had remarkable results not only in melanoma (23), but also in tumors such as lung cancer that have been previously thought to be 'immunologically silent' (24, 25). Even more encouraging, simultaneous blockade of two checkpoints (CTLA-4 and PD-1) in melanoma significantly improved the response rate and time to response over either therapy alone (26). The third TIL strategy is direct isolation and ex vivo activation of the tumor-infiltrating lymphocytes and has been tested in multiple early-phase studies and results in durable responses in melanoma (27). In the majority of presenting cases, however, this approach cannot be undertaken, either because surgical material is not available or contains insufficient numbers of TILs, or the patient cannot tolerate the conditioning regimen or the time required for manufacturing of their TIL product, preventing the ability to conduct randomized controlled studies.

As discussed below, recent advances in the use of genetically engineered T cells, and an understanding of the principles underlying effective T cell therapy, have produced encouraging results in the use of T cell therapies for viruses, hematologic malignancies, and solid tumors. T cell therapy is now poised to advance from Phase I trials to more Phase II and Phase III trials, and for the first time is being actively clinically developed by multiple biotech and pharmaceutical companies, with the goal of offering a standardized, quality-controlled, regulatory-body approved treatment for the integration of cell therapies to the treatment of patients worldwide.

Principles of T cell transfer: the soil, the fertilizer, the seed

T cell transfer and engraftment into the host is a complex biologic process, and can be optimized by a thorough understanding of the role of the host immune system (the soil), growth factors and the balance against inhibitory cells (the fertilizer), and the transferred T cell product (the seed) (Figure 1).

Preparation of the soil (host conditioning)

Evidence from bone marrow transplantation and adoptive therapy trials of TILs demonstrated that 'conditioning,' or lymphodepleting the host enhanced engraftment of the transferred T cells. There are multiple hypotheses as to why conditioning, or preparing the 'soil' for the incoming T cells, is attractive, particularly in the setting of malignancy. These include reduction of tumor burden (thus improving the effector:target ratio in vivo), reducing the population of inhibiting regulatory T cells (28), and inducing production of homeostatic cytokines to facilitate proliferation of the transferred T cells (29). Typical regimens for host conditioning include cyclophosphamide with or without fludarabine; some centers also use total body irradiation. All of these techniques, particularly the most intense ones that combine chemotherapies and irradiation (30), appear to improve persistence of the transferred T cells and the clinical responses in the setting of cancer. Notably, however, host conditioning has not been required in the setting of HIV infection to enable long term persistence of transferred T cells (5); similarly low numbers of virus-specific T cells can persist and expand in the post-transplant setting (31). Furthermore, recent reports with CAR-modified T cells administered in the absence of host conditioning have had clinical effects in both hematologic and solid tumors (32, 33).

Fertilizer: cytokines and T-cell modulating antibodies

Cytokines provide important growth and homeostatic signals to T cells, with IL-2, IL-7, and IL-15 being particularly well-studied. Recombinant human IL-2 as a single agent is FDA-approved for metastatic melanoma, though because of its toxicity, it is only administered in select centers. Furthermore, the biologic role of IL-2 is physiologically complicated; low-dose IL-2 is thought to maintain regulatory T cells and has been used to control graft vs host disease (34). Thus, it is not clear that administration of IL-2 will help the transferred cytotoxic T cells over the native regulatory T cells. Strong pre-clinical data support the use of IL-7 and IL-15, both of which are also being explored in clinical trials. IL-15 in particular is thought to relieve the inhibition of regulatory T cells while providing support for adoptively transferred T cells (35).

The combination of adoptive cell therapies with newer agents, including checkpoint blockade and/or small molecule targeted therapies is still in its nascent stages but is bound to generate excitement. Many combinations can be envisioned: co-administration of agonistic CD40 antibodies (36) or 4-1BB antibodies (37) to mediate co-stimulation, or with checkpoint blockade such as anti-PD-1 or anti-CTLA-4, is likely to improve both the effects of the transferred T cells and to stimulate the native T cell responses to tumors. Typically, small-molecule drugs aimed at aberrant signaling in the tumor effects rapid but short-lived tumor responses, while immunotherapy approaches take longer to eliminate tumor but are potentially long-lived. Combinations of treatment with T cell transfer coupled with small molecule drugs targeting tumor mutations (such as BRAF inhibitors in melanoma (38), or Bruton's tyrosine kinase or Bcl-2 inhibitors in lymphoma) have the exciting potential to make cancer treatment chemotherapy-free. (Tables 1-3 and Figure 2)).

Seed: the cell product

Because of the complexity of T cell activation, differentiation, and homeostasis, several groups have investigated the optimal cell population to serve as the 'seed' for adoptive cell therapy.

It is now clear that T cells that have been cultured extensively, whether stimulated with autologous dendritic cells, artificial antigen presenting cells (APCs), or cloned and passaged on allogeneic feeder cells, have a terminally differentiated phenotype with a loss of in vivo engraftment and proliferative capacity, and limited in vivo function. Reprogramming of the T cell (39, 40) may overcome these effects, but is associated with its own complex culture system that will be difficult to adopt widely. Currently, culture systems that rely on repetitive antigen stimulation to generate a T cell product are not easily scalable, efficient or reliable enough to generate functional T cells for immunotherapy, except perhaps as third party donor banks, which are expensive to generate due to the heterogeneity of HLA types in the population.

In an effort to maintain the persistence and function of adoptively transferred T cells, some investigators have used T cells specific to a chronic virus such as EBV or CMV and re-directed them to tumor-associated antigens (41, 42). Others have explored using phenotypically defined populations that are expected to proliferate and survive for longer,

such as central memory T cells (43) or naïve T cells (44), to improve engraftment in pre-clinical studies. Recent data has identified and characterized an early-differentiated, stem-cell memory T (T_{SCM}) (45). These cells constitute the most undifferentiated human T-cell compartment exhibiting bona fide memory functions, and can survive for extended periods even after the loss of cognate antigens. This type of T cell is thought to persist and support memory T cell functions, which would make it an ideal candidate for long-term control of cancer in addition to engaging it for viral vaccine purposes (46). However, it is not clear that the frequencies of the TSCM peripheral blood samples are consistent in large numbers of diverse cancer patients, and it will require validated and clinically approved systems to isolate these cells to form the basis of a cell therapy product. (Figure 3)

Finally, although the efficacy of adoptive cell therapy is most often attributed to CD8+ T cells, there are reports of pure CD4+ T cell populations mediating tumor regression (47). Furthermore, immune effector cell types other than T cells can and have been used in cell transfer protocols. For example, NK-based trials have been published in the autologous (48) and allogeneic settings (20), and re-direction or engagement of NK cells is an area of active research.

Strategies of ex vivo T cell culture

An inherent barrier to widespread clinical application remains the manufacturing difficulties and the access to robust and efficient methods for the expansion of input T lymphocytes. Our laboratory has developed methods for the efficient activation, expansion, and gene transduction of T lymphocytes. (Figure 4) Additionally, desired properties of adoptive immunotherapies include 1) demonstrated potency against tumor or infectious organism, 2) efficient engraftment enabling a high effector to target ratio, 3) long term persistence and memory.

T Cell Therapy and *Ex Vivo* Culture Methods

The clinical application of T-cell based therapeutics has gained extensive momentum within the past 30 years due to a number of critical discoveries that included the identification of T cell antigens that have also been tested as cancer vaccines (49). There have been a large number of studies that suggest that DCs, when appropriately activated and induced to present tumor-associated antigens can elicit tumor-specific T cell immunity. This dendritic cell therapeutic approach is currently being pursued by several biotechnology companies (50-53), but has limitations in that the ability to generate dendritic cells varies from patient to patient and this variability may result in short-term or insufficient T cell activation to generate an effective immune response.

Magnetic Bead-Based Artificial Antigen Presenting Cells

With recognition that both a primary specificity signal via the T Cell Receptor (TCR) (Signal 1) and a costimulatory/regulatory signal via the CD28 receptor (Signal 2) are simultaneously required for the generation of full T-cell effector function and a long-lasting immune response (54), we developed efficient and reproducible methods of mimicking the signal provided to T cells by dendritic cells, but without delivering a negative costimulatory

signal. With artificial Antigen Presenting Cells (aAPC), T cells to be grown rapidly ex vivo to clinical scale for therapeutic applications. The technology enables direct T cell activation, instead of indirect activation via vaccines, which can be modulated by the nature of cell dose as necessary to achieve a clinical response (55, 56).

The first generation of off-the-shelf aAPC covalently linked clinical grade anti-human CD3 and anti-CD28 monoclonal antibodies to magnetic Dynal beads (Life Technologies) which serve to crosslink the endogenous CD3 and CD28 receptors on the T cell. This bead-based aAPC enables the most efficient reported growth of human polyclonal naïve and memory CD4+ T cells (56). In terms of cell function, the expanded cells retain a highly diverse TCR repertoire and, by varying the culture conditions, can be induced to secrete cytokines characteristic of T helper 1 (Th1) or T helper 2 (Th2) cells (57). One important advantage of this bead-based system is that it does not cross-react with CTLA-4 and therefore provides unopposed CD28 stimulation for more efficient expansion of T cells. Another, unanticipated discovery was that crosslinking of CD3 and CD28 with bead-immobilized antibody renders CD4+ T lymphocytes highly resistant to HIV infection. This is due to the down-regulation of CCR5, a necessary co-receptor for the internalization of HIV, as well as the induction of high levels of β-chemokines, the natural ligands for CCR5 (58-60), and allows for the efficient culture of CD4+ T cells from HIV-infected study subjects. Ex vivo expansion may also indirectly enhance T cell activity by removing T cells from a tumor-induced immunosuppressive milieu (61-64). Other key features are that exogenous growth factors or feeder cells are not needed to enable the T cell stimulation and expansion, as with previous methods.

Cell-based Artificial Antigen Presenting Cells

Cell-based artificial Antigen Presenting Cell (aAPC's) lines have been derived from the chronic myelogenous leukemia line K562 (65-67). K562 cells do not express Major Histocompatibility Complex (MHC) or T costimulatory ligands, and these cells may represent a DC precursor that retains many other attributes that make DCs such effective APCs, such as cytokine production, adhesion molecule expression and macropinocytosis. These cells have been transduced with a library of lentiviral vectors that allows for the customized expression of stimulatory and costimulatory molecules that can used activate and expand different subset of T cells, and be further modified to amplify antigen specific T cells in culture. These aAPCs offer the advantage of expression of molecules additionally to CD3 and CD28 on their surface. The K562 aAPCs have been transduced with vector to express the antibody Fc-binding receptor and the costimulatory molecule 4-1BB. The expression of CD64, the high affinity Fc receptor, on K562 aAPC's allows the flexibility of loading antibodies directed against T cell surface receptors. CD3 and CD28 antibodies are added to the cells and are bound by the Fc receptor to yield a cell that expresses CD3, CD28 and 4-1BB. These cell-based aAPC's have proved to be more efficient at activating and expanding T cells, especially CD8+ and antigen-specific T cells, than the magnetic bead-based aAPC (66-68). In addition, the cells are capable of stimulating CD4 cells efficiently.

Thus, K562 cells may represent ideal cell scaffolds to which the desired MHC molecules, costimulatory ligands, and cytokines can be introduced in order to establish a DC-like

aAPC. Advantages of this artificial dendritic cell platform include high levels of MHC expression, a wide array of costimulatory ligands and the ability to engage in cytokine crosstalk with the T cell. This mimics the advantages of natural dendritic cells, without recognized disadvantages including the need to derive natural DCs from either G-CSF mobilized CD34+ cells or monocytes, patient specific differentiation, limited life span, and limited replicative capacity. Moreover, these cells have been injected into humans as part of a tumor vaccine (69), signifying that these cells can be used in a GMP manner. Additionally, our laboratory and our collaborators have now developed either bead or cell-based aAPCs optimized for Th2 cells (57) (70), and for T regulatory cells (71).

Manufacturing Process

Independent of which of the above aAPC's is used, the manufacturing procedure remains similar, starting with an apheresis product. Alternatively, T cells can be derived from a blood draw, bone marrow, ascites, or tumor infiltrating lymphocytes. The pheresis product may be washed out of collection buffer in a Haemonetics CellSaver5 or other automated cell washing device, or directly loaded in the Terumo Elutra™ Cell Separation System for depletion of monocytes and isolation of lymphocytes. The depletion of CD4+, CD8+, or CD25+ T cells can be accomplished using a Miltenyi CliniMACS®. This instrument is an electromechanical device intended to isolate certain cell subsets via large scale magnetic cell selection in a closed and sterile system. Before selection, the washed cells from a pheresis product are magnetically labeled by using particles conjugated with anti-CD4, anti-CD8, or anti-CD25 mAb. A single-use tubing set, including separation columns, is then attached to the CliniMACS® Instrument and the cell preparation bag, containing the labeled cells. After starting the selection program, the system automatically applies the cell sample to the separation column, performs a series of washing steps depending on the program chosen and finally elutes the purified target cells.

The lymphocyte fraction from the Elutra™ Cell Separation System, or enriched T cells are cultured in a nutrient media and stimulated to divide and grow via the addition of the antibody coated magnetic beads or of irradiated and antibody pre-loaded K562 aAPC's, each of which is described above. Gene transduction of anti-CD3/anti-CD28 aAPC stimulated T cells with retroviral, lentiviral, or adenoviral vectors is very efficient (72-74). The whole mixture of cells, growth media, vector and aAPC is added to a gas permeable plastic bag or alternative culture vessel. Tubing leads on the bags and a variety of connecting devices allow the cells to be grown in a closed system with minimal risk of contamination. After gene vector washout, if needed, on the Baxter CytoMate and during log phase cell growth, cultures are transferred to the WAVE Bioreactor, where cell concentrations may reach 1×10^7 cells/ml or higher. The advantage of the WAVE is that T cells can be grown at higher densities, which saves labor on processing and during the cell harvest. The cultures are maintained for 9-11 days prior to harvesting and preparation for reinfusion or cryopreserved for later infusion. At harvest, magnetic bead-based aAPC are removed, the cells are washed, resuspended and cryopreserved in an infusible solution. If the cells are to be infused fresh, in process samples are taken for microbiological testing, viability, and cell phenotype by flow cytometry for the release. Testing is repeated on the final product, although results for some tests are not available until after the cells are infused.

Genetic engineering platforms

T lymphocytes can be modified by gene transfer methods to permanently or transiently express therapeutic genes to enhance and expand the therapies. Importantly, genetic engineering can also be used to endow lymphocytes with several other features, including an increased proliferative potential (75), a prolonged in vivo persistence (76), an improved capacity to migrate to tumor tissues (77), or to recognize an entirely new antigen. Re-direction of antigen-specificity is usually based on either a TCR of known specificity (78) or a synthetic receptor such as a chimeric antigen receptor (CAR), which recognizes antigen through antibody-derived complementarity-determining regions, but signal through TCR-associated molecules (79).

Current clinical trials of permanently modified T cells employ viral and non-viral based approaches. Retroviral (gamma-retroviral and lentiviral) vectors can be used to transduce cells without producing any immunogenic viral proteins, with the transgene becoming a permanent part of the host cell genome. Retrovirus-based gene delivery has proven to be an extremely useful tool in gene therapy research and is commonly used in trials of T cell therapies. Non-viral DNA transfection or transposons are also used for permanent gene expression in gene-modified T cell based therapies. Gene delivery by using adenoviral vector or RNA transfection enables the transgene expression for up to 1 week; these approaches have promise for when transient transgene expression is desirable (Figure 5).

Gamma-Retroviral vectors

Currently, most retroviral vectors are derived from murine or avian retroviruses. The Moloney murine leukemia retrovirus (gamma-retrovirus) has been extensively studied as a vector, and can package up to 8 kb of genetic material. Vectors that are derived from gamma - retrovirus have been most useful for long-term gene expression because of their ability to integrate into the host genome, which results in permanent expression of the transgene with low intrinsic immunogenicity (80). The first human trial of immunotherapy with gene-modified T cells was reported in 1990 in patients with advanced melanoma using tumor-infiltrating lymphocytes modified by retroviral gene transduction (81); in this case, the retrovirus was used to encode neomycin resistance and served only to track the fate of the infused T cells. Also, in 1990, two girls suffering from adenosine deaminase severe combined immunodeficiency (ADA SCID) were treated by T lymphocytes transduced with a gammaretrovirus expressing the ADA gene, which led to the reconstitution of the patient's immune system with those gene-corrected T cells (82). Since then, retroviral vectors have been the major tools for permanent transgene expression, and have been widely used as vehicles to deliver genes into different type of cells for gene therapy, including T lymphocytes (83, 84). Hematopoietic stem cells (HSCs), which have the potential to self-renew and differentiate into all blood lineages, were initially thought to be the most desirable targets for retroviral gene modification for the treatment of genetic disorders and other diseases (85-91). Although initial results were encouraging, adverse events were observed in trials for SCID-X1 and X-chromosome linked chronic granulomatous disease (X-CGD) due to vector integrations in the vicinity of well-characterized proto-oncogenes (87, 92, 93). It is believed that the target cells most vulnerable to insertional-mediated

transformation are primitive progenitor cells, and that more mature cells are less prone to this event (94-97). T lymphocytes remain major targets for retroviral-based gene modification, not only to deliver therapeutic genes but also to be redirected for a specific tumor-associated antigens (3, 78, 98-100). Unlike HSCs, T lymphocytes are less susceptible to transformation (101). Although insertional mutagenesis can contribute to immortalization of retrovirally transduced mature T cells in vitro, it is a rare event, and occurs in the setting of a synergistic effect between activation of a proto-oncogene, such as LMO2, and robust signaling through T cell homeostatic cytokines, such as IL-2 or IL-15 (102, 103). Whether a T cell stimulating signal generated by a transduced TCR or CAR can synergize with activation of a proto-oncogene caused by retroviral insertional mutagenesis to mediate transformation of the transduced T cells remains to be elucidated. Malignant transformation has not been observed thus far in clinical trials of retroviral-based gene transfer into mature T cells (3, 5, 72, 104).

Lentiviral vectors

Lentiviral gene transfer is relatively new and shares many features of the retroviral system. Lentiviruses are distinct members of the retroviruses family. Lentiviral vectors have been constructed from several types of lentiviruses, but the most commonly used is the human immunodeficiency virus (HIV), because its molecular biology has been extensively studied (83, 105).

Lentiviral vectors resemble gamma-retroviral vectors in their ability to stably integrate into the target cell genome, resulting in persistent expression of the gene of interest; they can accommodate up to 10 kb transgene material, and the immunogenicity of the vector is low. However, in contrast to gamma-retroviral vectors, lentiviral vectors have the advantage of being able to transduce non-dividing cells (106), they have broader tissue tropisms, and have a potentially safer integration site profile (107, 108). Furthermore, lentiviral vectors are less susceptible to gene silencing by host restriction factors (109). These distinctive features broaden the possible applications of lentiviral vectors, especially in settings where gamma-retroviral vectors are not suitable. Lentiviral vectors have been safely used in human clinical trials to engineer HSCs and T lymphocytes and there have been no oncogenic events observed (110) (73, 89, 90, 111). However, clonal expansion and dominance of hematopoietic progenitors has been reported in a clinical trial in which HSCs were genetically modified with a lentiviral vector that expressed the beta-globin gene for the treatment of thalassemia (90). Therefore, genotoxicity of insertional mutagenesis is still a potential safety concern for lentiviral vectors.

From a manufacturing perspective, it is notable that stable packaging cell lines are easily established for gamma-retroviral vectors, whereas this is still challenging for lentiviral vectors due to the toxicity of envelope proteins; this limitation obligates researchers to generate vectors from inefficient, transient multi-plasmid transfections. In addition to the risk of insertional mutagenesis, another potential safety issue applicable to both gamma-retroviral and lentiviral vectors is the possibility of generating replication competent retroviruses (RCR). Although new generations of vectors have been designed to reduce the

production of RCR, these additional steps result in decreased efficiency of vector production.

Adenovirus vectors

Adenovirus vectors are capable of transducing both dividing and quiescent cells; they can accommodate relatively large transgenes, production of high-titer vector stocks is relatively easy, and the vectors are non-oncogenic due to their lack of integration into the host genome. Adenovirus vectors are widely used in clinical trials, especially for cancer targeted gene therapies. Application of adenovirus vectors in T cell-based therapy is limited by the transient transgene expression and the immunogenicity of the vector. Chimeric adenoviral vectors Ad5–F35 were reported to mediate gene transfer up to 10% of resting T cells and 30–45% of T cells after activation with phytohaemagglutinin (112). Ad5F35 vectors could result in gene transfer in more than 90% of T cells after activation by CD3 and CD28 specific antibodies (8, 74). Adenovirus vectors have promise as gene delivery vehicles to T cells in clinical situations where duration of transgene expression of less than a week is required, and there is no foreseeable need for repeated cell infusions. Examples of such situations can be envisioned for gene editing strategies such as zinc-finger nucleases (ZFN) or transcription activator-like effector nucleases (Talens), or Clustered Regularly Insterspaced Short Palindromic Repeats(CRISPR)-mediated specific gene silencing (8).

DNA transfection and Transposon-based gene delivery

Non-viral based DNA transfection methods remain popular as vectors for gene therapy due to their low immunogenicity and a low risk of insertional mutagenesis. The first clinical trial testing the adoptive transfer of T cells engineered using electroporation was recently reported (113), and although this approach was safe, the cells were short-lived after transfer, probably owing to the long-term culture of the cells that was required to select sufficient numbers of permanently integrated T cell clones for the treatment.

Transposon-based systems can integrate transgenes much more efficiently than plasmids that do not contain an integrating element (114, 115). Sleeping Beauty (SB) was shown to provide efficient stable gene transfer and sustained transgene expression in primary cell types, including human hematopoietic progenitors, mesenchymal stem cells, muscle stem/progenitor cells (myoblasts), pluripotent stem cells and T cells (116). Various transposase-based systems are now entering clinical trials to test the safety and feasibility of this approach to engineer T cells (117). Non-viral vectors have several advantages over viral vectors as a modality to engineer cells, including lower costs and perceived safety benefits. However, the safety profile of these approaches is still uncertain, since the relative genotoxicity of transposons is unknown. Approaches to achieve site-specific integration and DNA editing are under development, and if these prove to be efficient, they should allay concerns regarding lymphocyte engineering using non-site-specific integration approaches.

RNA transfection

Thus far we have discussed viral transduction or plasmid DNA transfection of T cells, which can result in stable genomic integration, allowing for constitutive, permanent expression of the transgenes. Safety concerns, such as genotoxicity, potential generation of RCR, and the

difficulty in predicting off-tumor toxicities are potential limiting factors for the widespread use of gene-modified T cells, particularly as a first approach in the clinical setting. Moreover, there are clinical situations in which multiple infusions of the engineered T cells would be required (i.e. to overcome the lack of persistence or the immunosuppressive influence of the tumor microenvironment), making manufacturing a clinical dose T cell product difficult to achieve and expensive. When transient expression of the transgene is desired, such as to identify potential off-tumor toxicities, or recursive infusions are planned, RNA transfection of T cells is an attractive approach because it is relatively inexpensive, fast, and the transfection efficiencies can easily approach 100%. RNA-based electroporation of human T lymphocytes using *in vitro*-transcribed mRNA mediates transient expression of proteins for approximately 1 week. The self-limiting transgene expression can provide a safety check for off-tumor, on-target or off-target toxicities, or other unwanted side effects, as the engineered T cells are essentially a 'biodegradable' product. Furthermore, there is no genotoxicity concern as the introduced mRNA does not integrate into the host genome. RNA electroporation has been used to deliver message for TCR or CAR, chemokine receptors or cytokines (118-121). In one study, T cells modified by CAR RNA were evaluated in a side-by-side comparison with retrovirus modified T cells; RNA engineering was at least as efficient as retroviral gene transfer (122). Alternatively, for transposon-based gene delivery systems, transposase enzymes can be delivered as mRNA thereby avoiding the possibility of genomic integration (123); gene editing strategies based on ZFN, Talens or CRISPR can be also delivered by RNA transfections. In pre-clinical animal studies, multiple injections of CAR RNA modified T cells can mediate regression of disseminated tumors (124-126). Clinical trials of treating solid tumors with RNA-electroporated CAR T cells have been initiated by several groups (33) and the safety and efficacy results will provide valuable information for further cancer treatments using genetically modified T cells.

T cells re-directed with specific T cell receptors (TCRs)

Transduction of T cells with a specific TCR has the advantage of redirecting the T cell to an intracellular antigen. Given that most oncogenic proteins are intracellular, development of a panel of TCRs specific to oncogenic driver proteins has great appeal. However, a library of MHC-restricted antigen-specific TCR reagents would need to be characterized and available to treat patients, who have diverse HLA alleles. Furthermore, the low-affinity of most tumor-directed TCRs is thought to significantly impact their efficacy. This is one reason that most peptide-cancer vaccines alone or in combination with adjuvants or professional antigen-presenting cells have produced underwhelming clinical responses despite in vitro evidence of tumor-directed T cell responses (127-129).

Several groups have explored retroviral transduction of native T cell receptors with the goal of re-directing a bulk of T cells to an intracellular antigen. Potentially significant obstacles were hypothesized for the reason that when a T cell transcribes the chains for two different TCRs, there are four potential combinations of T cell receptors that can be expressed at the cell surface (native-alpha/beta, transduced alpha/beta, and native/transduced 'mispaired' heterodimers). This is problematic for two crucial reasons: (1) the native/transduced heterodimers have unknown specificity and potential autoimmune consequences, which has been demonstrated to occur in some mouse models (130), and (2), there is dilution of the

signal transduction apparatus, since the availability of CD3 complex molecules is limiting. Early studies of HIV-directed TCRs encountered low levels of expression of the transduced TCR, along with mispairing; this combination resulted in decreased efficacy in vitro and heterogeneous populations of T cells (131). Several groups have described methods to favor pairing of the transduced TCRs by engineering the transduced TCR chains, including (1) partial murinization of the constant regions(132-134), (2) the addition of disulfide bonds (130, 135-138), (3) altering the knob-in-hole directional interaction of the endogenous TCR constant regions (139), and (4) adding signaling domains to the intracellular portions of the transduced TCR (140). Another interesting approach is to knockdown the endogenous TCR with gene editing or shRNA (141); a third party bank of T cells that also have endogenous HLA knocked down can also be envisioned (142). Most of these modified TCR designs are still in pre-clinical development. (Figure 6)

Nevertheless, trials of native TCR-transduced T cells have been reported, and though some have resulted in significant anti-tumor responses (99), others have noted significant on-target toxicity (78, 143) and off-target toxicity (144), particularly when the TCR has relatively high affinity for its cognate antigen. In one clinical study of a high affinity transduced TCR to the MART-1 melanocyte antigen, off-tumor toxicity was observed in the form of significant uveitis and otitis, based on destruction of pigmented cells in the eye and inner ear, respectively (78). In a second example of off-tumor on-target toxicity, a TCR specific for the MAGE-A3 cancer testis antigen was known to cross-react with an epitope derived from the related antigen MAGE-A12; in clinical trials, neurologic toxicity was observed and found to be a result of previously unrecognized expression of MAGE-A12 in the brain (143). The dose-limiting toxicity of TCRs directed to carcinoembryonic antigen (CEA) was colitis (145). Although testing for on-target toxicity can be relatively straightforward, for example by RT-PCR from archived normal tissues, testing for potential off-target toxicities of TCRs is significantly more challenging. Typically, the starting point for identifying off-target toxicity requires testing of the new TCR against a panel of live cultured cells that are representative of human tissues to serve as targets. In one case, despite extensive pre-clinical testing on panels of cell lines, cardiac toxicity of a MAGE-A3 directed TCR could not be replicated in vitro until beating cardiac myocytes derived from induced pluripotent cells were used as targets (144, 146); in this case, the cause of ‘off-target’ toxicity turned out to be the result of TCR cross-reactivity to an unrelated peptide derived from titin(144, 146). Interestingly, the effects of TCR-transduced T cells that have been encountered clinically have not been a result of the predicted effects of mispairing and poor signaling; rather, the toxicities have been related to TCR affinity and specificity, and demonstrate the high potency of TCR-transduced T cell products. Clinical trials with native-TCR and engineered-TCR transduced T cells directed to a number of HLA-restricted antigens are underway in hematologic malignancies and solid tumors (Table 3), with results showing early promise (147).

T cells re-directed with chimeric antigen receptors

The first generation of chimeric antigen receptors (CARs) were engineered receptors comprising an scFv (where the variable portions of the light and heavy chains of a high-affinity antibody are connected by a linker sequence), a transmembrane domain and the

signaling domain of CD3zeta (79, 148). Since then, second generation CARs have included the costimulatory domains derived from CD28, 4-1BB, or OX40 to optimize T cell activation, and these have improved function in vivo particularly against more aggressive tumors that do not express costimulatory molecules (149); third ‘generation ’ CARs also include the signaling domains of a third molecule such as TNF receptor family members such as 4-1BB or Ox40; these have less potent cytotoxic activity but persist longer in vivo (150-152). Because CARs are antibody-based, high-affinity single-chain variable fragments (scFv) derived from antibody sequences typically have been directed to native surface antigens, which restricts suitable targets to proteins or epitopes displayed on the surface of the target cell. (Figure 7)

The exact mechanisms of how CARs function are still unknown (153), but it does appear that CARs homodimerize independently of the TCR, and only become part of the CD3 complex if the transmembrane domain selected is that of CD3zeta (154). This is not entirely surprising given that the transmembrane or intracellular signaling domains typically dimerize (CD8alpha, CD28), although some of them (4-1BB) are thought to form trimers in their native conformation. There is also some evidence to suggest that the spacing and conformation of binding to the epitope between the target and the CAR-T cell is important in optimizing CAR-induced signaling (155), whereas affinity appears to have less of a role in CAR-T cells (particularly compared to TCR-redirected T cells).

Similarly to TCRs, CAR-directed T cells do seem to maintain an exquisite sensitivity to low levels of the cognate antigen. This is perhaps surprising because the signaling domains of CARs are not expected to amplify signal to the same degree as TCR triggering of the entire CD3 receptor complex. Indeed, in vitro data suggests that CAR-T cells have a threshold for signaling at about 50 molecules/target, whereas native T cells require engagement of 1-10 molecules for TCR triggering (156, 157). Recent results suggest that the TCR has multiple modes of downstream signaling that regulate discrete functional events, and that the number of ITAMS recruited to the synapse regulate these distinct signaling pathways (158). Clinical investigation of CAR-T cells have confirmed this low threshold for activation: in trials of CAR-T cells directed to the tumor antigen Her2/neu or carbonic anhydrase IX, subjects have experienced severe toxicity based on low-level expression of the target antigen in the lung or the biliary tract, respectively (159, 160).

Currently, most pre-clinical investigation of new forms of CAR-T cells involve xenogeneic immunodeficient models, where human tumor is implanted either subcutaneously or orthotopically, and human T cells are injected either into the tumor or intravenously, either simultaneously or after tumor engraftment. These models are limited in that the tumor microenvironment is not replicated in the animal, the remaining arms of the immune system are absent or debilitated, and there is generally no possibility of evaluating off-tumor expression of target. In the case of CD19, for example, pre-clinical models did not predict that CAR-T cells would cause the degree of cytokine release or macrophage activation that has been observed clinically (32). Although syngeneic models (161, 162) may overcome some of the limitations of the xenogeneic models, the active cell under evaluation is the engineered T cell, and mouse and human T cells do exhibit significant mechanistic differences that affect the evaluation of the engineered CAR molecule; for example, mouse

T cells are much more dependent on CD28 signaling, and 4-1BB signaling has modest effects at best (163, 164).

There are number of clinical trials of CAR-T cells directed to a variety of antigens underway (Tables 1-2) (165). Several centers have directed effort to CD19-directed CAR-T cells (Table 1), and to other B cell markers such as immunoglobulin light chains and CD20 (Table 2), in part because hematopoietic cells have been extensively characterized, and the expression of their surface molecules is often lineage-dependent. Multiple reviews of the CD19 CAR-T cell trials have been written (166-169), and are only discussed in aggregate here. One issue that has complicated the interpretation of the CD19 CAR-T cell trials is that each center has developed their own CAR, with different single-chain variable fragments to effect antigen binding, different signaling domains, different modes of introduction of the CAR gene into T cells, different conditioning regimens, and different post-CAR-T infusion interventions. However, in sum, it has become apparent that some B cell malignancies are more consistently clinically responsive to CD19-CAR T than others; for example, trials in chronic lymphocytic leukemia (CLL) have yielded very mixed clinical results (111, 170-173), whereas trials in acute lymphocytic leukemia (ALL) have yielded impressive responses in multiple centers (32, 100, 174). In one case of pediatric ALL, a patient relapsed with CD19-negative disease, indicating that the CD19-CAR T effected very strong selective pressure on cells expressing the CD19 target (32). In contrast, tumor cells from all the non-responding patients with CLL appear to retain CD19 expression. The fate and length of persistence of the CAR-T cells seems to have a significant impact on the clinical responses, but the determinants of these variables are still unclear. It is likely that factors such as the input cell population and the tumor microenvironment play a prominent role in determining CAR-T persistence and therefore on clinical efficacy, even when other variables (type of CAR-T and manufacturing process) are controlled.

There is also great interest in developing CAR-T against other hematologic malignancies. The carbohydrate antigen Lewis Y (carbohydrate antigen) is being tested as a potential target in AML, MDS and multiple myeloma patients (clinicaltrials.gov NCT01716364). Specific targets for AML (175) and multiple myeloma(176) are in pre-clinical development.

The development of CARs for solid tumors has been challenging, in part because of the lack of extensive literature on specific surface markers expressed on malignant epithelial cells. Furthermore, it is not clear that many surface markers are exclusively expressed on tumor cells, and more often, targets with merely higher levels of expression on tumor than normal tissue have been selected as CAR targets. Although this approach has safety concerns (160, 177), it is possible that a therapeutic window will be found. For example, despite the death that rapidly ensued after administration of 10^{10} dose of Her2/neu CAR-T in a patient aggressively conditioned, new trials directed against the same antigen but starting with lower doses are underway (NCT00902044, NCT01109095).

T cell products that employ a safety check mechanism, whether based on transient expression of the CAR (such as RNA electroporation (125)) or a suicide gene encoded into the transduced cells, are an attractive method to initiate clinical testing. This may be necessary, for example for FAP-directed CAR-T cells (178, 179), GD2-directed CAR-T

cells (180), or PSMA-directed CAR-T cells (clinicaltrials.gov NCT01140373), where pre-clinical testing or antibody-based testing in the clinic indicate some concerning potential tissue-directed toxicities. The choice of safety check mechanism may also affect the function of the CAR-T cells: transient expression of the CAR may sensitize the patient to the CAR (33); incorporating transient viral vectors or viral proteins may also be immunogenic. The humanized caspase9 suicide system is very attractive, and has been clinically tested in the setting of donor lymphocyte infusions after bone marrow transplant (181), but it is unclear whether all the transduced T cells are completely eliminated, should this be required. Alternatively, the synthetic biology of CAR-T cells may borrow more lessons from nature: that is, one way to increase specificity of the CAR-T cells is to separate T cell signal 1 (antigen) from signal 2 (costimulation). In one paper (182), this strategy was successfully implemented in in vitro and in mouse models, where the primary antigen-receptor had low affinity and delivered a weak (akin to a TCR signal), but a second engineered receptor that engaged separate antigen delivered the costimulatory signals (chimeric costimulatory receptor, CCR); only engagement of both engineered receptors generated a sustained T cell response.

The field of T cell engineering is now entering adolescence, and creative solutions to many of the current limitations are sure to emerge (Figure 8)). For example, one group of investigators hypothesized that in the setting of graft vs host disease following allogeneic bone marrow transplant, T cells could only cause damage to the tissues if they trafficked to those tissues; by reducing tissue trafficking with administration of a drug blocking CCR5 (maraviroc), GvHD was ameliorated in early studies (183), and confirmatory studies are underway. Incorporating chemokine receptors to alter tumor-specific T cell migration is also under investigation (77), as are mechanisms to improve T cell resistance to inhibitory signals and perhaps to avoid conditioning chemotherapy regimens (162).

The future of CAR therapies looks bright, in part because early studies have shown that CAR-T cells are quite potent; strategies to expedite the discovery of suitable surface antigens, and generating the single-chain variable fragments to perform rapid throughput testing on tumors and on normal tissues to identify potential off-tumor reactivity, have the potential to make CAR-T cells more widely applicable.

Biomarkers for T cell therapies

The field of biomarkers has undergone dramatic evolution over the past few years, with an increased realization that relevant and meaningful measures of biological activity are unlikely to be generated simply on the basis of hypothesis testing, and that endeavors to interrogate for biological activity need to be supplemented by broader and hypothesis generating studies (184). This concept is particularly relevant for strategies that seek to therapeutically manipulate the immune system through immune modulation or immune activation, where the inherent multidimensional and integrated complexity of the immune system inevitably confounds scientific reductionism. A parallel concept is the implementation of uniform data reporting guidelines to support more transparent and systematic analysis of data from T cell therapy trials (185).

In a general sense, biomarkers provide information about the bioactivity of the tested therapy. Beyond this point, in early stage trials principal objectives of biomarker studies are to identify parameters that reveal specific information about the mechanism of action of the treatment and to provide proof of the tested principle being operative. In more advanced trials, the focused principle objective of biomarker studies is to identify and eventually validate biomarkers that correlate with the efficacy of the treatment and can potentially be pursued as surrogate endpoints for the treatment.

Unlike traditional strategies, where the treatment modality is an inert chemical compound, cell therapies are characterized by the fact that the “drug” is a biologically viable, dividing, and evolving entity, which interacts with and responds to the myriad complexities of the host biology. As a result, biomarker strategies for cell therapies must focus on studying not only the impact of the infused cells on patient biology, but also the infused cells themselves (186). A fundamental starting point for these studies is a thorough understanding of the properties of the manufactured cell product, obtained through product release, potency, and characterization assays.

Arguably, the field of biomarker studies for T cell therapy trials was ushered in by the seminal reports from the NCI group which demonstrated cancer regression in melanoma patients following adoptive transfer of bulk TIL-derived lymphocytes (187) and subsequently gene-engineered MART-1 specific T cells (98). These studies were among the first to examine persistence of infused cells, characterize their surface phenotype, and indirectly demonstrate in vivo functionality through cancer regression and autoimmunity.

Perhaps predictably, persistence of infused cells has been shown to correlate with cancer regression and durability of remission (188). Indeed, a major limitation for maximal efficacy of T cell therapy-based approaches has been thought to be the lack of robust long-term persistence of transferred cells, a limitation that now appears to have been overcome in at least some settings (32, 111). More controversy exists with regard to the phenotype and functional status of T cells optimally needed for anti-tumor immunity. Earlier work suggested that TIL cells cultured minimally were less differentiated, more diverse phenotypically, and had superior efficacy following transfer (189). Other work from the surgery branch of the NCI has indicated that naïve rather than memory cells were superior for adoptive transfer, demonstrating better transduction, more robust expansion, enhanced proliferative potential and telomere length, and were less susceptible to terminal differentiation (190), while the same group has shown that adoptive T cell treatment efficacy may be related to the persistence of T cells that are or can convert in vivo to memory cells (191). More recently, work principally from the Seattle group in primate models has suggested that central memory cells might be more effective in adoptive T cell transfer strategies (43). Even more recent and provocative work from the Munich group has suggested that there is a phenotypic plasticity within at least some naïve and memory T cell subsets (192). Together these disparate results highligt the potential folly of interrogating differentiation phenotypes of persisting cells as an essential element of biomarker studies. Direct assessment of the in vivo functionality of infused cells has been difficult to accomplish, at least in part due to the aforementioned relatively poor persistence of infused cells, although recent studies have demonstrated directly ex-vivo the ability of long-term

persisting cells to recognize antigen-positive targets (111). Less direct measures of T cell bioactivity have included the measurement of systemic cytokine levels in patients post T cell infusion. Initial efforts in this area focused on specific cytokines directly associated with T cell effector functions; more recent and hypothesis agnostic efforts in the setting of potent clinical efficacy have revealed a broader profile of immune activation that might be an important element for ultimate efficacy of T cell-based immunotherapy strategies (32, 100, 111, 173).

The principal mechanism of action for T cells to effect anti-tumor activity is through direct engagement and cytolysis of target cells as well as production and secretion of soluble cytokine and chemokines which directly impact tumor cells and also orchestrate a more integrated anti-tumor inflammatory response. Accordingly, biomarker studies to interrogate T cell mechanism of action focus on detecting the presence and effector functionality of infused T cells. Although this objective is relatively straightforward to accomplish in cases where cancers are predominant in peripheral blood or bone marrow, it is a considerably more difficult challenge for cancers where disease is not readily accessible. Indeed, recent clinical data has provided compelling evidence to support the need to evaluate the T cell function at the site of disease (193, 194). Data principally accumulated in the context of adverse events demonstrate that infused T cells do in fact traffic throughout the body and home to sites where target antigen is expressed (144, 146), highlighting the critical need to develop innovative and sensitive approaches to enable the interrogation of tissues for T cell presence and functionality.

Approaches to monitor T cell persistence. In most adoptive T cell immunotherapy studies the total infused cell number corresponds to a small fraction of the total T cells, although lymphodepleting conditioning can skew this ratio at early timepoints post infusion. Homeostatic and antigen-driven expansion has been shown to drive high frequencies of infused T cells in patients at late times post infusion (32, 98, 111). Both molecular and flow-cytometry-based approaches have been developed to evaluate persistence and homing of infused T cells.

Quantitative PCR (Q-PCR)-based approaches have been developed in a number of cases to detect and quantify the persistence of infused T cells. Such approaches are feasible if the infused T cells have been genetically engineered prior to infusion, and typically afford for the ability to detect infused cells at frequencies as low as 0.01% of total cells, and provides important information about persistence, trafficking, and homing of infused T cells in patients. This approach is increasingly being applied in clinical studies to monitor T cell persistence see for example (32, 100, 111, 173), as well as to interrogate and demonstrate the contribution of infused cells to serious adverse events (SAE) (144). Recent technological advances in the ability to perform high throughput deep sequencing of CDR3 domains for TCR loci to detect and quantify individual TCR clonotypes in samples (195) opens up the potential to be able to obtain molecular signatures for individual clonotypes that persist in patients and correlate this signature to the original infusion product. A considerable limitation of molecular approaches is that they generate data from bulk populations of cells. The robust development of single cell multiplexed digital PCR-based approaches (196) opens up the exciting possibility for not only more sensitive detection of infused cells in

samples, but also the ability to interrogate functionality of infused engineered cells at the single cell level.

Flow cytometric based approaches depend on the availability of antibody reagents to detect gene–engineered and infused cells. Such reagents have included MHC class I multimers (dextramers, tetramers, pentamers) which have been employed in a large number of clinical trials; more recently in the context of CAR trials idiotype-specific antibodies that recognize CAR-engineered cells have been employed to detect infused cells with high specificity (32, 100, 111). This approach typically requires that the frequency of antigen specific cells is at least 0.2% of the total CD3 population, although reports of considerably more sensitive detection have been reported (197), as well as the development of higher-throughput combinatorial strategies that increase sensitivity and reduce sample usage (198). One advantage of flow-cytometry based approaches is that they can be readily combined with stains for surface phenotypic or functional markers allowing for the generation of more integrated data sets.

Technical advancements in polychromatic flow-cytometry combined with an increased understanding of T cell biology have precipitated a number of flow-cytometry-based approaches to interrogate T cell function; specific markers and strategies are summarized in a recent review (186). The continued development of mass cytometry based platforms and algorithm driven hierarchical clustering approaches which allow for the ability to simultaneously interrogate very large numbers of T cell molecules including surface and intracellular proteins, phosphoproteins and RNA species (199, 200) has the potential to render traditional flow cytometry-based approaches to interrogate T cell functionality as a stand-alone platform obsolete.

As discussed above, the integrated complexity of the immune system mandates that part of the evaluation of T cell therapy focus on the impact of the treatment on the broader immune system. Although this relatively new concept has not yet been broadly implemented in clinical trials, one approach that has been implemented with some success has been to evaluate systemic cytokine levels in patients during treatment. In studies targeting leukemias using CAR-engineered T cells this strategy has revealed that engineered T cell activation and anti-tumor activity results in broad and potent cytokine-driven effects, including cytokine release syndrome (CRS) (32, 111), as well as macrophage activation syndrome (MAS) and hemophagocytic lymphohistiocytosis (HLH). Notably, the agnostic interrogation of cytokines in these trials unexpectedly identified IL-6 as a major cytokine induced by the CAR therapy, an observation which has resulted in the successful experimental deployment of the anti-IL-6 receptor antagonist antibody Tocilizumab to mitigate the observed cytokine induced toxicity (32), a treatment now being applied more systematically to counteract CRS (201).

Building T cell therapies into a pillar of medicine

Now that adoptive T cell therapy is showing such dramatic effects, the question becomes, how can we move past offering this as boutique medicine in major medical centers, and offer it in communities all over the world? There are several logistical issues that need to be

addressed: shipping and tracking of autologous blood products, large-scale manufacturing of vectors and T cell products and validation of processes and immunologic assays necessary for quality control. This is likely to require adaptation of a variety of fields outside the scope of most physicians and immunologists, from blood banking, a field that has learned to manage cell collection and processing techniques in a standardized ways at the international level, to robotic manufacturing techniques used in high-throughput laboratories and systems such as building automobiles(202).

Understandably, some scientists have raised concerns about the complexity of this type of therapy; even performing multi-center studies with T cell products has been challenging at the academic level. Also, most regulatory health agencies are set up to handle the testing of drugs, which are manufactured centrally, the active ingredient is measured and controlled, and where one lot treats many patients. In contrast, the active 'ingredient' of a T cell product is challenging to define because it reflects a heterogeneous population of T cells; even the typical Phase I dose-escalation trial design is not necessarily the most appropriate method to test for safety of T cell products, given that the 'dose' is not static or even maximal immediately after infusion. Furthermore, each 'lot' produced can only be used for one patient, and thus far, T cell products have been manufactured locally. As these therapies move to Phase II or III studies to obtain an indication for use, it is not yet clear whether it will be possible to perform double-blind randomized controlled trials that are considered the gold standard in establishing a standard of care therapy.

Although these issues pose significant challenges, they are not necessarily insurmountable barriers; organ and bone marrow transplants are now considered fairly routine (203). As manufacturing of cell products becomes more automated, and as scientists better define the key components of what makes the most bioactive, optimal T cell product, it will become an absolute necessity to develop large-scale, centralized processes that can generate standardized quality-controlled T cell products. This endeavor will require scientists, physicians, and industry to work together in building the necessary infrastructure and adapting the current regulatory standards to reflect an entirely new pillar of medicine, comprised of personalized cell therapies (202).

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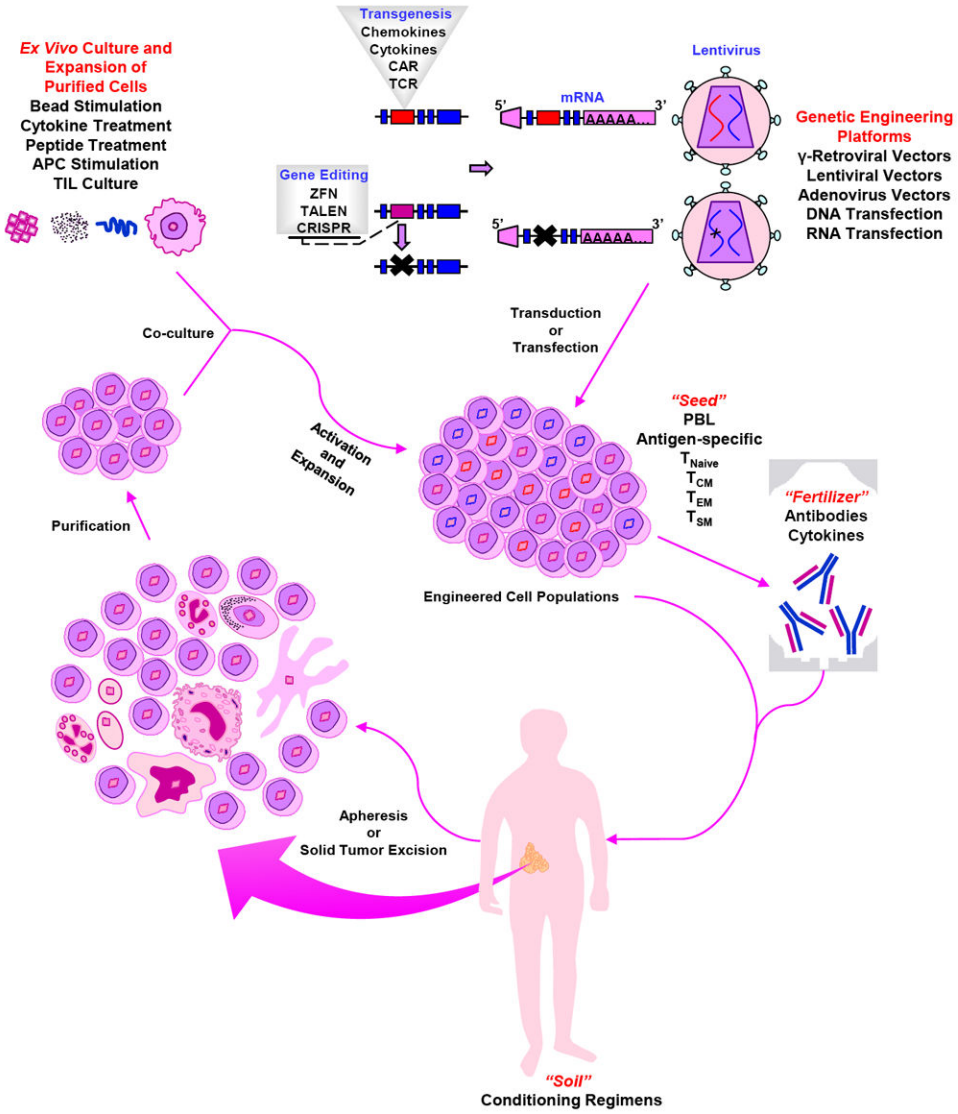

Figure 1. Adoptive transfer of autologous, genetically engineered, *in vitro*–expanded T cells: The "seed," the "soil" and the "fertilizer"

Autologous cells are harvested from the patient by apheresis. Following purification, cells undergo polyclonal *in vitro* activation and expansion, as well as genetic modification to form the "seed." Engineered cell populations ("seeds") are re-infused ("planted") into the pre-conditioned patient ("soil"), along with antibodies and/or cytokines ("fertilizer").

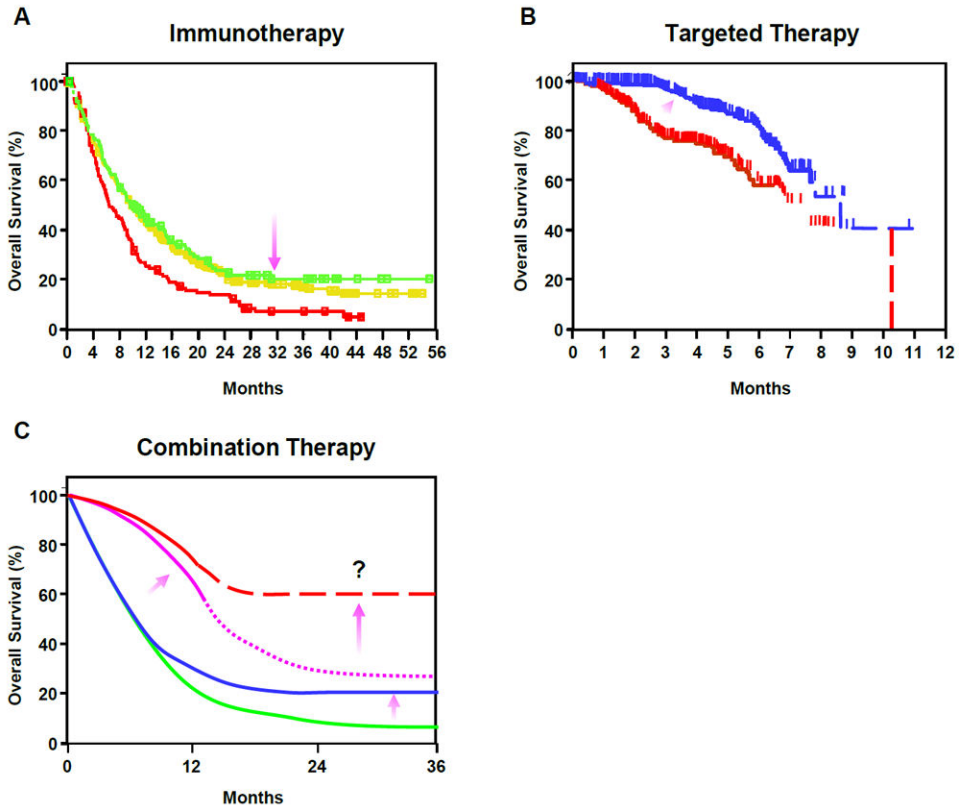

Figure 2. Durable tumor regression may be achieved by combining immunotherapy with targeted therapeutic strategies

(A) Patients with metastatic melanoma exhibit improved survival with Ipilimumab treatment(23) (Kaplan-Meir survival plot). Treatments are as follows: ipilimumab-alone (green line); gp100-alone (red line); ipilimumab + gp100 (yellow line). Purple arrow indicates how immunotherapy raises the tail of curve, indicating prolonged effects. (B) Kaplan-Meir survival plot showing that improved survival can be achieved in melanoma with Vemurafenib therapy (204). Patients were treated with either Dacarbazine (red line) or Vemurafenib (blue line). Purple arrow indicates direction of change effected by targeted therapy; overall survival is improved early but effects are transient. (C) Hypothetically, the combination of immunotherapy with targeted treatment may increase survival in patients with metastatic cancers. The green line depicts a typical survival curve for standard cancer therapy; the blue line represents survival with immunotherapy alone; the purple line represents the effect of a targeted therapy alone survival plot; the dashed red line depicts the potential enhanced survival that can be achieved using immunotherapy combined with targeted therapy. Arrows highlight the impact of the above therapeutic regimens on the tail of the curve or the spread between curves for different treatments. Thus, immunotherapy requires more time, but increases survival; Targeted therapy works rapidly, but is not durable. Combining these strategies may ultimately improve the fraction of patients with long-term survival.

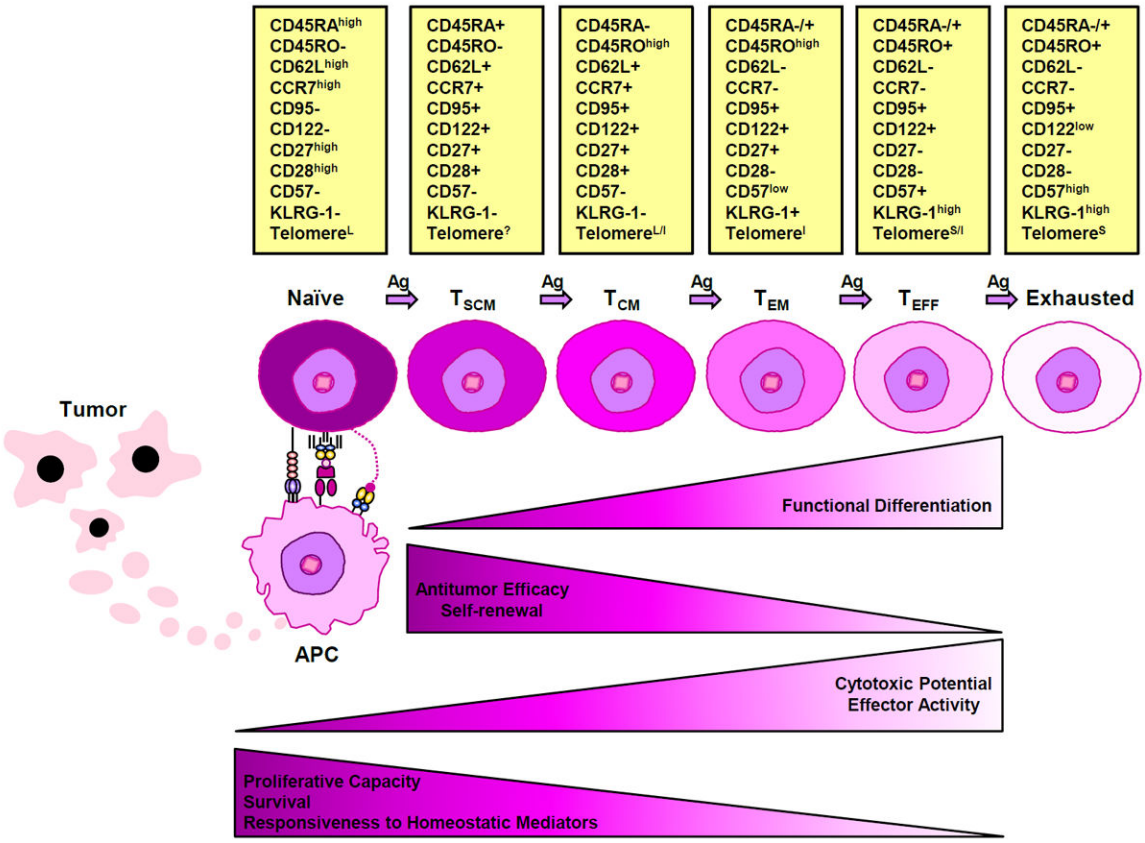

Figure 3. Phenotypic and functional changes in T cells during progressive differentiation driven by chronic antigen stimulation

A state of persistent and frequent antigenic stimulation, such as that induced by tumor burden, facilitates a progressive differentiation pathway whereby naïve T cells become terminally differentiated effectors. Changes in the phenotypic markers that characterize progressive T cell differentiation are depicted as: expressed (+), not expressed (-), expressed at high levels (high), expressed at low levels (low); long telomere length (L), unknown telomere length (?); long/intermediate telomere length (L/I), intermediate telomere length (I), short/intermediate telomere length (S/I) and short telomere length (S). Together with the gradual shortening of telomere length, T cells lose their proliferative and self-renewal capacities, as well as responsiveness to homeostatic mediators and ultimately become exhausted. Although cytotoxic potential/effector functions increase with persistent antigen stimulation and T cells must be fully differentiated to possess anti-tumor activity, experimental evidence suggests that in the context of adoptive cell therapy, increasing differentiation state is inversely correlated with anti-tumor efficacy (205).

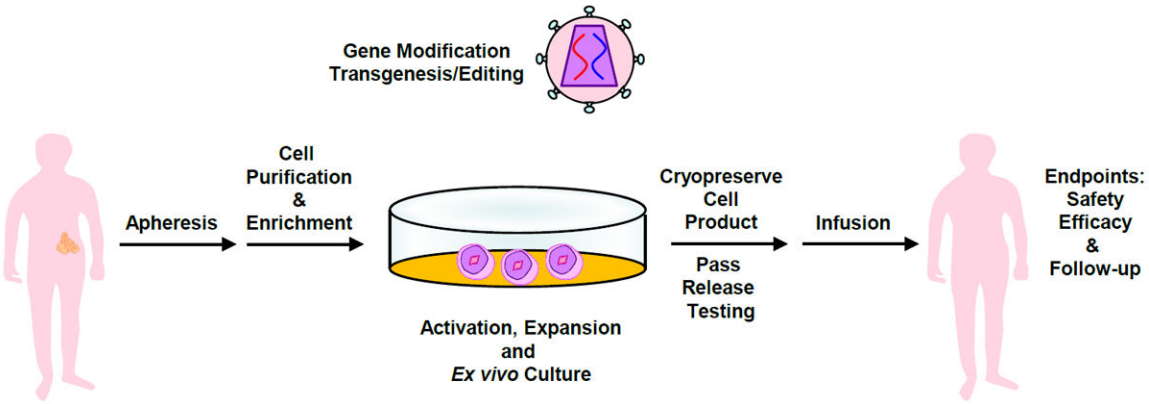

Figure 4. Clinical application of gene-modified cell therapies

Cells of interest are isolated from the whole blood of a patient, followed by enrichment, activation and expansion. At the time of activation, the lentiviral vector is added. On the final day of culture, cells are harvested and cryopreserved in an infusible media. The patient is infused with gene-modified cells and endpoint assays are conducted at designated time intervals. At the conclusion of active monitoring, in the US, the patient is transferred to a destination protocol for long-term follow-up as per FDA guidelines.

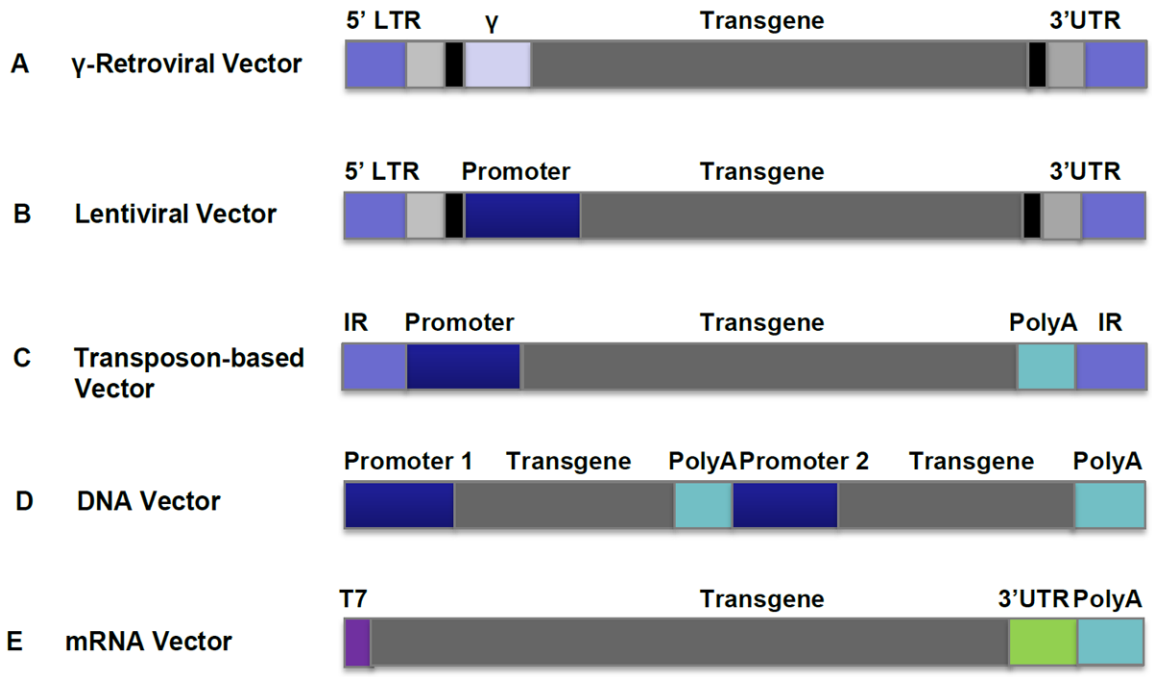

Figure 5. Vector systems for T adoptive therapy

(A) γ-retroviral vectors are one of the most commonly used vector systems for permanent gene expression in T cells for adoptive immunotherapy in the clinic. Clinical grade vector produced under Good Manufacturing Practices (GMP) viral vectors can be produced by stable packaging cell lines. vectors. GMP Viral vectors can be produced by stable packaging lines. (B) Lentiviral vectors are becoming more popular vector systems for permanent gene expression in T cells for adoptive immunotherapy clinical trials, due to their advantages of transducing non-dividing cells, high transduction efficiency and potentially safer integrating profile over γ-Retroviral vectors. (C) Non-viral transposase-mediated gene transfer, in which gene integration is achieved by the provision of the transposase enzyme, can mediate permanent gene expression in T cells. (D) DNA can be directly transfected into T cells by electroporation and integrated; genetically modified T cells can be cloned and expanded. (E) T cells can be directly transfected by electroporation with in vitro transcribed mRNA without integrating. This is a transient transgene system and the transgene can be expressed in T cells for up to 1 week. Repeated infusions of the mRNA-transfected T cells are required. All vectors are depicted as linear DNA. Internal repeat (IR); Long terminal repeat (LTR); Packaging Signal (ψ); Untranslated region (UTR); Poly(A) tail (PolyA).

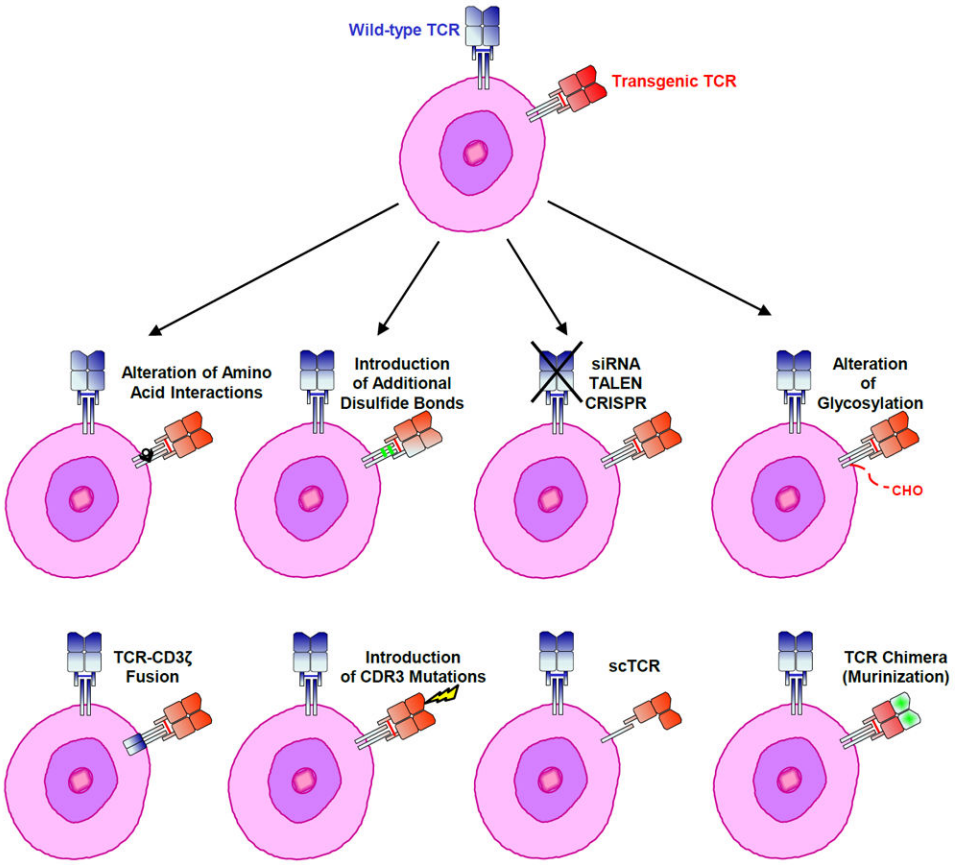

Figure 6. Strategies to improve the function of transgenic TCRs

Expression of a transgenic TCR may be improved by preventing the formation of mixed dimers between endogenous and ectopic engineered TCR chains. These strategies include the alteration of amino acid interactions, the creation of TCR-CD3 ζ fusions, single chain (sc) receptors, TCR chimeras (murinization) and the introduction of additional disulfide *bonds or glycosylation. Mutating amino acids in the* complementarity determining region 3 (CDR3) and knocking down/out endogenous TCR expression may also increase transgenic TCR activity.

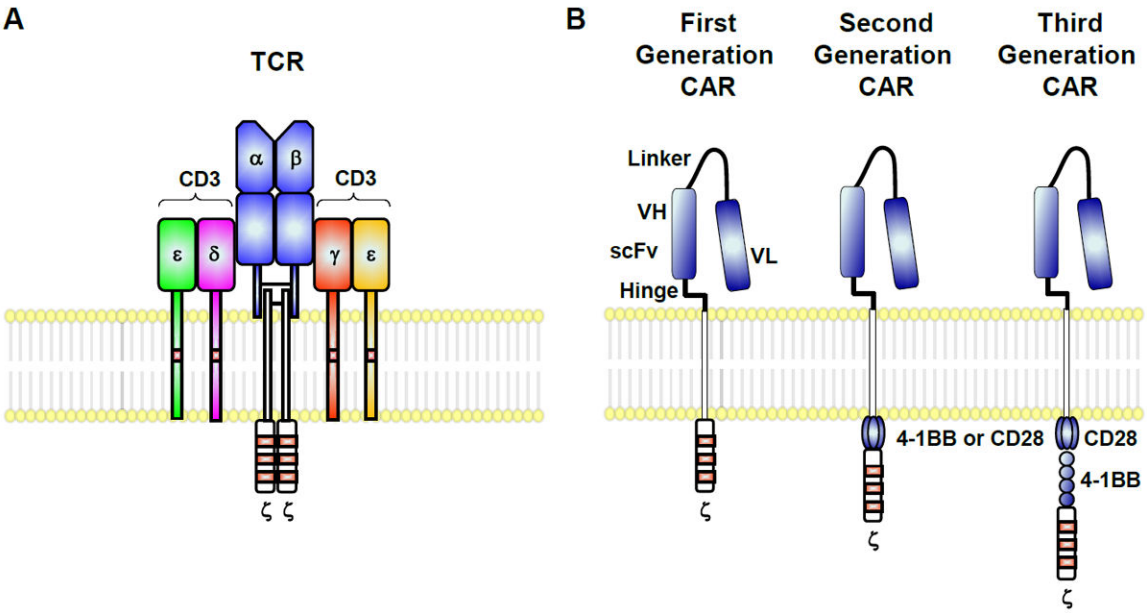

Figure 7. T cells can be redirected to possess specificity for tumors

(A) Endogenous T cells express a single TCR. (B) Alternatively, these genes can be engineered to express chimeric tumor antigen–specific receptors or "CARs" that target surface antigens in an MHC-independent manner. T bodies express an extracellular portion that is usually derived from an antibody and intracellular signaling modules derived from T cell signaling proteins. First generation CARs contain CD3ζ, while second generation CARs possess a co-stimulatory endodomain (e.g., CD28 or 4-1BB) fused to CD3ζ. Third generation CARs consist of two co-stimulation endodomains linked to CD3ζ.

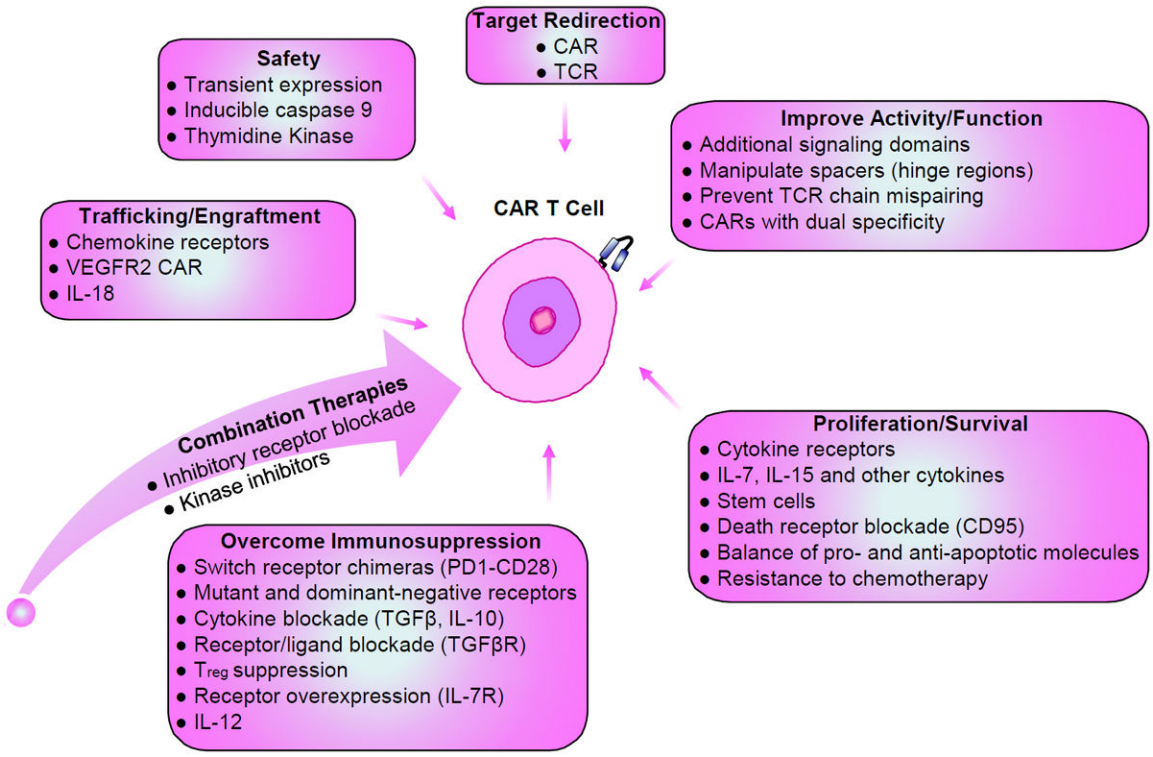

Figure 8. Strategies for engineering effective genetically-modified T cells

Various strategies can be undertaken to genetically modify T cells for adoptive therapy in an endeavor to enhance function and survival, proliferation, trafficking to tumor sites, and safety. Through genetic modification, these cells may also be armed to be efficacious in the immunosuppressive tumor microenvironment. Combination therapy can be used to improve the therapeutic efficacy of engineered T cells.

Table 1

Summary of current clinical trials of CD19 CAR T cells

Target Antigens	Cancers	CAR Signaling Domain	Combinatorial/ Engineering Strategies (Biologicals, Drugs)	Phase/ID	Sponsor
CD19	B cell malignancies relapsed post-allo-HSCT, T cells from donor	CAR: CD28-CD3ζ	None	I; NCT01087294	National Cancer Institute
CD19	NHL; CLL	CAR: CD28-CD137-CD3ζ or CD28-CD3ζ	None	I; NCT01853631	Baylor College of Medicine
CD19	Relapse/refractory CLL, NHL, or ALL	CAR: CD3ζ	None	I/II; NCT01865617	Fred Hutchinson Cancer Research Center
CD19	ALL; DLBCL; MCL; NHL; CLL relapsed post allo-HSCT	CAR: CD28-CD3ζ	CMV- or EBV-specific T cells derived from donor CD62L+ T_{CM}	I/II; NCT01475058	Fred Hutchinson Cancer Research Center
CD19	ALL; CLL; NHL	CAR: CD137-CD3ζ	None	NP; NCT01864889	Chinese PLA General Hospital
CD19	ALL; CLL; NHL	CAR: CD28-CD3ζ	Ipilimumab	I; NCT00586391	Baylor College of Medicine
CD19	CLL; Small lymphocytic lymphoma; MCL; Follicular lymphoma; Large cell lymphoma	CAR: CD28-CD3ζ	IL-2	I/II; NCT00924326	National Cancer Institute
CD19	Auto-HSCT for NHL followed by T cell infusion	CAR: CD28-CD3ζ	none	I; NCT01840566	Memorial Sloan-Kettering Cancer Center
CD19	B cell leukemia; B cell lymphoma	CAR: 4-1BB-CD3ζ	None	I; NCT01626495	CHOP/University of Pennsylvania
CD19	NHL; CLL	CAR: CD28-CD3ζ	CD19 CAR transduced PBLs and EBV-specific CTLs	I; NCT00709033	Baylor College of Medicine
CD19	Pediatric relapsed B cell ALL	CAR: CD28-CD3ζ	None	I; NCT01860937	Memorial Sloan-Kettering Cancer Center
CD19	CD19+ malignancies	CAR: CD28-4-1BBζ	None	NP; NCT01029366	University of Pennsylvania
CD19	ALL; CLL; NHL	CAR: CD28-CD3ζ	CD19 CAR transduced tri-virus-specific CTLs (CMV, EBV, and adenovirus)	I/II; NCT00840853	Baylor College of Medicine

Target Antigens	Cancers	CAR Signaling Domain	Combinatorial/ Engineering Strategies (Biologicals, Drugs)	Phase/ID	Sponsor
CD19	Pediatric leukemia and lymphoma	CAR: CD28-CD3ζ	None	I; NCT01593696	National Cancer Institute
CD19	Relapsed ALL post-allo-HSCT	CAR: CD28-CD3ζ	CD19 CAR transduced EBV-specific CTLs	I; NCT01430390	Memorial Sloan-Kettering Cancer Center
CD19	Auto-HSCT for NHL followed by T cell infusion	CAR: CD28-CD3ζ	T_{CM}-enriched CD8+ T cells	I/II; NCT01318317	City of Hope/National Cancer Institute
CD19	ALL	CAR: CD3ζ	CD19 CAR transduced EBV-specific CTLs	I/II; NCT01195480	University College, London
CD19	Leukemia	CAR: CD28-CD3ζ; 4-1BB-CD3ζ	None	I/II; NCT00466531	Memorial Sloan-Kettering Cancer Center/ University of Pennsylvania
CD19/EGFRt	Auto-HSCT for NHL followed by T cell infusion	CAR: CD28-CD3ζ	T_{CM}-enriched T cells; (cetuximab as possible suicide system)	I; NCT01815749	City of Hope
CD19/EGFTt	Pediatric ALL	CAR: CD28-CD3ζ	None	I; NCT01683279	Seattle Children's Hospital

allo-HSCT, allogeneic hematopoietic stem cell transplant; NHL, non-Hodgkin lymphomas; CLL, Chronic lymphocytic leukemia; ALL, Acute lymphoblastic leukemia; DLBCL, Diffuse large B-cell lymphoma; MCL, Mantle cell lymphoma; MALT, Mucosa-associated lymphoid tissue; CMV, Cytomegalovirus; EBV, Epstein–Barr virus; TCM, Central memory T cells; PBTLs, Peripheral blood T lymphocytes; CTLs, Cytotoxic T lymphocytes; NP, Information not provided.

Table 2

Summary of current clinical trials of CAR T cells for cancer

Target Antigens	Cancers	CAR Signaling Domain	Combinatorial/Engineering Strategies (Biologicals, Drugs)	Phase/ID	Sponsor
CD20	ALL; CLL; NHL	CAR: 4-1BB-CD3ζ	None	NP; NCT01735604	Chinese PLA General Hospital
CD30	NHL; HL	CAR: CD28-CD3ζ	None	I; NCT01316146	Baylor College of Medicine
CD30	NHL; HL	CAR: CD28-CD3ζ	CD30 CAR transduced EBV-specific CTLs	I; NCT01192464	Baylor College of Medicine
CD33	Relapsed adult myeloid leukemia; chemotherapy refractory adult myeloid leukemia	CAR: CD137- CD3ζ	None	I/II; NCT01864902	Chinese PLA General Hospital
CD138	Relapsed and/or chemotherapy resistant multiple myeloma	CAR: CD137-CD3ζ	None	I/II; NCT01886976	Chinese PLA General Hospital
cMet	Metastatic breast cancer; Triple negative breast cancer	CAR: 4-1BB-CD3ζ	None	I; NCT01837602	University of Pennsylvania
EGFRvIII	Malignant glioma; Glioblastoma; Brain cancer	CAR: CD28-4-1BB-CD3ζ	None	I/II; NCT01454596	National Cancer Institute
ErbB	Head and neck cancer	CAR: CD28-CD3ζ	ErbB CAR coexpressed with 4αβ chimeric cytokine receptor to enable ex vivo expansion of engineered T cells using IL-4	I; NCT01818323	King's College London
FAP	Malignant pleural mesothelioma	CAR: CD28-CD3ζ	None	I; NCT01722149	University of Zurich
GD-2	Neuroblastoma	CAR: CD28-OX40-CD3ζ	iCaspase9 safety switch/AP1903 dimerizing drug	I; NCT01822652	Baylor College of Medicine
HER2	HER2+ malignancies	CAR: CD28-CD3ζ	CD19 CAR-transduced EBV-specific CTLs expressing a dominant negative TGFβ receptor	I; NCT00889954	Baylor College of Medicine
HER2	Glioblastoma multiforme	CAR: CD28-CD3ζ	CMV-specific CTLs	I; NCT01109095	Baylor College of Medicine
Ig Kappa Light Chain	Lymphoma; Myeloma; Leukemia	CAR: CD28-CD3ζ	None	I; NCT00881920	Baylor College of Medicine
Mesothelin	Mesothelin-expressing cancers	CAR: CD28-CD3ζ	IL-2	I/II: NCT01583686	National Cancer Institute
Mesothelin	Metastatic pancreatic cancer	CAR: 4-1BB-CD3ζ	None	I; NCT01897415	University of Pennsylvania
PSMA	Prostate cancer	CAR: CD28-CD3ζ	HSV thymidine kinase (used for imaging and suicide gene)	I; NCT01140373	Memorial Sloan-Kettering Cancer Center

ALL, Acute lymphoblastic leukemia; CLL, Chronic lymphocytic leukemia; NHL, non-Hodgkin lymphomas; EBV, Epstein–Barr virus; CTLs, Cytotoxic T lymphocytes; iCaspase9, inducible caspase 9; CMV, Cytomegalovirus; HSV, Herpes simplex virus; NP, Information not provided.

Table 3

Summary of recent clinical trials involving genetically-redirected T cells

Target Antigens	Cancers	Receptor	Combinatorial/Engineering Strategies (Biologicals, Drugs)	Phase/ID	Sponsor
WT1	AML; CLL	TCR	None	I/II; NCT01621724	University College, London
WT1	AML, MDS, or CML	TCR	Aldesleukin; Virus-specific CD8+ T Cells	I/II; NCT01640301	Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium
NY-ESO-1	Melanoma	TCR	None	I/II; NCT01350401	Adaptimmune
NY-ESO-1/LAGE-1	Multiple myeloma	TCR	None	I/II; NCT01892293	Adaptimmune
NY-ESO-1/MAGE-A3/6	Multiple myeloma	TCR	None	I/II; NCT01352286	Adaptimmune
CEA	Metastatic cancers	IgCD28TCR	None	II; NCT01723306	Roger Williams Medical Center
MART-1	Metastatic melanoma	TCR	Administration of MART-126.35-Pulsed Dendritic Cells and IL-2	II; NCT00910650	Jonsson Comprehensive Cancer Center

AML, Acute myelogenous leukemia; CLL, Chronic lymphocytic leukemia; MDS, myelodysplastic syndromes; CML, Chronic myelogenous leukemia; TCR, T cell receptor.