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Going viral: chimeric antigen receptor T-cell therapy for hematological malignancies

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Summary: On July 1, 2014, the United States Food and Drug Administration granted 'breakthrough therapy' designation to CTL019, the anti-CD19 chimeric antigen receptor T-cell therapy developed at the University of Pennsylvania. This is the first personalized cellular therapy for cancer to be so designated and occurred 25 years after the first publication describing genetic redirection of T cells to a surface antigen of choice. The peer-reviewed literature currently contains the outcomes of more than 100 patients treated on clinical trials of anti-CD19 redirected T cells, and preliminary results on many more patients have been presented. At last count almost 30 clinical trials targeting CD19 were actively recruiting patients in North America, Europe, and Asia. Patients with high-risk B-cell malignancies therefore represent the first beneficiaries of an exciting and potent new treatment modality that harnesses the power of the immune system as never before. A handful of trials are targeting non-CD19 hematological and solid malignancies and represent the vanguard of enormous preclinical efforts to develop CAR T-cell therapy beyond B-cell malignancies. In this review, we explain the concept of chimeric antigen receptor gene-modified T cells, describe the extant results in hematologic malignancies, and share our outlook on where this modality is likely to head in the near future.

Keywords: *genetic engineering, chimeric antigen receptor T cells, CD19*

Introduction

Publication of the first results of human clinical trials using chimeric antigen receptor T-cell technology occurred in 2006 (1, 2) and was quickly followed by a spate of papers that have provided fascinating and valuable insights (3–8). Leading investigators in the field have shown remarkable energy and zeal in translating the lessons of early studies into second- and subsequent-generation trials, thus accelerating discovery in the field. To use an internet term, chimeric antigen receptor (CAR) T-cell therapy is 'going viral'.

The concept of introducing into a cytotoxic T-cell hybridoma the genetic material for an antibody recognizing a model antigen (a hapten, 2,4,6-trinitrophenyl) was described in 1989 by Gross, Waks, and Eshhar (9) and shortly thereafter by Goverman *et al.* (10). Another seminal

demonstration was the construction of chimeric receptors encoding CD4 or CD25 linked to signaling modules, demonstrating that a single polypeptide chain could replicate much of the signaling features of the T-cell receptor (TCR) (11–13). The above basic science advances demonstrated that it was possible to redirect T-cell signaling to an antigen of choice and independent of MHC restrictions. In their seminal work, Gross *et al.* (9) concluded that 'construction of chimeric T-cell receptors with anti-tumor specificity will enable testing of the feasibility of this approach in combating human tumors.' Given the prescience of the Eshhar group, it is worthwhile to break down their conclusion into its constituent parts and discuss these in turn.

'Construction of chimeric T-cell receptors' relates to the molecular biology of the genetically engineered immunoreceptor. At its simplest embodiment, this is a polypeptide representing the sequences of a light and heavy chain from an antibody, linked to the signaling machinery of the T-cell receptor, typically the ζ chain. Serial modifications and improvements on this basic design have led to the addition of costimulatory domains that are derived from one or more of the endogenous molecules used by T cells, such as CD27, CD28, CD134, or CD137. Despite strong preclinical and clinical evidence that introduction of a single costimulatory molecule leads to superior T-cell functions when compared to CARs that contain no costimulatory domain, we are far from identifying the optimal costimulatory molecule(s) (14, 15). In fact, we do not yet know whether there is one optimal way to construct CARs or whether different disease settings will be best served by different CAR constructs.

'Anti-tumor specificity', a seemingly simple statement, belies a huge underlying complexity. What are tumor-specific antigens? How specific must they be? How do we really know what antigens are expressed by tumors compared with normal tissues? Do all tumor cells within a given patient possess the same antigen? Can the tumor be eradicated if only some cells are susceptible to immune-mediated targeting? What is the potential for bystander toxicity? Adoptive transfer of bulk T cells that were expanded *ex vivo* from circulating peripheral blood mononuclear cells or from tumor-infiltrating lymphocytes has led to rapid reconstitution of lymphocyte numbers after high-dose chemotherapy, and these cells are clearly functional (16, 17). However, these T cells are not enriched for tumor specificity and simply represent a population of activated T cells. Most of the currently identified tumor-specific antigens are self-antigens that are normally expressed in early fetal development and that are aberrantly expressed during malignancy. Examples include NY-ESO1 and the MAGE family antigens. The affinity of T-cell receptors for these self-antigens is substantially lower than for viral antigens, likely reflecting the impact of central (thymic) tolerance on T-cell repertoire to self-antigens (18). In contrast, use of scFv from antibodies that are derived in other species or by techniques such as phage display yields a high-affinity receptor-ligand interaction (19).

'Testing of the feasibility of this approach' can only be carried out in clinical trials, as preclinical models have proven time and again to be insufficiently predictive of both efficacy and toxicity in humans (20, 21). Yet clinical trials of genetically engineered T-cell products are extraordinarily complex and expensive undertakings that are subject to onerous regulatory requirements. The first clinical trials with CAR T cells were conducted in patients with HIV infection in 1998 to 2000 (22, 23). The past 16 years have provided useful insights that have already led to refinements in next generation trials and have demonstrated that functional T cells can be grown from most patients with active malignancy, that gene transfer can be performed under good manufacturing practice (GMP) conditions, and that patients with aggressive rapidly progressive malignancies can be treated in a timely manner (24, 25). Novel complications, such as a cytokine release/macrophage activation syndrome, have been reported and their treatment described (25–27). Nevertheless, the pathogenesis and implications of the cytokine release syndrome remain to be fully elucidated, and it is likely that each new CAR target will uncover novel toxicities.

'Combating human tumors' utilizing CAR T cells represents an opportunity to eradicate cancer in some patients. There are patients who have been tumor-free by the most sensitive detection methods for years and may be cured of their disease (5,25). What is the relative contribution of the early profound depletion of all the cancer cells that are visible to the immune system, compared with a long-term immunosurveillance role? Obtaining an answer to this and other questions would have implications for how we think about CAR design.

Chimeric antigen receptors: form and function

Conceptually, the CAR endows the gene-modified cell (usually a T cell) with one or more designer features. The basic feature is novel specificity. Additional features can include built-in stimulation signals such as costimulatory molecules, cytokine production, or reversed inhibitory signals (28).

The CAR is designed in a modular fashion that typically consists of an extracellular target-binding domain, a hinge

region, a trans-membrane domain that anchors the CAR to the cell membrane, and one or more intracellular signaling domains (*Fig. 1*). The target-binding domain is typically derived from the light and heavy chain portions of a single chain variable fragment (scFv) linked in series via a polypeptide sequence. Target-binding can also occur if a ligand recognizes its receptor on the target cell, as first shown for HIV gp120 binding CD4:ζ CARs (11, 13) and later for example, that a membrane-bound interleukin-13 (IL-13) mutein on the CAR T cell can bind the IL-13 receptor on the target cell without an scFv (29).

The nature of the interaction between the CAR and its ligand differs from that which occurs between a TCR and its peptide-MHC ligand. Affinity and avidity are much higher between antibody-ligand than TCR-ligand. CARs recognize intact cell surface proteins, and therefore targeting is not MHC-restricted. Furthermore, unlike TCR-based recognition, CAR recognition is not dependent on processing and antigen presentation and hence is not susceptible to common tumor escape mechanisms such as HLA loss or altered processing mechanisms (30). The main disadvantage of CAR is that intracellular molecules cannot be recognized. However, a 'TCR-like' CAR that binds peptides from the intracellular antigen WT1 in the MHC groove has recently been described and appears to be sufficiently specific that it shows no reactivity against the MHC molecule (31).

The ligand-binding domain is supported by a hinge region that is usually derived from the CD8 or IgG4 molecules. The hinge is important for CAR expression on the cell surface. The hinge region affects flexibility of the scFv and hence its interaction with the ligand. There are few direct comparisons of different hinge regions, although it appears that the length of the hinge region may influence the quality of the interaction between T cell and target, depending on the location of the epitope on the target antigen. For example, CD22 epitopes that are close to the cell membrane trigger more potent lytic activity compared with distal epitopes (32). Similarly, longer spacer domains increase the potency of other CAR targeting NCAM or 5T4 recognizing membrane proximal epitopes, possibly by providing increased flexibility (33, 34). These findings imply that there may be an optimal distance between the CAR T cell and the target cell.

Engagement of the CAR transmits a signal to the intracellular T-cell machinery via a signaling domain, typically the CD3 ζ chain. Some groups have used signaling domains derived from the Fcγ receptor (2).

The incorporation of costimulatory molecules such as CD27, CD28, CD134 (OX40), CD137 (4-1BB), CD244, or ICOS can augment the effects of ζ chain signaling and hence enhance T-cell proliferation and persistence (14, 35–39). CD28 was the first costimulatory molecule to be included in the CAR construct and dramatically increased production of IL-2 as well as cytotoxicity (40, 41), and CARs with CD28 costimulation were relatively resistant to suppression by regulatory T cells (42). Incorporation of CD137 appears to result in improved *in vivo* persistence, antitumor activity, and tumor trafficking compared with CD28-costimulated CARs in carefully performed preclinical models. These findings were

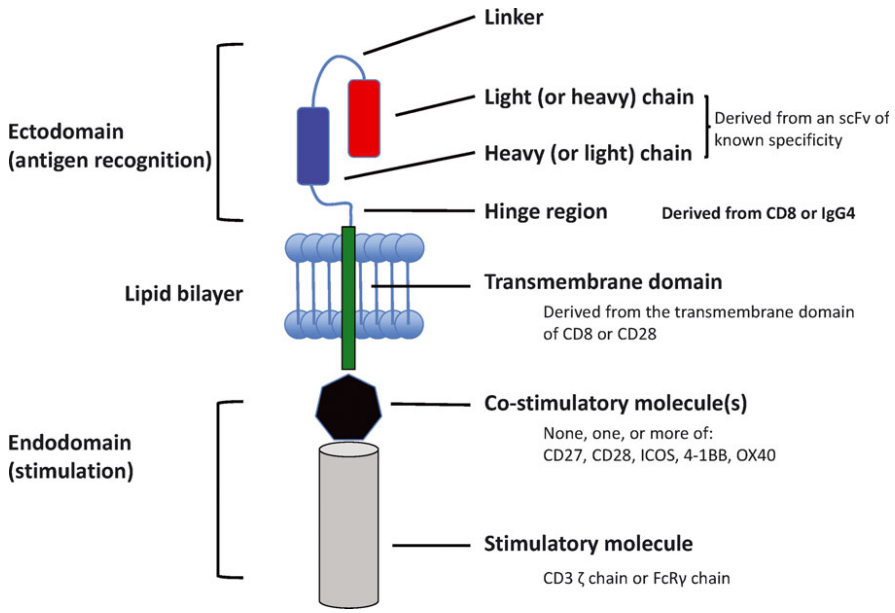

Fig. 1. Anatomy of a CAR.

instrumental in the design of the anti-CD19 CAR that was ultimately used by our group (14). Overexpression of the ICOS signaling domain drives human T-cell differentiation into a T-helper 17 (Th17) phenotype and in some settings leads to a superior anti-tumor effect compared with Th17 cells costimulated with CD28 (43, 39). CAR constructs with one costimulatory molecule are known as 'second generation', and those with more than one additional costimulatory molecule are known as 'third generation' CARs. It is clear that incorporation of a single costimulatory molecule leads to superior persistence and other T-cell functions, but whether the addition of secondary costimulation (third generation CARs) provides further benefit remains unclear (44, 45).

The mechanism by which CAR stimulation mediates T-cell activation remains incompletely elucidated but may act directly via the antigen-ligated signaling chain and indirectly via associated chains within the TCR complex (46). Whether the residual natural TCR remains fully active is currently unclear. We have not noted graft-versus-host disease after infusion of allogeneic donor-type CAR-modified T cells into patients with B-cell acute lymphoblastic leukemia (ALL) who had relapsed after prior allogeneic hematopoietic cell transplantation (HCT), suggesting that the TCRs were either anergic to host histocompatibility antigens or that TCR signaling was somehow impaired. In contrast, work by the Baylor group has shown that when CAR-modified T cells with antiviral TCR specificity are expanded, they retain that specificity and can expand in response to viral reactivation (47). An alternative approach to prevent TCR-based alloreactivity is to genetically eliminate expression of the endogenous $\alpha\beta$ TCR chains (48).

It is conceivable that different clinical settings may require different combinations of scFv, hinge, transmembrane, stimulatory, and costimulatory domains. The optimal design of a given CAR thus remains an area of active investigation and should be empirically evaluated for the treatment of different malignancies. The modular design of the CAR facilitates evaluation of a range of different configurations by replacing different components at will using simple cloning techniques. The ease with which new ideas on CAR design can be realized in the laboratory implies that progress in the CAR field may be more rapid than is typical for new molecular entities that are developed in the pharmaceutical industry.

Gene transfer into T cells

Genetic material encoding the chimeric antigen receptor can be transferred into the patient's T cells using viral or non-viral techniques. Gammaretroviral or lentiviral vectors integrate into the host cell genome and have low intrinsic immunogenicity and hence lead to permanent transgene expression. Although most groups currently use gammaretroviruses for clinical work, we employ a lentiviral [human immunodeficiency virus (HIV)-based] vector. In contrast with gammaretroviral vectors, lentiviral vectors can integrate into non-dividing cells (particularly when coupled with cytokine preactivation) (49, 50), are less susceptible to silencing by host restriction factors, and can deliver larger DNA sequences (51–54). Despite early concern about insertional mutagenesis following retroviral transduction of hematopoietic stem cells (53), long-term follow-up of clinical studies supports the safety of using these vectors in T cells. In particular, long-term follow-up consisting of over 500 patient-years from our institution confirms prolonged persistence without oncogenesis or persistent clonal expansion (55). Transduced T cells recovered from patients show that lentiviral integration sites are not random and do not favor proto-oncogenes or tumor suppressor genes (55, 56). Other viral vectors include adenovirus or adeno-associated virus. These can result in long-term episomal transgene expression and have been shown to infect human T cells with high efficiency but are very immunogenic (57).

The disadvantages of viral approaches are the expense and expertise required for production, as well as the regulatory requirements for follow-up. Non-viral approaches are typically cheaper and are regarded as potentially safer by regulatory agencies. These include transposon/transposase systems, such as Sleeping Beauty, that can deliver a large payload with persistent high-level transgene expression (58–61). There are preclinical data using the transposon/transposase system, and a clinical trial is being designed at the MD Anderson Cancer Center. Our group uses an alternative method for non-viral gene transfer that relies on RNA electroporation. Here, a DNA plasmid encoding the CAR is first transcribed *in vitro* under GMP conditions, resulting in messenger RNA that is then inserted into T cells by electroporation (*Fig.* 2). Careful titration of experimental conditions leads to high level transgene expression that begins as soon as the mRNA is translated and that lasts up to 3–5 days (62). The relative simplicity and efficiency of this approach mean that high-throughput iterative testing of novel constructs is feasible and is flexible enough to allow simultaneous transfer of several different constructs, stimulatory or homing molecules. The transient expression achieved serves as an important safety feature when first introducing a novel

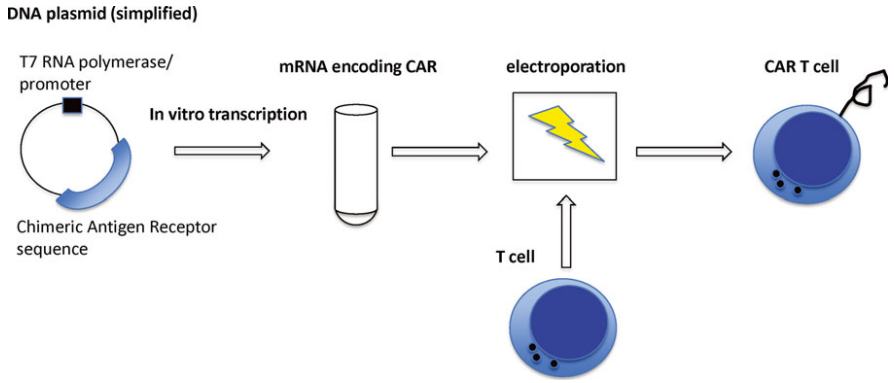

Fig. 2. Creation of a CAR T cell.

CAR into patients. We have used this in patients with solid tumors receiving anti-mesothelin CAR T cells as a safety switch in case of development of severe serositis. The favorable safety profile of the anti-mesothelin mRNA CAR cleared the way to proceeding to a trial based on lentivirally transduced CAR T cells (62, 63).

Although patients treated with anti-CD19 CAR T cells tolerate prolonged B-cell aplasia, the same may not be true for other targets. Thus, an important challenge for the field is to develop effective approaches to deplete the transgenic T cells when required. When T cells are transduced with the herpes simplex virus-derived thymidine kinase gene, they can be efficiently killed by administration of gancyclovir; however in patients, the viral TK-derived sequences have proven to be immunogenic (64). A more effective method is based on introduction of a fusion protein comprised of a FK506 binding domain linked to human caspase 9 signaling domains that induces apoptosis upon exposure to a small molecule dimerizing agent (65).

Other groups have incorporated the extracellular portions of the EGF receptor, CD34 or CD20, to facilitate depletion of the transgenic T cells upon infusion of a clinically available selection systems (66–68). Preclinical data at relatively short follow-up indicate that this modality could be clinically relevant. Whichever modality is used must be robust, reliable, and not 'leaky' to ensure complete ablation of the transgenic T cells in order to rescue patients with severe CART-related complications.

Expansion of genetically engineered T cells for infusion into patients

The steps required to generate clinically meaningful quantities of genetically engineered T cells for adoptive transfer are shown in Fig. 3. Peripheral blood mononuclear cells must first be collected from the patient by apheresis and

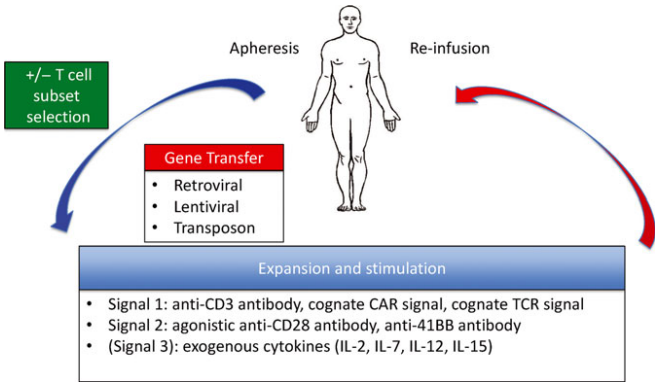

Fig. 3. Generation of genetically engineered T cells for adoptive transfer.

grown under conditions that will support the expansion of T cells. Apheresis is typically performed without granulocyte colony-stimulating factor mobilization, although there is no *a priori* reason why previously collected cytokine-mobilized peripheral blood products, that typically include high numbers of T cells, cannot be used.

T-cell expansion *ex vivo* requires activating signals, appropriate culture media, and extensive expertise. GMP-compliant T-cell expansion is carried out in certified laboratory settings. *Ex vivo* expansion is performed by stimulating T cells using the anti-CD3 clone OKT3 with or without additional costimulating antibodies such as anti-CD28 or with cytokines such as IL-2, IL-7, IL-12, IL-15, or IL-21 (69, 70). Careful comparison of CAR T cells expanded with soluble OKT3 and high dose IL-2 versus anti-CD3/CD28 beads shows superior persistence and anti-tumor activity with the latter approach (71). Alternatively, artificial antigen-presenting cells (aAPCs) such as irradiated K562 tumor cells or Epstein–Barr virus (EBV)-transformed cells can be used (4, 5, 72–76). A novel aAPC that can universally stimulate all CAR T cells has been described (77). Different centers tend

to utilize their 'home-grown' methods. Based on the preclinical expertise developed in their laboratories, these include different ways to deliver positive signals, whether and which cytokines are used to support growth, and for how long the T-cell culture lasts. Broad comparisons across different centers and under several different settings indicate that most groups achieve several hundred to several 1000-fold T-cell expansion during a culture period ranging from 10 days to 6 weeks (4, 5, 7, 8, 72, 74–76, 78–80) (*Table 1*). *Ex vivo* expansion using aAPCs appears to generate higher numbers of T cells, but the culture period may be longer. It is clearly important to generate T cells that are capable of further proliferation after *in vivo* transfer. It appears that T cells with a less-differentiated immunophenotype and/or those expanded in the presence of IL-12 or IL-15 have a better proliferative capacity (81). The combined use of IL-7 and IL-15 in the context of anti-CD3/CD28 bead-based stimulation has been shown to facilitate the *ex vivo* differentiation and expansion of gene modified $CD8^+$ with a stem cell memory phenotype (T_{SCM}) from naive T cells under GMP-compliant conditions (82). Use of IL-21 leads to relatively less-differentiated T cells that are capable of a superior anti-tumor effect *in vivo* (70, 76, 83). Modulation of T-cell metabolism in culture will likely also have an impact on their differentiation and memory subsets (84).

The optimal duration of *ex vivo* expansion is unknown, but longer expansion periods may result in more terminally differentiated T cells. Preclinical data support the use of T cells with a less differentiated phenotype, as acquisition of the full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of $CD8^+$ T cells (85). Longer culture periods also consume more reagents and are therefore more expensive and less practical. For example, the use of human serum as currently applied by most centers will probably not be sustainable in the future, owing to considerations of cost and availability, and will likely have to be replaced by alternative, serum-free methods (86).

The *ex vivo* manipulation of T cells that is a requisite part of the manufacturing process provides a unique opportunity to select particular T-cell subsets for expansion, so-called 'graft engineering'. Preclinical studies variously implicate naive T cells, central memory T cells, Th17 cells, or memory stem cells as possessing optimal characteristics for adoptive cellular therapy (87–91). Overall, it appears that naive or central memory cells, rather than terminally differentiated effector cells, exhibit superior anti-tumor efficacy *in vivo*, likely due to their capacity for proliferation and *in vivo* persistence (87, 92, 93). Indeed, studies using tumor-infiltrating lymphocytes have demonstrated that a short duration in culture, a rapid doubling time, CD27 expression, and longer telomere length correlate with better clinical outcomes (94–96).

Based on studies showing that central memory cells have enhanced persistence compared with effector memory cells, investigators at the Fred Hutchinson Cancer Research Center in Seattle currently separate T-cell subsets prior to expansion. Central memory $CD8^+$ T cells are sorted and expanded in culture with unselected $CD4^+$ T cells, leading to a final product that is capable of sustained proliferation (97, 98). This process adds complexity and expense to an already involved manufacturing process, and furthermore, there is

***Table 1. Ex vivo* expansion used in clinical trials in healthy donors and patients**

Method	Typical culture time	Comments	References
Anti-CD3/CD28 beads	12 days	Healthy donor T cell expansion 10^9 to 10^{11} fold over 60 days	Levine *et al.* (170)
Anti-CD3/CD28 beads + IL-2	11–17 days	HIV patients 37-fold Cancer patient typical expansion of T cells 49–385-fold	Levine *et al.* (171) Porter *et al.* (78) Porter *et al.* (5) Hollyman *et al.* (72) Brentjens *et al.* (8)
Virally infected APC + IL-2	5–6 weeks	EBV-transformed LCL pulsed with CMV and adenoviral peptides 21 ± 10-fold	Micklethwaite *et al.* (73)
Anti-CD3 and IL2 with irradiated feeder cells	6 weeks		Jensen *et al.* (4) Jensen *et al.* (79)
Anti-CD3 and IL2 +/− irradiated feeder cells	8–26 days		Johnson *et al.* (74) Kochenderfer *et al.* (7)
Artificial APC	10 days – 2 months as required	K562 cell line coated with anti-CD3, anti-CD28 antibodies and expressing CD137 and other ligands; Typical expansion 150-fold	Maus *et al.* (75) Suhoski *et al.* (172) Ye *et al.* (80)
Artificial APC		K562 cell line expressing CD19, CD137 ligand, CD86, CD64, and membrane-bound IL15 and IL21; Up to 40 000-fold expansion	Singh *et al.* (76)

Modified from S Gill and M Kalos, T cell-based gene therapy of cancer, in Translating Gene Therapy to the Clinic, editor J Laurence, Elsevier 2014

no clinical evidence that this approach yields superior outcomes. Therefore, at present our group and others do not preselect T-cell subsets. It is important to note that bulk unmanipulated T-cell population obtained by apheresis will usually contain naive, central, effector, and possibly memory stem cells, thus likely providing all the requisite cell subpopulations without the need for additional manipulation. Clinical trials will be required to determine the merit of various cell selection methodologies prior to deciding on the optimal cellular product.

The culture system must be robust and reproducible, and the process is underpinned by complex logistics. This involves leukapheresis capability, access to lentiviral vector manufacturing, validated cell-shipping network, and chain-of-custody procedures. The cell culture and gene transfer must be performed by highly skilled scientists and technicians, but for truly large-scale production in the future there will be a need to translate this resource-intensive procedure into a more automated, production line-like culture system (69).

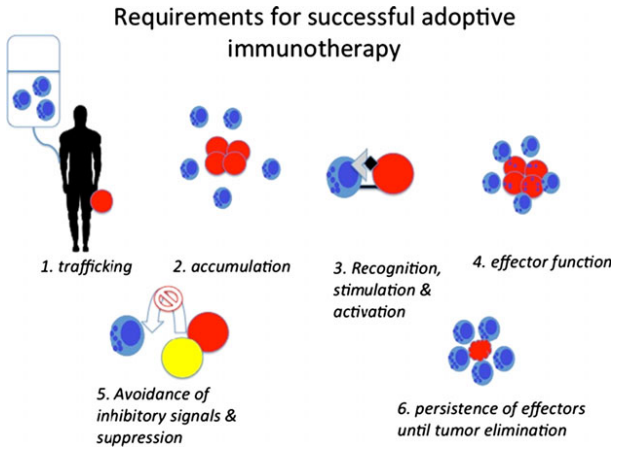

Fig. 4. Steps to successful adoptive T-cell immunotherapy. 1. Upon infusion into the patient's circulation, the T cells must traffic to the site of disease. 2. T cells must accumulate at the site of disease by a combination of trafficking and proliferation. 3. T cells recognize their cognate target and are activated, leading to 4. Induction of effector functions. 5. T cells must avoid inhibitory and suppressive signals from the target cells, from regulatory immune cells, and from the tumor microenvironment. 6. T cells must persist until elimination of the tumor. From Gill and Kalos, T cell-based gene therapy of cancer, Translational Research 2013, by permission.

What is the fate of CAR T cells after infusion?

Despite years of experience with cellular immunotherapy in the context of allogeneic HCT and donor lymphocyte infusions, there are still many unknown details about the kinetics, trafficking and site of activity of T cells in humans. Conceptually, upon infusion into a patient, T cells must traffic to the tumor site, engage with their cognate antigen, proliferate, avoid inhibitory signals from the tumor microenvironment, kill target cells, and persist long enough participate in immunosurveillance against residual tumor cells (*Fig.* 4).

Both preclinical and clinical studies clearly show that T-cell homeostatic proliferation and persistence is augmented by lymphodepletion, typically using chemotherapy and/or radiation (17, 99). Lymphodepletion is thought to facilitate T-cell expansion and persistence through the creation of homeostatic 'space' for expansion and through the depletion of other cells that would compete for the available cytokines (so-called 'cytokine sinks'). The conditioning regimen itself may induce production of homeostatic cytokines such as IL-15 as a consequence of the inflammatory reaction (100). Available data suggest that T-cell infusion should occur early after conditioning. In both adult and pediatric settings, we have shown that functional immune reconstitution is superior when T-cell infusion occurs on Day 2 after high dose conditioning compared with later time-points (101, 102).

In the setting of clinical CAR T-cell therapy, we are not aware of direct comparisons with or without lymphodepletion, but the group at the Memorial Sloan Kettering Cancer Center reported improved responses in patients receiving anti-CD19 CAR T cells if preceded by cyclophosphamide conditioning (8). It is unknown whether a single infusion is superior to serial infusions of CAR T cells. Our first patients were given fractionated (split dose) infusions of CART19 cells to mitigate potential infusional toxicity. With a single exception, we have not observed infusion reactions and have therefore started to administer the total cell dose as a single infusion (5, 103).

The optimal dose of total T cells or indeed total CAR-positive cells remains unknown. Typically the T-cell dose is reported as total number of viable cells per body surface area or per kilogram of ideal body weight. However, the level of T-cell engraftment and expansion may not correlate with the infused dose and may be related to other factors such as tumor burden and antigen exposure (6, 25).

Upon intravenous infusion, adoptively transferred leukocytes tend to redistribute rapidly from the blood into the tissues and can be seen in the lungs in the first few hours, followed by accumulation in the liver and spleen (2, 104, 105). The potential importance of low level antigen expression in the lungs was highlighted in a case report where low level antigen expression in the lung endothelium led to rapid and lethal pulmonary toxicity after infusion of high doses of Her2-specific CAR T cells (106). Once they migrate

beyond the lungs, it is clear that adoptively transferred T cells distribute to multiple tissues. In two patients, who unfortunately died of unexpected off-target recognition by transgenic T cells of a myocardial protein, autopsy revealed widespread low-level distribution of the infused T cells in the majority of organs evaluated (107).

T-cell trafficking is dependent upon an array of soluble factors, receptors, and adhesion molecules (108). Recruitment of effector T cells into the tumor microenvironment may be impeded by sub-threshold expression of homing and trafficking molecules on tumor microvessels (109) and conversely may be enhanced in the context of fever by IL-6 trans-signaling (110). These issues may be more relevant in the setting of solid tumors than hematologic malignancies. In the setting of CAR T-cell therapy, we typically note an initial early peak in CAR T-cell numbers in the peripheral blood, followed by a reduction within the first few days, which is then followed by an increase in CAR T-cell numbers in the blood as the cells proliferate (5, 6). T cells can be transduced or electroporated to overexpress the relevant chemokine and homing molecules to promote homing to desired tissues (111, 112). For example, co-expression of CCR4 led to increased tumor homing in a Hodgkin's lymphoma tumor model (113), and enhanced tumor trafficking was seen when anti-GD2 CAR T cells were co-transduced with the chemokine receptor CCR2b (114).

Poor persistence of infused T cells may be caused by several factors. These include *ex vivo* culture conditions, lack of long-term transgene expression, poor effector functionality, exhaustion (115), and replicative senescence and the development of cellular or humoral anti-transgene immune responses (116, 117). The development of anti-transgene immune responses was dramatically highlighted in a recent clinical trial at our institution in which a patient developed anaphylaxis after receiving several infusions of mesothelin-specific CAR T cells derived from a murine anti-human mesothelin scFv (103). This observation spurred a change in the clinical trial protocol to avoid prolonged breaks between exposure to the murine CAR construct and such toxicity will also be mitigated by use of humanized or human-derived CAR constructs in the future. It is interesting to speculate that similar anaphylactic reactions have not been reported after infusion of CART19 cells because of the associated humoral immune ablation.

Strategies to enhance T-cell persistence after transfer include exogenous cytokine administration, overexpression of pro-survival signals, or the reversal of anti-survival signals. Historically, IL-2 has been used to enhance the proliferation of transferred T cells, particularly $CD8^+$ T cells. However, IL-2 may be deleterious to the persistence of memory T cells, and it also increases the number of regulatory T cells due to their high-level expression of CD25 (theαchain for the IL-2 receptor) (118, 119). In contrast, IL-7 and IL-15 play important roles in T-cell expansion and persistence of memory T cells and do not selectively enhance regulatory T-cell numbers (120). Engineering T cells with cytokine 'switch' receptors, whereby the extracellular portion of one cytokine receptor is linked to the intracellular signal transduction of another cytokine, is a particularly interesting approach first applied more than 15 years ago but that is now finally reaching clinical translation (121, 122).

Additional methods include overexpression of the anti-apoptotic molecule Bcl-xL, leading to enhanced T-cell survival in pro-apoptotic conditions, and overexpression of the telomerase gene hTERT, which extends the replicative potential of $CD8^+$ T cells (123, 124). Virus-specific T cells lacking the apoptosis-inducing molecule Fas were resistant to apoptosis after exposure to FasL yet remained dependent on antigen-specific stimulation and did not display autonomous growth (125). Introduction of a dominant-negative TGFβ receptor into cytotoxic T cells rendered them invulnerable to inhibition by TGFβ secreted from tumor (28). Each of these strategies must be evaluated carefully prior to translation into clinical trials, due to the potential to generate autoimmunity or a T-cell lymphoproliferative disorder.

After adoptive transfer and trafficking, CAR T cells undergo local accumulation at the sites of target recognition, though the extent to which this accumulation is due to proliferation or trafficking is unclear (111). Within the tumor microenvironment, CAR T cells must resist inhibitory and immunosuppressive stimuli (126). CAR T-cell function can be inhibited by cellular agents such as regulatory T cells and myeloid derived suppressor cells (127) as well as by molecular signals such as the immune checkpoint PD-1 (128–130). At present there are no clinical data showing that 'checkpoint inhibitor' antibodies can augment CAR T-cell function, but they likely represent a fruitful area for future study.

Despite clear evidence that CAR T cells mediate a robust and prompt anti-tumor effect, it is unknown how long CAR T cells must persist to prevent relapse. Evidence from the allogeneic HCT literature that chronic rather than acute GVHD is among the strongest predictors of long-term relapse-free survival indicates that years of persistent T-cell activity may in some settings be required to ensure cure. It

is therefore likely that CAR T cells too must persist long-term as agents of immunosurveillance to prevent relapse. That CAR T cells are capable of this long-term persistence is becoming clearer every day. Memory T cells have a lifespan of many years, and gammaretroviral or lentiviral integration can lead to stable integration of the transgene. Patients with HIV who were infused at our institution with gene-modified T cells over 10 years ago still show evidence of T-cell persistence, and patients treated with anti-CD19 CAR T cells up to 4 years ago still have circulating CART19 cells and B-cell aplasia (5, 25, 55).

Extensive preclinical and exciting clinical experience indicate that CAR T cells possess the requisite characteristics that define successful adoptive cellular immunotherapy. CAR T cells are clearly capable of trafficking, expansion, proliferation, effector function, and long-term persistence as memory cells. CAR T cells have been used to treat both hematologic and solid malignancies, with impressive outcomes in the treatment of $CD19^+$ B-cell leukemias and lymphomas (5, 7, 8). In the following sections, we describe the results of preclinical and clinical studies in hematologic malignancies.

CAR T cells for hematologic malignancies

Hematologic malignancies represent a relatively small proportion of the total cancer burden but have played a prominent role in advancing cancer treatment. The attempt to improve outcomes in patients with hematologic malignancies led to the development of novel therapeutic approaches such as multi-agent chemotherapy, monoclonal antibodies, and tyrosine kinase inhibitors. In the setting of CAR T-cell therapy, some of the earliest trials and the most important lessons were drawn from the field of solid tumors (1,2), but to date, the most exciting results from CAR T-cell therapy have been derived from trials of patients with B-cell malignancy (5,7,8). There is a wealth of new antigen-specific immunotherapeutics in addition to CAR T cells. In *Table* 2, we have provided a conceptual comparison of CAR T cells, antibody-drug conjugates (ADCs), and bispecific T-cell engaging antibodies (BiTEs) using B-cell malignancies as an example.

Adoptive T-cell transfer elicits quantitatively different responses to those engendered by therapeutic tumor vaccines and immune checkpoint blockade, likely because the latter rely on stimulation of endogenous polyclonal T cells with low precursor frequency and relatively low TCR affinity. The response to tumor vaccines and checkpoint blockade occurs within several months whereas the response to T-cell transfer usually occurs within days to weeks and can be accompanied by tumor lysis syndrome (however, there are several fascinating examples in the literature of slow or delayed response to CART19) (6, 131). The quality of the immune response is different as well. As a result of antigen-driven activation of the T cells, there is widespread cytokine production and secondary immune activation. Several groups have reported cytokine release syndrome (CRS) after infusion of anti-CD19 CAR T cells. The CRS refers to the production of inflammatory cytokines as a direct consequence of T-cell activation and is characterized by high fevers, hypotension, and hypoxia (6, 7, 132). The CRS has been observed in children as well as in adults and is positively correlated with tumor burden. Most cases of severe CRS have occurred in patients with B-ALL and lie on a continuum with a severe macrophage activation syndrome. High levels of IL-6 have been observed, and treatment with tocilizumab, an anti-IL6R monoclonal antibody, generally

***Table* 2. Comparison of antigen-specific immunotherapy approaches to B-cell malignancies**

Technology	CART	ADC	BiTE
Example	CART19 (Penn) CTL019 (Novartis) (autologous *ex vivo* expanded T cells transduced with an anti-CD19 scFv)	Inotuzumab (anti-CD22 Mab linked to calicheamycin)	Blinatumumab (anti-CD3 anti-CD19 bispecific antibody)
Dosing	One infusion	Once every 3 weeks; or weekly	Continuous infusion 28 days on, 14 days off
Complete responses (relapsed/refractory B-ALL)	90% (173)	19% (174)	66% (175)
Survival	78% 6 months OS	5–6 months median	9 months median
Major toxicity	Cytokine release syndrome, encephalopathy	Fever, hepatotoxicity	Cytokine release syndrome, encephalopathy
Antigen-loss relapses noted?	Yes	No	Yes
Major challenges	Complex process to manufacture an individualized product	Relatively lower response rates	Burdensome infusion regimen

leads to rapid resolution when combined with high-dose steroids (25).

CAR T-cell therapy for B-cell malignancies

Patients with B-cell malignancy were first treated using DNA-electroporated B-cell specific autologous T cells in 2008 by investigators from the Fred Hutchinson Cancer Research Center and from the City of Hope National Medical Center (3). Seven patients with indolent or mantle cell lymphoma were treated with anti-CD20 redirected T cells and achieved a partial response (one patient), stable disease (four patients), or maintained a previous complete response (two patients). T cells persisted up to 9 weeks. In 2010, the City of Hope group published a follow-up study on patients with relapsed diffuse large B-cell lymphoma treated with anti-CD20 (two patients) or anti-CD19 (two patients) CAR T cells, but in this study, T cells persisted no longer than 7 days, perhaps due to a cellular anti-transgene immune response in some of the patients (4).

These results were followed by a spate of publications from the National Cancer Institute, Memorial Sloan Kettering Cancer Center, the Baylor College of Medicine and the University of Pennsylvania, describing over 100 patients in various settings (*Table* 3). There were several differences between the groups, including patient population, type of B-cell malignancy, costimulatory molecule, gene transfer technique, T-cell expansion methodology, and use of lymphodepletion. All these trials were single-arm studies, thus precluding direct comparisons between them. Nonetheless, we draw the following conclusions from the collective experience of these patients: (i) patients should receive lymphodepleting chemotherapy; (ii) second generation CAR constructs are superior to first generation constructs; (iii) responses are more dramatic among acute lymphoid rather than chronic lymphoid leukemia; (iv) patients with dramatic responses tend to develop a severe cytokine release syndrome; (v) there is no clear dose-response relationship between the number of CAR T cell infused and the depth of response; and (vi) there appears to be no correlation between initial tumor burden and response, in that patients with marked bone marrow infiltration by leukemia can and do experience complete responses.

There may be an indication that bulky lymphadenopathy may be more difficult to eradicate than heavy marrow disease, yet it is clear that CAR T cells can enter extramedullary sites such as the central nervous system (5, 24, 47, 131). A novel cytokine release/macrophage activation syndrome has been described by several groups (24, 25, 133). This entity is characterized by high persistent fever, hypoxia, and hypotension accompanied by markedly elevated ferritin and c-reactive protein. Real-time evaluation of serum cytokines in the first patients revealed a unique cytokine signature that led to the discovery that a single dose of the anti-IL6 receptor antibody tocilizumab can usually extinguish CRS/MAS promptly (25). Neurological toxicity has also been described, although the etiology of this remains unclear, as CAR T cells are often but not always found in the CNS at the time of neurotoxicity (24). Intriguingly, similar neurotoxicity has been described after blinatumumab infusion (134).

Another fascinating observation has been a handful of patients who initially appear to clear B-ALL but relapse with CD19-negative disease. This is an example of the potent immune effect of anti-CD19 CAR T cells and of the capacity of a rapidly progressive malignancy for immune escape (25). This is an important lesson when treating patients with highly proliferative, immature malignancies such as acute leukemias.

Other B-cell antigens have been targeted in preclinical models. These include CD22, CD23, ROR1, and the light chain. CD22 is highly expressed on mature lymphoid malignancies as well as on B-ALL. This is a rather long molecule and preclinical results have been informative in demonstrating that for maximal anti-tumor effect it is important to target a proximal epitope on CD22 (34). CD23 is expressed on CLL cells but not on normal B cells, and preclinical studies have shown that anti-CD23 CAR T cells have some activity against growth of a CLL-like cell line (135). Clinical translation of this construct could therefore spare normal B cells. ROR1 is a transmembrane tyrosine kinase that is detected on malignant B cells in CLL and MCL, and at lower levels on normal adipose cells and some B-cell precursors; successful targeting of this antigen could theoretically spare normal B cells unless the expression of ROR1 on B-cell precursors compromises B-cell regeneration (136). A clinical trial targeting ROR1 is planned. Targeting one of the two light chains would be useful in mature B-cell malignancies with surface light chain expression and is another way to spare some of the normal B-cell population; preclinical results show that free light chains do not interfere with the function of the CAR T cells and may in fact sustain their proliferation (137). Preliminary clinical results suggest that this approach is safe and effective (138). Currently open clinical trials for the treatment of B-cell malignancies are shown in *Table* 4.

Table 3. **Clinical trials with anti-CD19 T-cell therapy**

Patient	Disease (age)	Disease state	Lympho-depletion	Total T cell dose	CAR %	Exogenous cytokines	Toxicities	Best Disease response	Time to response	Time to relapse/ Progression (months)	CAR persistence (days)
City of hope, retroviral FMC63 anti-CD19 scFv-CD3ζ with thymidine kinase suicide gene [Jensen *et al.* (4)]											
1	FL (NR)	Refractory	Flu after dose #1	$10^8/m^2$ (#1) $10^9/m^2$ (#2, #3) $2 \times 10^9/m^2$ (#4, #5)	NR	IL-2	Lymphopenia	NE		1	1
2	FL (NR)	Refractory	Flu after dose #1	$10^8/m^2$ (#1) $10^9/m^2$ (#2, #3) $2 \times 10^9/m^2$ (#4)	NR	IL-2	Lymphopenia	NR		5	1
Baylor, retroviral FMC63 anti-CD19 scFv-CD3ζ and anti-CD19 scFv – CD28-CD3ζ [Savoldo *et al.* (15)]											
3	SLL (53)	Relapsed	No	$2 \times 10^7/m^2$	20–60	No	NR	SD		10	6 weeks
4	FL/DLBCL (56)	Relapsed	No	$2 \times 10^7/m^2$	20–60	No	NR	PD		1	6 weeks
5	DLBCL (46)	Relapsed	No	$1 \times 10^8/m^2$	20–60	No	NR	SD		3	6 weeks
6	DLBCL (57)	Relapsed Refractory	No	$1 \times 10^8/m^2$	20–60	No	NR	PD		1.5	6 weeks
7	FL/DLBCL	Relapsed	No	$2 \times 10^8/m^2$	20–60	No	NR	PD		1.5	6 weeks
8	DLBCL	Relapsed	No	$2 \times 10^8/m^2$	20–60	No	NR	PD		0.5	6 weeks
University of Pennsylvania, lentiviral FMC63 anti-CD19 scFv-41BB-CD3ζ [Porter *et al.* (5) and Kalos *et al.* (6)]											
9	CLL (65)	Relapsed	B	1.6×10^7/kg CAR^+ cells		No	Fever, hypotension	CR		11+	>24 weeks
10	CLL (77)	Relapsed	BR	1.0×10^7/kg CAR^+ cells		No	Fever, dyspnea, cardiac dysfunction	PR		7	>24 weeks
11	CLL (64)	Relapsed	PentoCy	1.5×10^5/kg CAR^+ cells		No	TLS, fever, transaminitis	CR		10+	>24 weeks
MSKCC, retroviral SJ25C1 anti-CD19scFv-CD28-CD3ζ [Brentjens *et al.* (8)]											
12	CLL (51)	Relapsed	No	2.5×10^9 CAR^+ cells		No	Fever	PD		NA	35
13	CLL (72)	Relapsed	No	1.2×10^9 CAR^+ cells		No	Fever	PD		NA	NR
14	CLL (73)	Relapsed	No	1.1×10^9 CAR^+ cells		No	Fever	PD		NA	NR
15	CLL (69)	Relapsed	Cy	3.2×10^9 CAR^+ cells		No	Fever, ARF, hypotension	NE (Died)		NA	1
16	CLL (68)	Relapsed	Cy	4×10^8 CAR^+ cells		No	Fever	PR (delayed)	1 month	9	8
17	CLL (68)	Relapsed	Cy	4×10^8 CAR^+ cells		No	Fever, hypotension	PD		NA	8
18	CLL (62)	Relapsed, progressive	Cy	7.6×10^8 CAR^+ cells		No	Fever	SD		4 months	20
19	CLL (61)	Relapsed, progressive	Cy	1.4×10^9 CAR^+ cells		No	Fever	SD		2 months+	30
20	ALL (67)	Relapsed	Cy	1.8×10^8 CAR^+ cells		No	Diarrhea, hypotension, neutropenia	NE (AlloHCT at 8 weeks)		NE	15
21	ALL (48)	Relapsed		NA							6 weeks
NCI, retroviral FMC63 anti-CD19 scFv-CD28-CD3ζ, [Kochenderfer *et al.* (7)]											
22a*	FL (47)	Relapsed	CyFlu	0.5×10^7/kg	64	IL-2	Cytopenias, fever	PR		7	20
22b	FL (48)	Relapsed	CyFlu	2.1×10^7/kg	63	IL-2	Fatigue, zoster	PR		18+	20

Table 3. (continued)

Patient	Disease (age)	Disease state	Lympho-depletion	Total T cell dose	CAR %	Exogenous cytokines	Toxicities	Best Disease response	Time to response	Time to relapse/ Progression (months)	CAR persistence (days)
23	FL (48)	Relapsed	CyFlu	0.5 × 10^7/kg	65	IL-2	Bacteremia, pneumonia, stroke	NE		NE (died, influenza)	20
24	CLL (61)	Relapsed	CyFlu	2.5 × 10^7/kg	45	IL-2	Hypotension, ARF, hypoxemia, capillary leak, hyper-bilirubinemia	CR		15+	20
25	SMZL (55)	Relapsed	CyFlu	2.0 × 10^7/kg	53	IL-2	Diarrhea, fatigue	PR		12	20
26	CLL (54)	Relapsed	CyFlu	0.6 × 10^7/kg	50	IL-2	Fever, fatigue, hypotension	SD		6	20
27	CLL (57)	Relapsed	CyFlu	5.5 × 10^7/kg	30	IL-2	Hypotension, capillary leak	PR		7	20
28	CLL (61)	Relapsed	CyFlu	5.4 × 10^7/kg	51	IL-2	Obtundation, ARF, capillary leak, hyper-bilirubinemia	PR		7+	132+
29	Follicular (63)	Relapsed	CyFlu	4.2 × 10^7/kg	71	IL-2	Hypotension, obtundation, ARF, capillary leak	PR		8+	132+
University of Pennsylvania, lentiviral FMC63 anti-CD19 scFv-41BB-CD3ζ [Grupp *et al.* (25) and Maude *et al.* (27)]											
30	ALL (7)	Relapsed, refractory	No	1.2 × 10^7/kg CAR^+		No	Fever, respiratory failure, hypotension	CR (molecular)		9+	180+
31	ALL (10)	Relapsed refractory post AlloHCT and blinatumumab	Cy, VP16	1.4 × 10^6/kg CAR^+ of donor origin		No	Fever, myalgias, confusion	CR (MRD^+)		2 (relapse with $CD19^-$ disease)	70
MSKCC, retroviral SJ25C1 anti-CD19scFv-CD28-CD3ζ [Brentjens *et al.* (132) and Davila *et al.* (24)]											
32	ALL (66)	Relapsed, MRD^+	Cy	1.8 × 10^8 CAR^+		No	Neutropenia, diarrhea, hypotension	MRD^-	D28	NA (AlloHCT)	35
33	ALL (56)	MRD^-	Cy	3.2 × 10^8 CAR^+		No	Febrile neutropenia, hypotension	MRD^-		NA (AlloHCT)	28
34	ALL (59)	Refractory	Cy	3.2 × 10^8 CAR^+		No	Fatigue, febrile neutropenia, hypotension, seizure	MRD^-	CR D11, MRD^- D59	1.5 (relapse with $CD19^+$ disease)	47
35	ALL (58)	Refractory	Cy	2.9 × 10^8 CAR^+		No	Hypoxia, altered mental state, febrile neutropenia, hypoxia, seizure	MRD^-	CR and MRD^- by D8	NA (AlloHCT)	47
36	ALL (23)	MRD^+	Cy	1.4 × 10^8 CAR^+		No	Fever	MRD^-	D30	NA (AlloHCT)	55
37	ALL (30)	Refractory	Cy			No		MRD^-			
38	ALL (74)	Extramedullary disease	Cy			No		NR			
39	ALL (23)	MRD^+	Cy			No		MRD^-			
40	ALL (27)	MRD^-	Cy			No		MRD^-			
41	ALL (32)	MRD^+	Cy			No		MRD^-		NA (AlloHCT)	
42	ALL (42)	Refractory	Cy			No		NR			

Table 3. (continued)

Patient	Disease (age)	Disease state	Lympho-depletion	Total T cell dose	CAR %	Exogenous cytokines	Toxicities	Best Disease response	Time to response	Time to relapse/ Progression (months)	CAR persistence (days)
43	ALL (36)	Refractory	Cy			No		MRD^-			
44	ALL (60)	Refractory	Cy			No		MRD^-		NA (AlloHCT)	
45	ALL (27)	Refractory	Cy			No		MRD^-		NA (AlloHCT)	
46	ALL (63)	MRD^+	Cy			No		MRD^+			
47	ALL (59)	Refractory	Cy			No		MRD^-			
Baylor, retroviral FMC63 anti-CD19 scFv – CD28-CD3ζ [Cruz *et al.* (47)], relapsed post allogeneic HCT											
48	ALL (23)	Molecular relapse	No	3.2×10^7		No	No GVHD	CR		3 months	12 weeks
49	CLL (59)	Refractory	No	3.4×10^7		No	No GVHD	PR		8 weeks	1 week
50	CLL (57)	Refractory	No	3.3×10^7		No	No GVHD	SD		15 months	2 weeks
51	ALL (9)	Relapsed MRD	No	1.9×10^7		No	No GVHD	PD		NA	12 weeks
52	CLL (49)	Refractory	No	1×10^8		No	No GVHD	PD		NA	8 weeks
53	CLL (59)	Refractory	No	1.1×10^8		No	No GVHD	PD		NA	4 weeks
54	ALL (40)	CR	No	9.7×10^7		No	No GVHD	CCR		8 months+	12 weeks
55	ALL (12)	CR	No	5.8×10^7		No	No GVHD	CCR		2 months+	8 weeks
NCI, retroviral FMC63 anti-CD19 scFv-CD28-CD3ζ [Kochenderfer *et al.* (131)]											
56	SMZL (56)	†	CyFlu	5×10^6/kg CAR^+		No	Hypotension, confusion, AKI, fever	PR			23+
57	PMBCL (43)	Refractory	CyFlu	5×10^6/kg CAR^+		No	Confusion, aphasia, facial nerve palsy, UTI, fever	CR			22+
58	CLL (61)	Refractory	CyFlu	4×10^6/kg CAR^+		No	Confusion, feverhypotension	CR			23+
59	PMBCL (30)	Refractory	CyFlu	2.5×10^6/kg CAR^+		No	Hypoxia, fever, bacteremia, vascular leak, death	NE			
60	CLL (63)	†	CyFlu	2.5×10^6/kg CAR^+		No	None >gr 3	CR			15+
61	CLL (48)	Refractory	CyFlu	2.5×10^6/kg CAR^+		No	None >gr 3	CR			14+
62	DLBCL (42)	Refractory	CyFlu	2.5×10^6/kg CAR^+		No	Influenza, fever, bacteremia	CR			9+
63	PMBCL (44)	Refractory	CyFlu	2.5×10^6/kg CAR^+		No	Pneumonitis, bacteremia, AKI, fever, obtundation	CR			12+
64	PMBCL (38)	Refractory	CyFlu	2.5×10^6/kg CAR^+		No	Aphasia, fever, myoclonus	SD			1
65	Low-grade NHL (57)	Relapsed	CyFlu	1×10^6/kg CAR^+		No	Bacteremia, fatigue, fever	CR			11+
66	DLBCL (58)	Refractory	CyFlu	1×10^6/kg CAR^+		No	Bacteremia, UTI, fever	PR			1
67	DLBCL (60)	Refractory	CyFlu	1×10^6/kg CAR^+		No	UTI, fever, bacteremia, DVT	NE			
68	CLL (68)		CyFlu	1×10^6/kg CAR^+		No	Dyspnea, hypotension, DVT, UTI, AKI	PR			4
69	DLBCL (43)	Refractory	CyFlu	1×10^6/kg CAR^+		No	None other than fever	CR			6

Table 3. (continued)

Patient	Disease (age)	Disease state	Lympho-depletion	Total T cell dose	CAR %	Exogenous cytokines	Toxicities	Best Disease response	Time to response	Time to relapse/ Progression (months)	CAR persistence (days)
70	DLBCL (64)	Relapsed	CyFlu	1×10^6/kg CAR^+		No	Aphasia, fever, encephalopathy, neuropathy, gait disturbance	PR			6+

+Indicates ongoing response at time of report.
*This patient was treated twice.
†These patients were previously reported.
AlloHCT, allogeneic hematopoietic cell transplantation; B, bendamustine; BR, bendamustine and rituximab; CLL, chronic lymphoid leukemia; CR, complete response; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; NE, non-evaluable; NR, no response; PentoCy, pentostatin and cyclophosphamide; PR, partial response; SD, stable disease; SMZL, splenic marginal zone lymphoma; FluCy, fludarabine and cyclophosphamide; VP16, etoposide.

CAR T-cell therapy for acute myeloid leukemia (AML)

Relapsed or refractory AML carries an extremely poor prognosis. The fact that AML is a partially immune-responsive disease suggests that optimism may be warranted in translating the results of B-cell targeting CAR T cells to the AML arena. However, as AML is a malignancy of the hematopoietic stem cell, it is truly challenging to find a target that is present on AML and absent from normal hematopoietic cells. In fact, most validated AML or leukemic stem cell surface markers are shared to some extent with normal tissues. Nonetheless, some AML membrane antigens at an early stage of evaluation. The IL-3 receptor α chain, also known as the early hematopoietic antigen CD123, has been shown by both the City of Hope and the University of Pennsylvania groups to be expressed on the majority of AML patients. However, CD123 is also present on normal marrow precursors, suggesting that CAR T cells targeting this antigen could lead to severe hematopoietic toxicity (139, 140). This observation indicates that clinical trials using anti-CD123 CAR T cells should be carefully designed to mitigate the possible consequences of myeloablation, possibly with a backup bone marrow donor. This is the approach taken by the City of Hope in a recently announced clinical trial (NCT02159495). The apparent safety of a CD123-directed diphtheria toxin-conjugated reagent in patients with blastic plasmacytoid dendritic cell neoplasm (BPDCN) is only moderately reassuring in this regard, as the avidity of CAR T cells for their target is likely much higher than that of such cytokine-conjugated therapeutics (141).

Among the more obvious targets for the treatment of AML is the myeloid antigen CD33, which is the target of the antibody-drug conjugate gemtuzumab ozogamicin. Gemtuzumab led to hepatotoxicity and veno-occlusive disease in a minority of patients, although it is unclear whether that was related to specific targeting of Kupffer cells or to release of the calicheamcyin toxin (142, 143). CD33 is expressed on immature myeloid cells and therefore prolonged and potent attack on $CD33^+$ cells by CAR T cells has the potential to cause profound myeloablation. A recently published preclinical study compared anti-CD33 CAR T cells with anti-CD123 CAR T cells and demonstrated these to have equivalent anti-AML efficacy but in this particular study anti-CD33 CAR T cells induced more severe myeloablation than T cells redirected to CD123 (144). Nonetheless, a study in China is currently recruiting patients to an anti-CD33 CAR T-cell trial (NCT01864902) that is based on a lentivirally transduced, CD137 costimulated construct; one

Table 4. **Currently recruiting CAR T-cell therapy trials by antigen**

Center	Disease	Patient population	Co-stimulation	Gene transfer	Notes	Clinicaltrials.gov identifier
CD19						
MSKCC	CLL	>18 years old	*CD28*	RV	Dose-escalation	NCT00466531
BCM	B-cell malignancy	Any	*CD28*	RV	With ipilimumab	NCT00586391
BCM	B-cell malignancy	Any	*CD28*	RV	Dose escalation	NCT00608270
BCM	B-cell malignancy	Any	*CD28*	RV	After AlloHCT, viral co-specificity	NCT00840853
NCI	B-cell malignancy	18–68	*CD28*	RV	With IL2	NCT00924326
MDACC	B-cell lymphoma	18–65			With or without IL2	NCT00968760
MSKCC	B-ALL	>18 years old	*CD28*	RV		NCT01044069
NCI	B-cell malignancy	18–75	*CD28*	RV	Active GVHD not allowed	NCT01087294
MSKCC	CLL	>18 years old	*CD28*	RV	Upfront therapy	NCT01416974
MSKCC	B-ALL	<19 years old	*CD28*	RV	After AlloHCT, viral co-specificity	NCT01430390
Manchester, UK	B-cell malignancy	>18 years old	*None*			NCT01493453
MDACC	B-cell malignancy	1–65			After AlloHCT	NCT01497184
NCI	B-cell malignancy	1–30 years old	*CD28*	RV		NCT01593696
CHOP	$CD19^+$ leukemia and lymphoma	1–24 years old	*4-1BB*	LV		NCT01623495
Seattle Children's	$CD19^+$ ALL	Age 1–26			$EGFR^+$ construct (may allow deletion)	NCT01683279
Penn	CLL/SLL	>18 years	*4-1BB*	LV	2 dose level comparison	NCT01747486
MSKCC	Aggressive B-NHL, relapsed/refractory	18–70	*CD28*	RV	After autologous SCT	NCT 01840566
BCM	B-cell malignancy	Up to 75 years old	$CD28^{+/-}$ *4-1BB*	RV		NCT01853531
MSKCC	B-ALL	<26 years old	*CD28*	RV		NCT01860937
Beijing	B-cell malignancy	5–90 years old	*4-1BB*			NCT01864889
FHCRC	B-cell malignancy	>18 years		LV		NCT01865617
Penn	B-cell NHL	>18 years old	*4-1BB*	LV		NCT02030834
Seattle Children's	B-ALL		*4-1BB*	LV	$EGFR^+$ construct (may allow deletion)	NCT02028455
Penn	B-ALL	>18 years old	*4-1BB*	LV		NCT02030847
BCM	B-cell malignancy		*CD28*	RV	After AlloHCT	NCT02050347
Beijing	Mantle cell lymphoma	50–80				NCT02081937
Sweden	B-cell malignancy	>18 years old	*CD28 and 4-1BB*	RV		NCT02132624
Japan	B cell NHL	20–70	*CD28*	RV		NCT02134262
Kappa light chain						
BCM	B-cell malignancy or myeloma		*CD28*	RV		NCT00881920
ROR1						
MDACC	CLL/SLL	>18 years old				NCT02194374

patient has been treated so far with transient decrease in marrow blasts and tolerable toxicity (145).

CD44 is an adhesion molecule that is broadly expressed on normal tissues. One of its isoform variant, CD44v6 is expressed on some AML blasts and on some myeloma cells. Anti-CD44v6 CAR T cells mediated potent anti-tumor effects in mouse models (146). The presence of CD44v6 on keratinocytes and the report of lethal epithelial toxicity in a patient treated with an anti-CD44v6 monoclonal antibody suggests that caution is warranted in any CAR clinical trial that is based on targeting CD44v6 (147).

The literature contains references to other antigens that may be of relevance in AML, in particular putative leukemic stem cells (LSC) (148). Notably, the existence of LSC is still in question, and while CD123, CD47, or CD96 may enrich for improved engraftment in immunodeficient mice, all these antigens are also widely expressed on other normal tissues and would therefore likely be problematic as CAR targets. CLL-1, also known as CLEC12A, is thought to have no expression outside the hematopoietic system and has been reported to be a marker of LSC (149). A BiTE to CLEC12A is being designed. Theoretically, if a LSC-specific CAR T cell could be constructed, its major role would likely be in treating minimal residual disease to prevent relapse.

The feasibility of an AML CAR T-cell trial has already been established. The group at the University of Melbourne treated four patients with a CD28-costimulated retrovirally transduced CAR T-cell product targeting the Lewis Y antigen, a carbohydrate antigen that is expressed by many

human tumors. Minimal toxicity was observed, and two patients experienced minor responses. Of interest, radiolabeling of the infused CAR T cells clearly showed trafficking to sites of disease. Furthermore, in one patient, a deposit of leukemia cutis was observed to undergo inflammation, and CAR T cells were found in the involved skin, confirming that intravenously infused CAR T cells can localize to sites of disease (104). Responses were less than dramatic, despite prolonged persistence of the transgenic T cells, implying that additional factors need to be addressed before this particular construct could be used for treating AML.

The description of these efforts to apply CAR T-cell therapy to high-risk relapsed or refractory AML illustrates the scope of the issues associated with targeting a hematopoietic stem cell-derived malignancy. Clearly, clinical development of CAR T-cell therapy for AML will have to be undertaken very carefully. Robust and precise assessment of normal non-hematopoietic tissue expression of the targeted antigen should be performed and due consideration given to the consequences of damage to bystander cells and tissues, as has already been done by some groups (139, 146). The development of approaches to limit the persistence of the infused CAR T cells will be very helpful in this regard. Current approaches include the use of mRNA-electroporated, 'biodegradable' CAR T cells, introduction of a suicide gene, or the inclusion of an EGFR moiety on the surface of the T cells to render them potentially susceptible to depletion by anti-EGFR antibodies (139, 140, 146).

CAR T-cell therapy for Hodgkin's lymphoma

Although the malignant cell in Hodkin's lymphoma (HL) is thought to be derived from a crippled B cell, Hodgkin Reed-Sternberg (HRS) cells no longer express B-cell-associated surface markers. They are instead characterized by bright, uniform expression of CD30. This antigen is the target of the recently approved antibody-drug conjugate brentuximab vedotin (BV). In relapsed HL, BV leads to response rates that are impressively high but unfortunately are not durable, and the major role of BV is probably as a bridge to subsequent curative therapy. Importantly, patients who relapse after prior BV appear to retain CD30 expression on HRS cells, suggesting that they could be candidates for a CAR T-cell approach. There are preclinical data that $CD30^+$ Hodgkin cell lines can be targeted by CAR T cells (150, 151). Notably, CD30 is present on some activated T cells; thus, anti-CD30 CAR T cells could theoretically induce fratricide and impair their own expansion. Two trials targeting CD30 are ongoing at the Baylor College of Medicine (NCT01192464 and NCT01316146), and results are eagerly awaited.

CAR T-cell therapy for T-cell malignancies

Although relatively less common than B-cell malignancies in the Western hemisphere, T-cell malignancies are difficult to treat, and T-cell non-Hodgkin's lymphoma (NHL) carries a poorer prognosis than B-cell NHL, stage-for-stage. The only potentially curative modality for relapsed T-cell NHL is allogeneic HCT. Therefore, the development of a CAR T-cell based therapy for T-cell malignancies would represent a vertical advance in the field. Unfortunately, targeting abnormal T cells with antigen-specific CAR T cells represents arguably the most difficult application of the CAR concept. A specific antigen would have to be found on the malignant T cells and not on the transgenic T cells, and one particular target could be the clonotypic TCR in those tumors that retain expression of the TCR. As some T-cell lymphomas express CD30, theoretically an anti-CD30 CAR T cell could be used for this indication. One possible approach would be to use natural killer cells to treat T-cell malignancies targeting non-shared antigens (152), but the persistence of the NK cells would have to be closely regulated to prevent prolonged T-cell lymphopenia. We are not aware of preclinical data targeting CAR T cells against T-cell malignancies. This therefore represents a challenging but important avenue of investigation.

CAR T-cell therapy for myeloma

Almost 25 000 people per year are diagnosed with myeloma in the United States, and most of these are over 60 years old. Despite the advent of exciting new agents for the treatment of myeloma over the last decade, the disease remains incurable, spurring great interest in developing CAR T-cell therapy for myeloma. Myeloma is well-known to express CD138 and CD38 at high levels. CD138, also known as syndecan-1, is a cell membrane heparin sulfate proteoglycan. Although its expression within the hematopoietic system is limited to plasma cells, it is also widely expressed on epithelium (153–155). Clues to potential toxicity can be obtained from clinical trials of an anti-CD138 antibody-drug conjugate (BT062) where dose-limiting toxicities included inflammation of the palms and soles (likely an on-target effect) (156). Nonetheless, there is a phase I/II clinical trial of a second-generation, CD137-costimulated CAR against CD138 in Beijing (NCT01886976). The results of this are eagerly awaited. CD38 is an inhibitory signaling molecule on many lymphoid and myeloid subsets (including multipo-

tent progenitors), though it is particularly highly expressed on myeloma cells. The anti-CD38 antibody daratumumab has interesting single-agent activity in early phase clinical trials and does not seem to be associated with significant myelosuppression. Nonetheless, as CART-mediated targeting of CD38 is likely to be more potent than a monoclonal antibody, the main concern with CD38 as a CAR target remains the potential for myelotoxicity (157, 158).

Other potential targets have been identified in myeloma. In our opinion, the most interesting of these is BCMA, a tumor necrosis factor receptor (TNFR) family protein found on mature B and plasma cells that promotes survival of long-lived bone marrow plasma cells (159). BCMA is clearly expressed in multiple myeloma and does not appear to be expressed on any non-lymphoid tissues by immunohistochemistry (160). The NCI group has published preclinical data on targeting BCMA using CAR T cells (161).

The cell surface glycoprotein CS1 is expressed in most multiple myeloma cells and normal plasma cell samples. Importantly, it is also present at lower levels on other lymphocytes, activated monocytes, and activated dendritic cells (162). A particular concern is its expression on activated T cells, which suggests that anti-CS1 CAR T-cell therapy may be complicated by fratricide during the manufacturing culture or *in vivo*. Nonetheless a monoclonal antibody targeting CS-1, elotuzumab, is in advanced phases of clinical development (163), and a group at Ohio State has published preclinical data on the efficacy of a CS1-redirected CAR introduced into both NK cells and T cells (164, 165).

There are preclinical data on anti-CD44v6 CAR T cells in myeloma. As outlined above, the main concern with CD44v6 as a CAR target is its expression on keratinocytes. A radioisotope-labeled antibody bivatuzumab caused myelosuppression and a fatality due to skin toxicity, though it is interesting to note that CAR T cells based on a the bivatuzumab antibody did not kill keratinocytes *in vitro* (146, 147).

Conclusions

The design and implementation of novel CAR T-cell products are accelerating, and the cycle from antigen discovery to clinical trial is likely to get shorter and shorter. However, several important questions remain to be answered, and we should devote as much effort to optimizing CAR T-cell therapy as we do to extending its reach.

The optimal CAR design is as yet undefined. Ideally, careful clinical comparisons of CARs based on the different costimulatory molecules would inform this, in the way that a comparison of first-generation and second-generation clearly showed enhanced persistence from the latter (15). Similar consideration should be given to the other components of the CAR, such as the hinge and transmembrane regions. Indeed, different epitopes on the target may be best targeted using different length CAR (34). In addition, most CARs are currently derived from murine antibodies. Humanization or primary human derivation will probably abrogate the development of clinically relevant HAMA reactions (166).

We do not yet know the optimal gene-transfer method. Lentiviral and retroviral gene delivery may be equivalent but both are complicated, expensive, and onerous. On the other hand, there is more clinical experience with these methods than with alternative, non-viral techniques such as the transposon/transposase system (59). Transient gene delivery such as RNA electroporation is safe, efficient, and relatively less complicated, but multiple infusions are required to ensure consistent T-cell-mediated immune pressure and may not be as powerful as viral strategies (63, 167).

T-cell expansion is currently carried out as a cottage industry. Suitable ways to scale production horizontally (or scale out) must be found to increase throughput and decrease variability. This will require training of technicians as well as adoption of new technologies and instrumentation by cell production facilities. Importantly, T-cell expansion protocols vary between institutions and should ideally be standardized in the way that blood banking or hematopoietic stem cell products are currently harvested, stored, and administered.

The optimal dose of T cells to administer is unknown. Some patients have clearly had profound responses with a relatively small aliquot of infused T cells, and others have failed to respond despite a relatively larger transgenic T-cell infusion. Thus, there is no clear dose–response association. In the mouse, the adoptive transfer of a single central memory T cell is sufficient to protect from lethal infection (168). It is likely that the degree of T-cell expansion is more important than the infused dose.

CAR T-cell therapy represents a shift away from conventional biologics in several ways: first, this approach is truly patient-specific in that a new, autologous product must be manufactured for each patient. Currently this is feasible only in the context of large academic centers with extensive expertise and resources. The decades-long experience with hematopoietic cell transplantation (approximately 1 million patients transplanted to date) indicates that transformative therapy provided solely within the context of academic cen-

ters can be meaningful on a population level. Second, we think of CAR T cells as a 'living drug'. One infusion of T cells is clearly sufficient, in most cases, to induce extensive proliferation that leads to expansion on the order of 3–4 logs. This implies that the anti-tumor effect and other manifestations of T-cell activation can take several days to weeks to become apparent. The kinetics of T-cell expansion and tumor rejection appear to vary depending on the tumor type, although this has not been systematically studied as yet.

Third, although CAR T cells represent targeted therapy in terms of antigen recognition, the mechanism by which they lead to target cell death is unclear and is likely multi-factorial. T cells have a panoply of weapons at their disposal, including perforin-granzyme-mediated killing, death receptor engagement, production of cytokines, and recruitment of additional immune effectors. Thus, in contrast with small molecules, tumor escape from CAR T-cell killing likely cannot occur through modulation of susceptibility pathways. It can only occur through antigen loss escape, as has already been described (25). This escape pathway can most readily be addressed by the infusion of a T-cell product that contains two (or more) specificities (29).

Fourth, in contrast with bispecific T-cell engaging antibodies, CAR T cells have novel, designer specificity and are not limited by the endogenous TCR repertoire. In addition, costimulation is built in and may provide a more potent response to cellular targets.

Fifth, with great power comes great responsibility. The sequence encoding single chain variable fragments of clinically used antibodies can and has been inserted into CAR T cells. This has taught us that CAR T cells are much more powerful than antibodies. Although the affinity of the scFv is identical, the functional avidity that results from the immune synapse forming around a CAR-ligand interaction leads to potent T-cell activation. Therefore, CAR T cells can be more toxic than their progenitor antibodies. This means that we cannot be reassured by the toxicity profile of the parental monoclonal antibodies. We believe that each new CAR must be put through a strenuous toxicity assessment prior to completing translation into the clinic (1, 169). There is a dearth of preclinical models to predict off-target effects or on target effects consequent to low level antigen detected by CAR T cells that is not detected by conventional assays.

This is an exciting time in cellular immunotherapy. Years of basic research in the fields of virology, molecular biology, and T-cell culture methodology have coalesced in an avalanche of preclinical research that continues to deliver new insights on a regular basis. This preclinical research is being translated into clinical trials in both liquid and solid malignancies. CAR T-cell treatment of B-cell malignancies in particular is clearly feasible and can lead to impressive and profound responses in patients with otherwise treatment-refractory cancer. It is likely that CAR T-cell therapy has leapfrogged over monoclonal antibody therapy as an antigen-specific immunotherapy. The power of CAR T cells is truly unprecedented and should not be underestimated. Clinical trials targeting antigens other than CD19 in HL, myeloma, and AML have already begun or are in advanced stages of design. The number of centers performing or planning to perform these trials is expanding dramatically. Thus, CAR T-cell therapy for hematologic malignancy is going viral. We look forward to seeing this transformative therapy being delivered to more patients as the academic community learns where and how best to deploy these new weapons.

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