

REVIEW ARTICLE

Genetic modification of human T lymphocytes for the treatment of hematologic malignancies

Valentina Hoyos,^{1,2} Barbara Savoldo,^{1,2} and Gianpietro Dotti^{1,2,4}¹Center for Cell and Gene Therapy, Baylor College of Medicine, Houston; ²Department of Medicine, Baylor College of Medicine, Houston; ³Department of Pediatrics, Texas Children's Hospital, Houston; and ⁴Department of Immunology, Baylor College of Medicine, Houston, USA

ABSTRACT

Modern chemotherapy regimens and supportive care have produced remarkable improvements in the overall survival of patients with hematologic malignancies. However, the development of targeted small molecules, monoclonal antibodies, and biological therapies that demonstrate greater efficacy and lower toxicity remains highly desirable in hematology, and oncology in general. In the context of biological therapies, T-lymphocyte based treatments have enormous potential. Donor lymphocyte infusion in patients relapsed after allogeneic hematopoietic stem cell transplant pioneered the concept that T lymphocytes can effectively control tumor growth, and this was then followed by the development of cell culture strategies to generate T lymphocytes with selective activity against tumor cells. Over the past decade, it has become clear that the adoptive transfer of *ex vivo* expanded antigen-specific cytotoxic T lymphocytes promotes sustained antitumor effects in patients with virus-associated lymphomas, such as Epstein-Barr virus related

post-transplant lymphomas and Hodgkin's lymphomas. Because of this compelling clinical evidence and the concomitant development of methodologies for robust gene transfer to human T lymphocytes, the field has rapidly evolved, offering new opportunities to extend T-cell based therapies. This review summarizes the most recent biological and clinical developments using genetically manipulated T cells for the treatment of hematologic malignancies.

Key words: immunotherapy, cytotoxic T lymphocytes, gene transfer, chimeric antigen receptor, suicide gene.

Citation: Hoyos V, Savoldo B, and Dotti G. Genetic modification of human T lymphocytes for the treatment of hematologic malignancies. *Haematologica* 2012;97(11):1622-1631. doi:10.3324/haematol.2012.064303

©2012 Ferrata Storti Foundation. This is an open-access paper.

Introduction

The modern concept of targeted therapy in patients with hematologic malignancies, and cancers in general, aims at generating new agents committed to targeting tumor cells more specifically and with less toxicity than conventional chemoradiotherapy. Immunotherapy in general, and T-cell based immunotherapies which are the specific focus of this review, are excellent examples of these new agents. Physiologically, T lymphocytes recognize antigens through a unique antigen-specific $\alpha\beta$ -T cell receptor (TCR), promote the elimination of the targeted antigen (effector function) and amplify the attack to the antigen by recruiting other components of the immune response (helper function). Although tumor specificity can be accomplished clinically using monoclonal antibodies, T lymphocytes have additional critical properties that make their clinical application attractive. T lymphocytes can, indeed, actively biodistribute themselves within tissues and the tumor environment, and have the potential for *in vivo* expansion and self-maintenance, as they can establish a memory compartment. New bi-specific antibodies also have the properties of selective antigen specificity and T-cell activation.¹ Although preliminary clinical studies are very encouraging, the antitumor

effects provided by these molecules may not be long-lasting, as no specific T-cell memory is generated. There is also a concern for potential induction of T-cell anergy, as recruited T cells will not receive appropriate co-stimulation.

Donor lymphocyte infusion (DLI) and adoptive transfer of antigen-specific cytotoxic T lymphocytes (CTLs) targeting Epstein Barr virus (EBV) associated antigens can control hematologic malignancies, and EBV-specific CTLs in particular represent a cost effective treatment modality for EBV-associated post-transplant lymphomas and Hodgkin's lymphoma.²⁻⁴ Recent advances in the field have allowed genetic modifications of T cells to provide robust, personalized T lymphocytes that target specific tumor-associated antigens. In this review article, we will discuss the development of the clinical grade methodologies that allowed efficient gene transfer to T cells, and how such gene transfer has armed T lymphocytes with enhanced anti-tumor activity, while retaining an acceptable safety profile.

Gene transfer to T lymphocytes

Gene transfer in human T lymphocytes can be accomplished by several means (Table 1). DNA plasmids can be inserted by electroporation or nucleoporation, and transgenic T cells can

Funding: the authors declare no competing financial interests. This work was supported in part by R01 CA142636 National Institutes of Health-NCI, W81XWH-10-10425 Department of Defense Technology/Therapeutic Development Award, NIH P50CA126752 and Leukemia and Lymphoma Society Translational Research grants. We would like to thank Dr Malcolm Brenner for the critical revision of the manuscript.

Manuscript received on May 3, 2012. Revised version arrived on July 11, 2012. Manuscript accepted on August 20, 2012

Correspondence: Gianpietro Dotti, MD, Center for Cell and Gene Therapy, Baylor College of Medicine, 6621 Fannin Street, MC 3-3320, Houston, Texas 77030, USA. Phone: international +832.8246891. Fax: international +832.8254732. E-mail: gdotti@bcm.tmc.edu

then be selected based on the accompanying insertion of drug resistance genes.⁵ Although relatively inexpensive, this approach is not efficient as naked DNA only integrates in a very low percentage of T cells. As a consequence, several weeks of culture are required to reach sufficient numbers of manipulated T cells for clinical use, which may significantly compromise their capacity to survive long-term *in vivo*. In addition, the inclusion of genes encoding antibiotic resistance in T cells may generate immunogenicity to the proteins produced, with premature elimination of these cells *in vivo*.⁶ Gene delivery can also be achieved through gammaretroviral vectors, which have been used in the majority of T-cell based clinical studies. These vectors can be manufactured on a large scale and they produce stable integration into the genome of the T cell and its progeny, formed by cell division. Current major concerns raised by the use of these viruses are the risks of generating replication competent retroviruses (RCR) and insertional mutagenesis. Modern packaging cell lines have significantly reduced the risk of generating RCR, as confirmed by a recent extensive analysis of more than 800 samples collected from different trials in the USA, all of which were free from RCR contaminants.⁷ Similarly, adverse consequences due to insertional mutagenesis in T cells have not yet been reported; a very different safety profile compared to the use of these same vectors to genetically modify hematopoietic stem cells.^{3,8,9} Lentiviral vectors have also been recently used to engineer T cells in non-HIV patients.¹⁰ These vectors are particularly attractive when less differentiated T-lymphocyte phenotype needs to be preserved, as they have the unique ability to infect T cells even upon a minimal activation, a property lacking in gammaretroviral vectors.¹¹ Unfortunately, the large-scale production of lentiviral vectors for clinical use remains problematic. Novel non-viral methods to deliver genes into T cells are based on transposon/transposase systems (Sleeping Beauty¹² and PiggyBac)¹³ which have a higher integration rate than naked DNA and allow the insertion of larger fragments of DNA than many viral vectors. Clinical trials using the Sleeping Beauty-based strategy to genetically modify T lymphocytes with a chimeric antigen receptor have recently been approved by the US Food and Drug Administration (FDA).

Donor lymphocyte infusions and suicide genes

Donor lymphocyte infusion (DLI) is the simplest form of T-cell based immunotherapy after allogeneic hematopoietic stem cell transplant (HSCT). DLIs have induced complete remissions in relapsed patients with chronic myelogenous leukemia (CML) and other hematologic malignancies.^{2,14}

However, the graft-versus-leukemia (GVL) effect mediated by T lymphocytes is frequently associated with a more generalized immune attack on the recipient known as graft-versus-host disease (GvHD). This complication remains a significant cause of morbidity and mortality, especially if DLI are obtained from donors who are non HLA-matched siblings.¹⁵

The genetic manipulation of donor lymphocytes to incorporate a suicide gene for their prompt elimination upon occurrence of severe GvHD represents an elegant approach, and a study using the gene transfer of the Herpes Simplex virus-derived enzyme thymidine kinase (HSV-tk) is now in a phase III clinical trial.^{16,17} HSV-tk phosphorylates nucleoside analogs that ultimately inhibit DNA synthesis, thereby killing dividing cells. This gene has been inserted in T lymphocytes to act as a suicide gene in the presence of gancyclovir (Figure 1). To date, more than 120 patients have been infused with HSV-tk modified T cells after receiving matched or haploidentical HSCT. None of these patients had evidence of severe adverse effects from the transgene, including those associated with insertional mutagenesis, further underlining the safety of the gammaretroviral-mediated gene transfer in T cells. When GvHD occurred, it was efficiently controlled by the administration of gancyclovir in all patients. Importantly, the infusion of HSV-tk modified T cells had relevant clinical benefits since it improved the rate of immune reconstitution, which is usually significantly delayed following haploidentical HSCT, and appeared to have anti-leukemic activity.^{17,18} The current phase III study should provide definitive information concerning the efficacy of this treatment. Nonetheless, the use of HSV-tk modified T cells has some limitations. The first drawback is that gancyclovir administration to these patients with the intent to prevent or treat cytomegalovirus (CMV) reactivation can not be accomplished without premature elimination of the transgenic T cells, so that alternative and potentially more toxic drugs are required to control infections or diseases caused by this virus. Secondly, the elimination of HSV-tk T cells by gancyclovir requires several days. As a consequence, this suicide gene may not be highly efficient in promptly controlling severe GvHD. Finally, there has been evidence of immune responses against the *HSV-tk* gene, leading to the elimination of transgenic T cells, especially when these T cells are infused in patients with spontaneous T-cell immune reconstitution.¹⁹ New variants of the *HSV-tk* gene are currently being explored to avoid alternative splicing that makes non-functional proteins.

At our institution, we have developed a novel suicide gene based on the expression of an inducible caspase-9

Table 1. Gene delivery systems for T cells.

Gene delivery method	Packaging capacity	Manufacturing	Host range	Genomic integration frequency	Expression	Clinical trials	Safety concern
Gammaretrovirus	4kb	Good packaging cell line. High titer. Expensive	Dividing cells	High	Long-term	Yes	Insertional mutagenesis
Lentivirus	8kb	Poor packaging cell line. Low titer. Expensive	Dividing and non-dividing cells	High	Long-term	Yes	Potential insertional mutagenesis
Plasmids	"Unlimited"	Easy production. Selection needed	Dividing and non-dividing cells	Very low	Transient	Yes	Unknown
Transposone/Transposase	"Unlimited"	Easy production. Selection needed	Dividing and non-dividing cells	Intermediate	Long-term	Yes	Unknown

(iC9) gene in T cells (Figure 1).²⁰ The innovation of this suicide approach relies on the expression of an inducible human molecule (iC9) that activates the cell's physiological apoptotic pathway in response to a specific small molecule. The native caspase-9 molecule that acts as a key player in the mitochondrial apoptosis is modified to include a motif that allows its dimerization (and hence activation) in the presence of a chemical inducer of dimerization (CID) (Figure 2).²¹ Unlike HSV-tk, the function of iC9 is not dependent on cell cycle and allows the rapid (within a few hours) induction of apoptosis in T cells. In a phase I dose escalation clinical trial, 10 patients who had undergone haploidentical HSCT received T cells expressing iC9²² (MK Brenner, unpublished data, 2012). As previously observed in the HSV-tk clinical trials, infusion of iC9-modified T cells was well tolerated up to 1×10^7 cells/kg, and induced rapid immune reconstitution. Four patients who developed grade I-II GvHD received a single intravenous infusion of CID that determined the elimination of more than 90% of iC9-T cells from the peripheral blood within 30 min of completing drug infusion, and complete and sustained resolution of the GvHD. Non-alloreactive T cells expressing iC9 were still detectable in the peripheral blood of patients more than one year after infusion.²² This preliminary experience showing the rapid induction of cell death in T lymphocytes expressing the iC9 suicide in response to CID indicates that this strategy may hold important advantages when compared to HSV-tk. Clinical trials in other centers are currently validating this approach. Two other suicide genes are based on the expression of CD20²³ and inducible CD95 (Fas).²⁴ These approaches have not yet been tested clinically. While the Fas-based approach relies on the same principle of the iC9 system, the forced expression of CD20 by T lymphocyte provides the advantage of using a single molecule (anti-CD20 antibodies) to select the transgenic cells *in vitro* and to eliminate them *in vivo*. Although the simplicity of this

approach continues to make it an attractive option, CD20 antibodies such as rituximab will also deplete normal B lymphocytes when they are infused to control GvHD induced by CD20-transgenic T cells.

T lymphocytes with redirected specificity

The *ex vivo* generation of antigen-specific CTLs relies on culture conditions in which polyclonal T cells isolated from peripheral blood are progressively enriched for those recognizing antigens loaded and presented by professional antigen-presenting cells (APCs).²⁵ Cultured CTLs are mostly composed of effector-memory T lymphocytes, with cytotoxic activity (mediated by their TCRs) against tumor cells expressing the antigens that have been presented by the APCs *ex vivo*. Using this methodology, EBV-specific CTLs have been successfully generated and expanded *ex vivo*, and safely and effectively infused in over 100 patients to prevent or treat EBV-associated malignancies.^{3,4} Although similar strategies have been implemented to generate T cells targeting minor histocompatibility antigens²⁶ or other relevant tumor-associated antigens (TAAs) expressed by hematologic malignancies, such as survivin,²⁷ hyaluronan-mediated motility receptor (HMMR/Rhamm),²⁸ Wilms tumor gene product 1 (WT1),^{29,30} proteinase 1 (PR1),³¹ preferentially expressed antigen in melanoma (PRAME)^{32,33} and MAGE,³⁴ the production of these cells for clinical purposes remains largely suboptimal due to its complexity and the low frequency of CTLs with adequate TCR affinity.³²

While the introduction of suicide genes provides the perfect example of how gene transfer contributes to dramatically improving the safety of conventional DLI after allogeneic HSCT, gene transfer can also be used to reshape the antigen specificity of T lymphocytes. Thus, polyclonal lymphocytes can be manipulated to express artificial antigen receptors (chimeric antigen receptors CARs), or ectopic α - and β -TCR chains, thereby conferring a second antigen

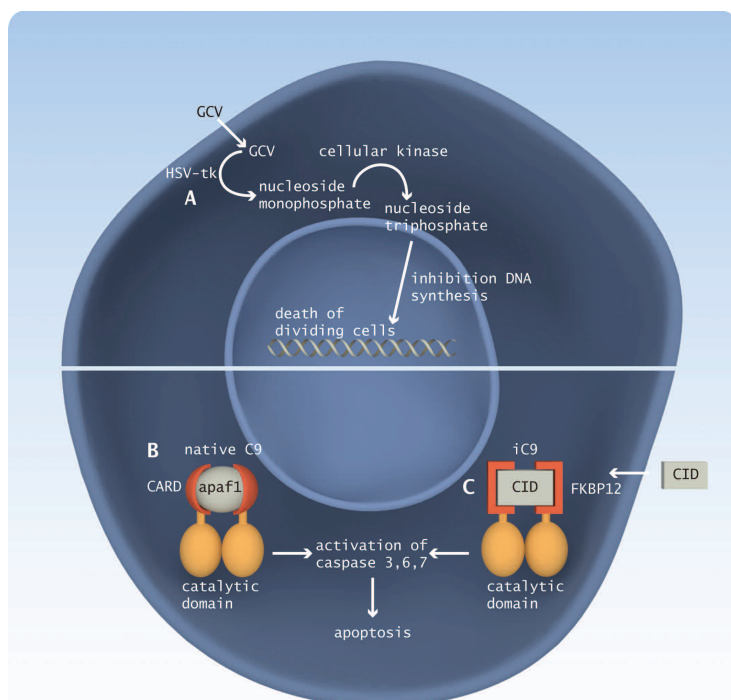


Figure 1. Expression of suicide genes in T lymphocytes. The figure illustrates the two suicide systems that have been tested in clinical trials. (A) HSV-tk is an enzyme that phosphorylates specific nucleoside analogs (gancyclovir) to nucleoside monophosphate. A second cellular kinase phosphorylates this product into nucleoside triphosphate, a molecule that inhibits DNA synthesis and therefore leads to death of dividing cells. (B) Caspase-9 is a key player in the mitochondrial apoptosis pathway. This molecule dimerizes upon binding with apaf1 and leads to cleavage of the effector caspases 3, 6 and 7. (C) The inducible caspase-9 (iC9) is a fusion protein in which the native CARD domain has been replaced by an FKBP12 domain that dimerizes in the presence of a specific chemical inducer of dimerization (CID).

specificity to T cells in addition to their native TCR (Figure 3).^{35,36} Both strategies provide a means of generating large numbers of T cells with defined antigen specificity that can be used for adoptive T-cell immunotherapy trials, thus overcoming the time consuming and highly inefficient process of selecting and expanding T cells that are naturally reactive against TAAs.

CAR-modified T lymphocytes

CARs are generated by fusing two distinct functional fragments. The most widely used combination is the antigen binding specific fragment composed of the single chain (scFv) obtained from the variable domains of the heavy and light chains of a monoclonal antibody, and the signaling domain derived from the CD3 ζ chain (Figure 3).³⁵ These simple molecules are currently defined as first generation CARs, and when expressed by CD8 and CD4 T-cell subsets, they provide specific binding to antigens expressed on the cell surface of tumor cells and simultaneous activation of the cytotoxic machinery (granzyme-B and perforin) of T cells through the CD3 ζ chain signaling immunoreceptor tyrosine-based activation motif (ITAM). Due to the anti-

body-mediated antigen recognition, the cytotoxic function of CAR-redirection T cells does not depend on the canonical antigen processing and MHC-restricted presentation typical of native TCRs. However, several pre-clinical models showed that first generation CARs do not fully activate modified T cells upon engagement with the antigen, as these molecules lack co-stimulatory properties. To overcome this obstacle, second generation CARs have been generated in which intracytoplasmic domains, derived from co-stimulatory molecules such as CD28^{37,38} or CD134/OX40³⁹ or CD137/4-1BB,⁴⁰ are incorporated within CARs to fully activate T cells. Third generation CARs, in which two co-stimulatory molecules (CD28 and 4-1BB or CD28 and OX40)^{39,41} are encoded in tandem, have also been generated, and pre-clinical experiments suggest that these molecules may be even more potent than second generation CARs (Figure 3).

T lymphocytes expressing CD19- and CD20-specific CARs

For hematologic malignancies, CAR technology allows targeting of lineage restricted antigens, such as CD19⁴² and CD20⁴³ that are conserved in the malignant counterpart, by

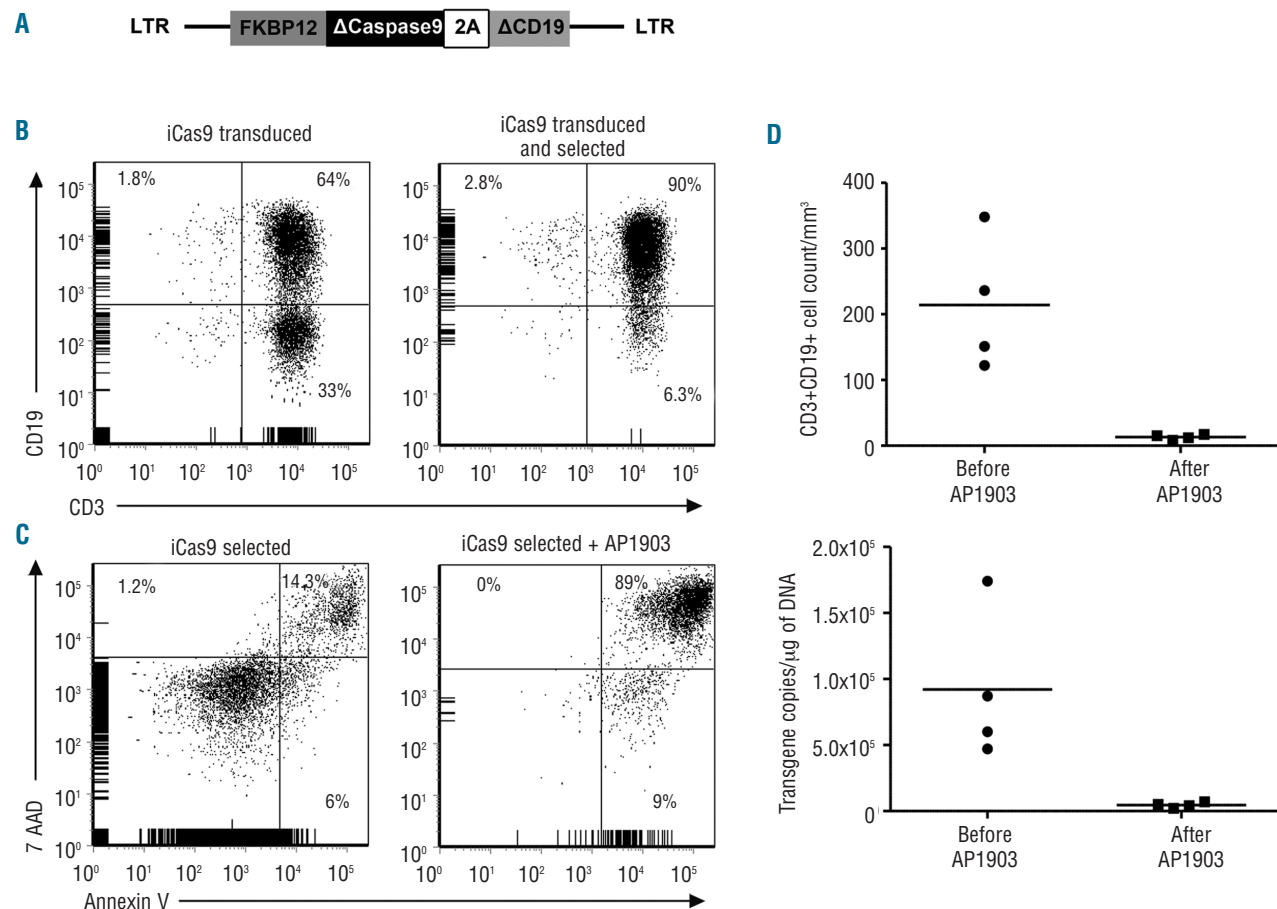


Figure 2. Inducible caspase 9 suicide gene. (A) Schematic representation of the bicistronic vector that encodes the inducible caspase 9 (iC9) and the truncated form of human CD19. The two genes are linked through the inclusion of a 2A-like peptide sequence. (B) Expression of CD19 in activated T lymphocytes transduced with the bicistronic vector and enrichment of CD19⁺ cells obtained after positive selection using a CD19-specific antibody. (C) Induction of apoptosis of T cells 24 h after exposure to the AP1903 (CID) that activates the inducible caspase 9. (D) Elimination of transduced T cells in 4 patients after one single dose of AP1903 as measured by phenotypic analysis and copy numbers of the iC9 transgene in peripheral blood mononuclear cells.

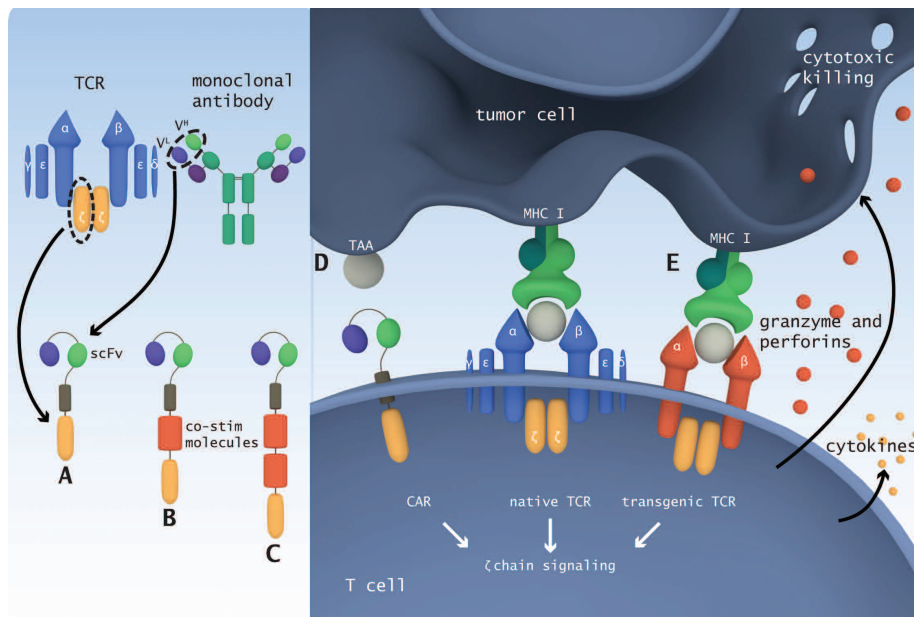


Figure 3. Redirecting T-cell specificity. T-cell specificity can be redirected by the expression of chimeric antigen receptors (CARs) or transgenic α - and β -TCR chains. (A) CARs are composed of two distinct fragments. The first is obtained by linking the variable heavy and light regions of a monoclonal antibody of known specificity into a single chain (scFv) and the second is the signaling region of the native TCR ζ -chain. (B) Second generation CARs incorporate co-stimulatory molecules such as CD28 and 4-1BB. (C) Third generation CARs include 2 or more co-stimulatory molecules. (D) CARs are activated after the scFv portion binds to an extracellular antigen thus the antigen recognition is MHC-independent. (E) Transgenic TCRs recognize specific peptides presented in the context of MHC class I molecules.

combining the binding specificity from the available monoclonal antibody targeting these surface molecules with the potent effector function of T lymphocytes. More than 20 clinical trials have been registered in the USA for infusing T lymphocytes expressing CD19- or CD20-specific CARs in patients with B-cell derived malignancies,⁴⁴ and results from many of these studies have recently been reported (Table 2).^{5,10,45-49} When analyzing the results of these trials, it is important to appreciate that few patients have so far been enrolled in each study, and each study cohort is heterogeneous with regards to patient age and type/stage of disease. There is also heterogeneity in the CAR constructs in terms of the co-stimulatory endodomains used, and the gene transfer systems and culture conditions employed to generate and expand these CAR-modified T cells. Although these variables make study comparisons difficult, it is possible to extrapolate guidelines for future applications of this technology: a) infusions of CAR-modified T cells are generally well tolerated, with only one serious reported adverse event which may, however, not have been directly associated with the T-cell infusion;⁵⁰ b) virus-mediated gene transfer seems to be the most efficient gene delivery method, as it allows robust expression of CARs by T cells, and minimizes the time of their *ex vivo* culture; c) the incorporation of co-stimulatory endodomains (CD28 or 4-1BB) within CARs is essential in supporting their *in vivo* persistence;^{10,45} d) trials that include lymphodepleting chemotherapy regimens prior to the T-cell infusion may have better clinical outcomes, as they reduce the tumor burden and provide an environment for the infused T cells that favors homeostatic expansion.⁴⁸ For example, impressive results were reported in 3 patients with aggressive, heavily pre-treated chronic lymphocytic leukemia (CLL) who received chemotherapy 1-4 days prior to the infusion of T cells transduced with a lentiviral vector encoding a CD19-specific CAR, incorporating the 4-1BB co-stimulatory endodomain.¹⁰ In this trial, the infused cells showed logarithmic expansion *in vivo* and migration to the site of disease with remarkable cytotoxic effects against tumor cells (2 complete and one partial remission), and persistence for more than 180 days. In contrast, CAR-T cell expansion observed *in vivo* in clinical trials

using CD19-specific CARs that incorporate the CD28 signaling endodomain have produced less striking results,⁴⁵ suggesting that the 'late activation' signaling pathways recruited by 4-1BB may be critical for functional, long-term expansion and engraftment of CAR-redirection T cells. However, given the diversity of the trials developed so far, systematic assessment of whether 4-1BB is responsible for the better outcome observed in one clinical trial may require a formal comparison between CAR-T cells encoding CD28 and CAR-T cells encoding 4-1BB infused in the same patient, in a similar way to the direct comparison of first and second generation (CD28 co-stimulation) CD19-specific CAR reported previously.⁴⁵

Several significant issues still remain to be resolved before designing large phase II studies using CAR technology.

Is the current design of CAR molecules ideal? Potent third generation CARs have been generated to combine 'early' (CD28) and 'late' co-stimulatory signals (4-1BB or OX40) for complete T-cell activation.^{39,41} However, combinations of these molecules raise safety concerns since they may induce T cells to produce excessive amounts of cytokines and promote severe cytokine storms as observed in a patient treated at the National Cancer Institute.⁵¹ One trial has been published using a CD19-specific third generation CAR (CD28 and 4-1BB).⁵² The infusion of these cells did not produce significant side effects, but the CAR for this study was delivered in T cells by DNA electroporation, and required extensive *ex vivo* culture before infusion, which may exhaust these T cells and thus underestimate their potential toxicities. Many CARs may also be immunogenic, as the scFv are usually derived from mouse hybridomas. Although CAR-T cells can still be detected for months after infusion, humanized scFv may be preferable in the future to reduce the immunogenicity.

How much lymphodepletion is required before the infusion of CAR-modified T cells? Regimens inducing profound lymphodepletion used by the group at the National Cancer Institute are based on their experience in melanoma patients infused with tumor infiltrating lymphocytes.^{56,48,53} However, because significant clinical responses were obtained in patients infused with CAR-T cells after less pro-

Table 2. Results of clinical trials using CARs targeting CD19 and CD20.

Reference	Targeted disease/antigen	Construct/generation	Gene transfer	Cell activation	Concomitant therapy	Cell dose	N. patients	Outcomes	CAR-T cell persistence
Till <i>et al.</i> ⁵	B-NHL/CD20	scFv-CH2CH3-CD4TM-CD3 ζ First	Electroporation	OKT3	Cyclophosphamide or fludarabine +/- IL-2	1x10 ⁶ /m ² to 3.3x10 ⁶ /m ²	7	2 maintained CR 1 PR, 4 SD	1-3 weeks alone./5-9 weeks with IL-2
Jensen <i>et al.</i> ⁶	NHL/CD19 or CD20	scFv-CH2CH3-CD4TM-CD3 ζ First	Electroporation	OKT3	ASCT/fludarabine IL-2	5 at 10 ⁶ /m ² , 7 at 10 ⁷ /m ² , 3 at 2x10 ⁷ /m ²	4	2 maintained CR after ASCT	1-7 days, cellular immune response against CAR-T cells
Savoldo <i>et al.</i> ⁴⁵	NHL/CD19	scFv-CH2CH3-CD3 ζ First vs. scFv-CH2CH3-CD28-CD3 ζ Second	Gammaretrovirus	OKT3/CD28	None	2x10 ⁷ /m ² to 2x10 ⁸ /m ²	6	2 SD	Persistence of second generation for more than 6 months
Brentjens <i>et al.</i> ⁴⁶	CLL or ALL/CD19	scFv-CD28-CD3 ζ Second	Gammaretrovirus	CD3/CD28 Beads	Cyclophosphamide	1.2-3x10 ⁷ /m ² then 0.4-1x10 ⁷ /m ²	10	1 Death, 3 SD	40 days
Kalos <i>et al.</i> ¹⁰ Porter <i>et al.</i> ⁴⁷	CLL/CD19	scFv-41BB-CD3 ζ Second	Lentivirus	CD3/CD28 Beads	Bendamustine or pentostatin/cyclophosphamide	1.4x10 ⁷ /m ² to 1.1x10 ⁸ /m ²	3	2 CR, 1 PR	>6 months >1,000-fold expansion of CAR-T cells
Kochenderfer <i>et al.</i> ⁴⁸	B-NHL/CLL/CD19	scFv-CD28-CD3 ζ Second	Gammaretrovirus	OKT3	Cyclophosphamide/fludarabine IL-2	0.5-5.5x10 ⁷ /Kg	8	6 PR, 1 CR, 1 SD	14 weeks
Till <i>et al.</i> ⁴⁹	B-NHL/CD20	scFv-CH2CH3-CD4TM-CD28-41BB-CD3 ζ Third	Electroporation	OKT3	Cyclophosphamide IL-2	1x10 ⁶ /m ² to 3.3x10 ⁶ /m ²	4	2 SD, 2 PR	12 months

found immunodepletion induced by treatment with fludarabine, bendamustine or pentostatin, profound lymphodepleting regimens may not be necessary if robust costimulation is provided to CAR-modified T cells.¹⁰

Are all B-cell malignancies susceptible to control by CD19-specific CAR-T cells and which stage of disease should be targeted? Chronic lymphocytic leukemias and low-grade lymphomas appear highly responsive to CAR-mediated immunotherapies,^{10,48,54} while relapsed/refractory high-grade lymphomas are rarely controlled,^{45,48} likely due to the more rapid progression of the latter. We still lack data from clinical trials targeting acute leukemia. Of note, tumor lysis syndrome has been observed in responding patients with bulky or advanced stage diseases,¹⁰ suggesting that CAR-T cell-based therapy may be better suited to minimal residual disease or as an adjuvant for patients at high risk of relapse, who respond to salvage treatment or after transplant. In this regard, our group recently initiated a phase I clinical trial in patients with B-cell malignancies who had relapsed after allogeneic HSCT. In this trial, patients are infused with donor-derived CTLs specific for opportunistic viruses (CMV, EBV and adenoviruses) that have also been genetically modified to express a CD19-specific CAR.^{55,56} The aim of this study is to provide a T-cell product that simultaneously protects against viral infection and produces anti-leukemia activity, without causing GvHD. In addition, virus specificity should promote the long-term persistence of these cells, following physiological activation and costimulation when their native TCRs engage latent virus

(EBV and CMV)-associated antigens presented by professional APCs.⁵⁷ So far, infusions of these products have been well tolerated without occurrence of GvHD or viral infections in 3 patients.

Other CARs for hematologic malignancies

While targeting CD19/CD20 by CAR-modified T cells may have benefit for B-cell derived malignancies, long-term persistence of CD19-specific CAR-T cells is associated with elimination of normal B lymphocytes, with resulting impairment of humoral immunity.¹⁰ Although this complication may be compensated by the administration of pooled immunoglobulins, more selective CARs targeting B-cell associated antigens have been generated that eliminate tumor cells while sparing normal B lymphocytes. For example, mature B-cell malignancies express monoclonal immunoglobulins carrying either the λ - or κ -light chain. Our group, therefore, developed a CAR that targets the κ -light chain, and demonstrated that T cells expressing this CAR can eliminate leukemic and normal κ -restricted B cells whilst sparing normal λ -chain restricted B lymphocytes, thus preserving at least a subset of the normal B-cell compartment.⁵⁸ A phase I clinical trial using this approach is open at our institution. Alternative selective targets for B-cell malignancies that have been explored in pre-clinical models include CD23,⁵⁸ CD70⁵⁹ and ROR1.⁶⁰ CD23, a B-cell activation marker, is highly expressed by CLL cells but only at relatively low levels in normal B lymphocytes. Pre-clinical studies have confirmed that CD23-CAR redirected T cells

can control CLL growth *in vitro* and *in vivo* with minimal toxicity to normal B lymphocytes.⁵⁸ CD70 is the ligand of CD27 receptor and is only transiently expressed by highly activated T and B cells, as well as by dendritic cells, but aberrantly over-expressed by many leukemias and lymphomas.⁵⁹ The CD70-specific counter receptor CD27 has been fused to the CD3 ζ chain and expressed by T lymphocytes, resulting in the elimination of CD70-expressing lymphoma cells.⁵⁹ The receptor tyrosine kinase-like orphan receptor 1 (ROR1) is highly expressed in CLL and mantle cell lymphoma cells. Unlike CD23 and CD70, expression of ROR1 is not up-regulated with B-cell activation, and expression in non-hematopoietic tissues is limited to adipose tissue, immature B cells and pancreas. ROR1-CAR redirected T cells are cytotoxic to CLL cells, and ongoing studies in non-human primates are addressing the potential toxicities of this strategy, especially in view of shared expression by pancreatic cells.⁶⁰

CD30 antigen is also being targeted by CAR-redirection T cells in an effort to extend the application of the technology to non-B cell lymphomas.^{61,62} CD30 is aberrantly expressed by most Hodgkin's and some non-Hodgkin's lymphomas. CD30 has been validated as a therapeutic target since monoclonal antibodies directed to this antigen have produced objective clinical responses.⁶³ CD30-specific CARs have also been extensively evaluated in pre-clinical studies, and two clinical trials with CD30-CAR redirected T cells in CD30⁺ lymphoma patients are currently open at our institution. For the treatment of myeloid malignancies, a CD33-specific CAR has been developed.⁶⁴ Although effective in pre-clinical experiments, this approach needs further evaluation as CD33 is a pan-myeloid marker, and CD33-CAR redirected T cells may, therefore, lead to profound and prolonged myeloid depletion. The isoform variant 6 of CD44 (CD44v6) represents another possible target for CAR-T cells in myeloid leukemias and in myeloma. Preliminary results show that CD44v6-CAR redirected T cells had anti-tumor effects against CD44v6⁺ malignancies.⁶⁵ However, the expression of this antigen by keratinocytes and the skin toxicity reported in patients treated with the corresponding monoclonal antibody are a potential cause for concern.⁶⁶

T lymphocytes expressing ectopic α - and β -TCR chains

The antigen specificity of T lymphocytes can also be redirected by the expression of a second TCR with known specificity. This is the only strategy available to target intracytoplasmic proteins that are processed and presented in association with MHC molecules (Figure 3). This category includes the great majority of tumor-associated/cancer testis antigens. For this approach, the antigen-specific TCR is usually derived from human T-cell clones with high affinity for the specific epitope or from murine T cells obtained by immunization of HLA-2 transgenic mice. Transgenic TCR chains can be expressed in polyclonally activated T cells, to provide them with a second HLA-restricted tumor specificity in addition to the native antigen specificity (Figure 3). This strategy has been pioneered by the group at the National Cancer Institute, in patients with melanoma or other solid tumors by targeting MART-1 and NY-ISO antigens.^{36,67} Complete and sustained clinical responses have been observed in these patients. For hematologic malignancies, transgenic TCRs have been prepared that target the Wilms tumor gene product 1 (WT1), which is a zinc finger transcriptional regulator present in acute myeloid leukemia, CML and myelodysplastic syndrome.³⁰ A phase I clinical trial

using this approach is currently ongoing in Europe and, if successful, this approach can be extended to many antigens that are highly expressed in leukemia or lymphomas, such as hyaluronan-mediated motility receptor (HMMR/Rhmm), melanoma antigen preferentially expressed in tumors (PRAME), proteinase 3 (PR3), MAGE-antigens and survivin.

Unlike CARs, the cytotoxic activity of transgenic TCRs remains HLA-restricted, and the great majority of cloned TCRs are HLA-A2 restricted, since this is the most frequent polymorphism in Caucasians. However, since technologies are now available to screen immunodominant epitopes presented by other HLA antigens, cloned TCRs will soon be available for many more HLA molecules. A concern associated with the ectopic expression of TCR in T lymphocytes is mispairing between native and transgenic α - and β -chains. This event reduces the expression of desired TCR (loss of function) and may also create unknown TCR specificities with potential autoreactivity (gain of function). Although animal models have shown that this mispairing can have off-target effects,⁶⁸ no such side effects have yet been observed in patients. However, strategies to enhance the expression of transgenic TCR or to silence the endogenous TCR have been developed to minimize this concern.^{69,71}

Enhancing persistence and trafficking of tumor-specific T lymphocytes

T cells with defined antigen-specificity are a fundamental requirement for T-cell based therapies of human malignancies. Once infused, T cells must accomplish several other tasks to achieve robust and sustained tumor regression. For instance, adoptively transferred T cells must expand and persist to reach the critical mass required to eliminate the tumor, in a similar way to their activity against pathogens. T cells must also reach the tumor site, and overcome the inhibitory mechanisms developed by tumor cells to block immune responses. It is likely that the clinical efficacy of adoptively transferred antigen-specific T cells will be enhanced by combination with systemic immunomodulatory molecules, such as the check-point modulators CTLA-4 or PD-1 blocking antibodies.⁷² However, to avoid the non-specific toxic effects of these antibodies produced by enhancing autoreactive clones, the clinical benefits of adoptively transferred T cells can be enhanced by incorporating genes into the modified cells that optimize their persistence, tumor-trafficking and resistance to tumor-mediated immunosuppression (Figure 4).

Ex vivo expanded T cells are highly dependent on cytokines for their continued growth and survival. To overcome the frequent toxicities associated with the systemic administration of cytokines such as IL-2, genetic modifications have allowed T cells to produce their own IL-2^{73,74} or IL-15,^{73,75} or to respond to homeostatic cytokines such as IL-7 through the constitutive expression of IL-7R α .⁷⁶ These modifications enhance anti-tumor effects in pre-clinical models. T-cell trafficking to the tumor microenvironment can also be improved. For example, specific chemokine receptors can be ectopically expressed by T cells to improve their homing along the chemokine gradients produced by tumor cells. This approach is effective in pre-clinical models of melanoma, Hodgkin's lymphoma, and neuroblastoma in which overexpression of CXCR2,⁷⁷ CCR4⁷⁸ or CCR2b⁷⁹ by T cells enhanced their trafficking (and subsequent anti-tumor effects) along CXCL1, TARC and CCL2 gradients,

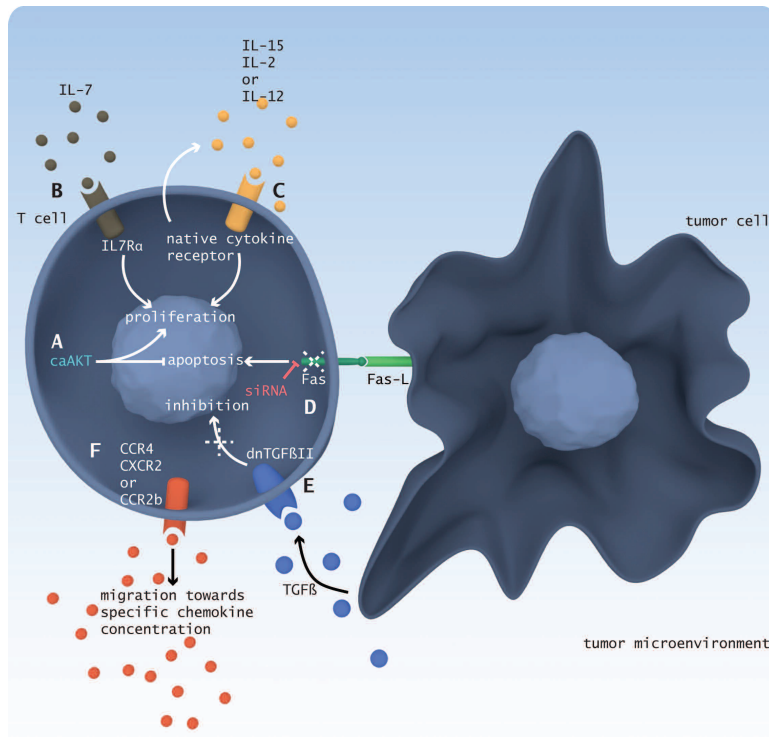


Figure 4. Enhancing T-cell persistence and trafficking. The figure illustrates some of the genetic modifications that have been implemented with the purpose of improving the anti-tumor effects of adoptively transferred T cells. (A) Expression of constitutively activated Akt (caAkt) supports cytokine production and upregulation of anti-apoptotic molecules in T cells. (B) IL-7R α expression that is lost in effector-memory T cells can be restored rendering effector-memory T cells responsive to the homeostatic cytokine IL-7. (C) T cells can be modified to produce cytokine such as IL-2, IL-15 and IL-12 to support their own proliferation and function. (D) CD95(Fas) that leads to apoptosis of activated T lymphocytes by engaging its ligand Fas-L can be down-regulated by siRNA in T cells in order to provide them with resistance to Fas-L-mediated apoptosis. (E) T cells genetically modified to express a dominant negative TGF- β receptor (dnTGF- β II) are resistant to TGF- β inhibition. (F) T cells forced to express specific chemokine receptors show enhanced migration towards tumor chemokine gradients.

respectively. Genetic modifications have also been exploited to counter inhibitory molecules present in the tumor microenvironment. T cells can be rendered resistant to transforming growth factor beta (TGF- β) by the expression of a dominant-negative TGF- β type II receptor (dnTGF- β II),^{80,81} or to be resistant to Fas ligand-induced apoptosis by selective downregulation of Fas/CD95.⁸² IL-12 secretion^{83,84} or/and expression of constitutively active Akt (caAkt)⁸⁵ by T cells also overcome an immunosuppressive tumor environment, and clinical trials evaluating the effects of expressing dnTGF- β II or IL-12 in T cells are ongoing. In patients receiving treatments that inhibit T-cell function, it is also possible to give antigen-specific T cells that are engineered to be resistant to the administered agent. For example, antigen-specific T cells can be made resistant to immunosuppressive drugs such as FK506,⁸⁶ cyclosporine⁸⁷ and rapamycin,⁸⁸ and used to reconstitute anti-EBV specificity in transplant recipients at risk of PTLD or in combination with rapalogs in lymphoma patients.

Conclusions

Cure of many cancers, including hematologic malignancies, will require the combination of multiple therapeutic approaches, including adoptive T-cell immunotherapy,

which will likely become a key player in integrated and personalized cancer therapy. T cells with redirected antigen specificity are one of the most attractive approaches, and it is likely that the clinical efficacy of redirected T cells will be further enhanced by the addition of multiple genetic modifications. However, such potent T cells can also induce dramatic and even lethal adverse effects, and therefore the antigens targeted and the activation signals incorporated in the T cells need careful evaluation. The inclusion of safety switches as an 'exit strategy' can also be considered to minimize damage to normal tissues. The many developments in this field, the striking clinical outcomes and the introduction of simpler, cheaper and faster methods to generate these genetically modified T cells should all help insure they will make a major contribution to the battle against hematologic malignancies.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

- Bargou R, Leo E, Zugmaier G, Klinger M, Goebeler M, Knop S, et al. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. *Science*. 2008;321(5891):974-7.
- Dazzi F, Szydlo RM, Goldman JM. Donor lymphocyte infusions for relapse of chronic myeloid leukemia after allogeneic stem cell transplant: where we now stand. *Exp Hematol*. 1999;27(10):1477-86.
- Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood*. 2010;115(5):925-35.
- Bollard CM, Gottschalk S, Leen AM, Weiss H, Straathof KC, Carrum G, et al. Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood*. 2007;110(8):2838-45.
- Till BG, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA, et al. Adoptive

- immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood*. 2008;112(6):2261-71.
6. Jensen MC, Popplewell L, Cooper LJ, DiGiusto D, Kalos M, Ostberg JR, et al. Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans. *Biol Blood Marrow Transplant*. 2010;16(9):1245-56.
 7. Bear AS, Morgan RA, Cornetta K, June CH, Binder-Scholl G, Dudley ME, et al. Replication-competent retroviruses in gene-modified T cells used in clinical trials: is it time to revise the testing requirements? *Mol Ther*. 2012;20(2):246-9.
 8. Bonini C, Grez M, Traversari C, Ciceri F, Marktel S, Ferrari G, et al. Safety of retroviral gene marking with a truncated NGF receptor. *Nat Med*. 2003;9(4):367-9.
 9. Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*. 2008;118(9):3132-42.
 10. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med*. 2011;3(95):95ra73.
 11. Cavaliere S, Cazzaniga S, Geuna M, Magnani Z, Bordignon C, Naldini L, et al. Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood*. 2003;102(2):497-505.
 12. Singh H, Manuri PR, Olivares S, Dara N, Dawson MJ, Huls H, et al. Redirecting specificity of T-cell populations for CD19 using the Sleeping Beauty system. *Cancer Res*. 2008;68(8):2961-71.
 13. Nakazawa Y, Huye LE, Dotti G, Foster AE, Vera JF, Manuri PR, et al. Optimization of the PiggyBac transposon system for the sustained genetic modification of human T lymphocytes. *J Immunother*. 2009;32(8):826-36.
 14. Mandigers CM, Verdonck LF, Meijerink JP, Dekker AW, Schattenberg AV, Raemaekers JM. Graft-versus-lymphoma effect of donor lymphocyte infusion in indolent lymphomas relapsed after allogeneic stem cell transplantation. *Bone Marrow Transplant*. 2003;32(12):1159-63.
 15. Frey NV, Porter DL. Graft-versus-host disease after donor leukocyte infusions: presentation and management. *Best Pract Res Clin Haematol*. 2008;21(2):205-22.
 16. Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science*. 1997;276(5319):1719-24.
 17. Ciceri F, Bonini C, Stanghellini MT, Bondanza A, Traversari C, Salomoni M, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol*. 2009;10(5):489-500.
 18. Ciceri F, Bonini C, Marktel S, Zappone E, Servida P, Bernardi M, et al. Antitumor effects of HSV-TK-engineered donor lymphocytes after allogeneic stem-cell transplantation. *Blood*. 2007;109(11):4698-707.
 19. Traversari C, Marktel S, Magnani Z, Mangia P, Russo V, Ciceri F, et al. The potential immunogenicity of the TK suicide gene does not prevent full clinical benefit associated with the use of TK-transduced donor lymphocytes in HSCT for hematologic malignancies. *Blood*. 2007;109(11):4708-15.
 20. Straathof KC, Pule MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, et al. An inducible caspase 9 safety switch for T-cell therapy. *Blood*. 2005;105(11):4247-54.
 21. Tey SK, Dotti G, Rooney CM, Heslop HE, Brenner MK. Inducible caspase 9 suicide gene to improve the safety of alodepleted T cells after haploidentical stem cell transplantation. *Biol Blood Marrow Transplant*. 2007;13(8):913-24.
 22. Di Stasi A., Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med*. 2011;365(18):1673-83.
 23. Introna M, Barbui AM, Bambacioni F, Casati C, Gaipa G, Borleri G, et al. Genetic modification of human T cells with CD20: a strategy to purify and lyse transduced cells with anti-CD20 antibodies. *Hum Gene Ther*. 2000;11(4):611-20.
 24. Thomis DC, Marktel S, Bonini C, Traversari C, Gilman M, Bordignon C, et al. A Fas-based suicide switch in human T cells for the treatment of graft-versus-host disease. *Blood*. 2001;97(5):1249-57.
 25. Rooney CM, Smith CA, Ng CY, Loftin SK, Sixbey JW, Gan Y, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood*. 1998;92(5):1549-55.
 26. Warren EH, Fujii N, Akatsuka Y, Chaney CN, Mito JK, Loeb KR, et al. Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. *Blood*. 2010;115(19):3869-78.
 27. Rapoport AP, Aqui NA, Stadtmauer EA, Vogl DT, Fang HB, Cai L, et al. Combination immunotherapy using adoptive T-cell transfer and tumor antigen vaccination on the basis of hTERT and survivin after ASCT for myeloma. *Blood*. 2011;117(3):788-97.
 28. Schmitt M, Schmitt A, Rojewski MT, Chen J, Giannopoulos K, Fei F, et al. RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma elicits immunologic and clinical responses. *Blood*. 2008;111(3):1357-65.
 29. Bornhauser M, Thiede C, Platzbecker U, Kiani A, Oelschlaegel U, Babatz J, et al. Prophylactic transfer of BCR-ABL-, PR1-, and WT1-reactive donor T cells after T cell-depleted allogeneic hematopoietic cell transplantation in patients with chronic myeloid leukemia. *Blood*. 2011;117(26):7174-84.
 30. Xue SA, Gao L, Hart D, Gillmore R, Qasim W, Thrasher A, et al. Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood*. 2005;106(9):3062-7.
 31. Mollredm JJ, Clave E, Jiang YZ, Mavroudis D, Raptis A, Hensel N, et al. Cytotoxic T lymphocytes specific for a nonpolymorphic proteinase 3 peptide preferentially inhibit chronic myeloid leukemia colony-forming units. *Blood*. 1997;90(7):2529-34.
 32. Quintarelli C, Dotti G, De Angelis B, Hoyos V, Mims M, Luciano L, et al. Cytotoxic T lymphocytes directed to the preferentially expressed antigen of melanoma (PRAME) target chronic myeloid leukemia. *Blood*. 2008;112(5):1876-85.
 33. Quintarelli C, Dotti G, Hasan ST, De Angelis B, Hoyos V, Errichiello S, et al. High-avidity cytotoxic T lymphocytes specific for a new PRAME-derived peptide can target leukemic and leukemic-precursor cells. *Blood*. 2011;117(12):3553-62.
 34. Fujie T, Tahara K, Tanaka F, Mori M, Takesako K, Akiyoshi T. A MAGE-1-encoded HLA-A24-binding synthetic peptide induces specific anti-tumor cytotoxic T lymphocytes. *Int J Cancer*. 1999;80(2):169-72.
 35. Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci USA*. 1993;90(2):720-4.
 36. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*. 2006;314(5796):126-9.
 37. Maher J, Brentjens RJ, Gunset G, Riviere I, Sadelain M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. *Nat Biotechnol*. 2002;20(1):70-5.
 38. Vera J, Savoldo B, Vigouroux S, Biagi E, Pule M, Rossig C, et al. T lymphocytes redirected against the kappa light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived malignant cells. *Blood*. 2006;108(12):3890-7.
 39. Pule MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. *Mol Ther*. 2005;12(5):933-41.
 40. Imai C, Mihara K, Andreansky M, Nicholson IC, Pui CH, Geiger TL, et al. Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia*. 2004;18(4):676-84.
 41. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhai M, Suhoski MM, et al. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci USA*. 2009;106(9):3360-5.
 42. Cooper LJ, Topp MS, Serrano LM, Gonzalez S, Chang WC, Naranjo A, et al. T-cell clones can be rendered specific for CD19: toward the selective augmentation of the graft-versus-B-lineage leukemia effect. *Blood*. 2003;101(4):1637-44.
 43. Jensen M, Tan G, Forman S, Wu AM, Raubitschek A. CD20 is a molecular target for scFvFc:zeta receptor redirected T cells: implications for cellular immunotherapy of CD20+ malignancy. *Biol Blood Marrow Transplant*. 1998;4(2):75-83.
 44. Jena B, Dotti G, Cooper LJ. Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. *Blood*. 2010;116(7):1035-44.
 45. Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G, et al. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *J Clin Invest*. 2011;121(5):1822-6.
 46. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood*. 2011;118(18):4817-28.
 47. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med*. 2011;365(8):725-33.
 48. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, et al. B-cell

- depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood*. 2012;119(12):2709-20.
49. Till BG, Jensen MC, Wang J, Qian X, Gopal AK, Maloney DG, et al. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. *Blood*. 2012;119(17):3940-50.
 50. Brentjens R, Yeh R, Bernal Y, Riviere I, Sadelain M. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Mol Ther*. 2010;18(4):666-8.
 51. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther*. 2010;18(4):843-51.
 52. Till BG, Jensen MC, Wang J, Qian X, Gopal AK, Maloney DG, et al. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. *Blood*. 2012;119(17):3940-50.
 53. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol*. 2008;26(32):5233-9.
 54. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood*. 2011;118(18):4817-28.
 55. Leen AM, Myers GD, Sili U, Huls MH, Weiss H, Leung KS, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med*. 2006;12(10):1160-6.
 56. Micklethwaite KP, Savoldo B, Hanley PJ, Leen AM, Demmler-Harrison GJ, Cooper LJ, et al. Derivation of human T lymphocytes from cord blood and peripheral blood with antiviral and antileukemic specificity from a single culture as protection against infection and relapse after stem cell transplantation. *Blood*. 2010;115(13):2695-703.
 57. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med*. 2008;14(11):1264-70.
 58. Giordano Attianese GM, Marin V, Hoyos V, Savoldo B, Pizzitola I, Tettamanti S, et al. In vitro and in vivo model of a novel immunotherapy approach for chronic lymphocytic leukemia by anti-CD23 chimeric antigen receptor. *Blood*. 2011;117(18):4736-45.
 59. Shaffer DR, Savoldo B, Yi Z, Chow KK, Kakarla S, Spencer DM, et al. T cells redirected against CD70 for the immunotherapy of CD70-positive malignancies. *Blood*. 2011;117(16):4304-14.
 60. Hudecek M, Schmitt TM, Baskar S, Lupo-Stanghellini MT, Nishida T, Yamamoto TN, et al. The B-cell tumor-associated antigen ROR1 can be targeted with T cells modified to express a ROR1-specific chimeric antigen receptor. *Blood*. 2010;116(22):4532-41.
 61. Hombach A, Heuser C, Sircar R, Tillmann T, Diehl V, Pohl C, et al. Characterization of a chimeric T-cell receptor with specificity for the Hodgkin's lymphoma-associated CD30 antigen. *J Immunother*. 1999;22(6):473-80.
 62. Savoldo B, Rooney CM, Di Stasi A, Abken H, Hombach A, Foster AE, et al. Epstein Barr virus specific cytotoxic T lymphocytes expressing the anti-CD30(zeta) artificial chimeric T-cell receptor for immunotherapy of Hodgkin disease. *Blood*. 2007;110(7):2620-30.
 63. Younes A, Bartlett NL, Leonard JP, Kennedy DA, Lynch CM, Sievers EL, et al. Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas. *N Engl J Med*. 2010;363(19):1812-21.
 64. Marin V, Pizzitola I, Agostoni V, Attianese GM, Finney H, Lawson A, et al. Cytokine-induced killer cells for cell therapy of acute myeloid leukemia: improvement of their immune activity by expression of CD33-specific chimeric receptors. *Haematologica*. 2010;95(12):2144-52.
 65. Casucci M, Falcone L, Nicolis di Robilant B, Camisa B, Genovese P, Genter B, et al. Dual Transgenesis of T cells with a novel CD44v6-specific chimeric antigen receptor and a suicide gene for the safe and effective targeting of chemoresistance in hematopoietic tumors. *Blood*. 118[21]. 12-11-2011. Ref Type: Abstract.
 66. Riechelmann H, Sauter A, Golze W, Hanft G, Schroen C, Hoemann K, et al. Phase I trial with the CD44v6-targeting immunconjugate bivatuzumab mertansine in head and neck squamous cell carcinoma. *Oral Oncol*. 2008;44(9):823-9.
 67. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol*. 2011;29(7):917-24.
 68. Bendle GM, Linnemann C, Hooijkaas AI, Bies L, de Witte MA, Jorritsma A, et al. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nat Med*. 2010;16(5):565-70, 1p following 570.
 69. Okamoto S, Mineno J, Ikeda H, Fujiwara H, Yasukawa M, Shiku H, et al. Improved expression and reactivity of transduced tumor-specific TCRs in human lymphocytes by specific silencing of endogenous TCR. *Cancer Res*. 2009;69(23):9003-11.
 70. Robbins PF, Li YF, El-Gamil M, Zhao Y, Wargo JA, Zheng Z, et al. Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. *J Immunol*. 2008;180(9):6116-31.
 71. Ochi T, Fujiwara H, Okamoto S, An J, Nagai K, Shirakata T, et al. Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety. *Blood*. 2011;118(6):1495-503.
 72. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363(8):711-23.
 73. Quintarelli C, Vera JF, Savoldo B, Giordano Attianese GM, Pule M, Foster AE, et al. Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor-specific cytotoxic T lymphocytes. *Blood*. 2007;110(8):2793-802.
 74. Liu K, Rosenberg SA. Transduction of an IL-2 gene into human melanoma-reactive lymphocytes results in their continued growth in the absence of exogenous IL-2 and maintenance of specific antitumor activity. *J Immunol*. 2001;167(11):6356-65.
 75. Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia*. 2010;24(6):1160-70.
 76. Vera JF, Hoyos V, Savoldo B, Quintarelli C, Giordano Attianese GM, Leen AM, et al. Genetic manipulation of tumor-specific cytotoxic T lymphocytes to restore responsiveness to IL-7. *Mol Ther*. 2009;17(5):880-8.
 77. Kershaw MH, Wang G, Westwood JA, Pachynski RK, Tiffany HL, Marincola FM, et al. Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. *Hum Gene Ther*. 2002;13(16):1971-80.
 78. Di Stasi A, De Angelis B, Rooney CM, Zhang L, Mahendravada A, Foster AE, et al. T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. *Blood*. 2009;113(25):6392-402.
 79. Craddock JA, Lu A, Bear A, Pule M, Brenner MK, Rooney CM, et al. Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. *J Immunother*. 2010;33(8):780-8.
 80. Wieser R, Attisano L, Wrana JL, Massague J. Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region. *Mol Cell Biol*. 1993;13(12):7239-47.
 81. Bollard CM, Rossig C, Calonge MJ, Huls MH, Wagner HJ, Massague J, et al. Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity. *Blood*. 2002;99(9):3179-87.
 82. Dotti G, Savoldo B, Pule M, Straathof KC, Biagi E, Yvon E, et al. Human cytotoxic T lymphocytes with reduced sensitivity to Fas-induced apoptosis. *Blood*. 2005;105(12):4677-84.
 83. Wagner HJ, Bollard CM, Vigouroux S, Huls MH, Anderson R, Prentice HG, et al. A strategy for treatment of Epstein-Barr virus-positive Hodgkin's disease by targeting interleukin 12 to the tumor environment using tumor antigen-specific T cells. *Cancer Gene Ther*. 2004;11(2):81-91.
 84. Zhang L, Kerkar SP, Yu Z, Zheng Z, Yang S, Restifo NP, et al. Improving adoptive T cell therapy by targeting and controlling IL-12 expression to the tumor environment. *Mol Ther*. 2011;19(4):751-9.
 85. Sun J, Dotti G, Huye LE, Foster AE, Savoldo B, Gramatges MM, et al. T cells expressing constitutively active Akt resist multiple tumor-associated inhibitory mechanisms. *Mol Ther*. 2010;18(11):2006-17.
 86. De Angelis B, Dotti G, Quintarelli C, Huye LE, Zhang L, Zhang M, et al. Generation of Epstein-Barr virus-specific cytotoxic T lymphocytes resistant to the immunosuppressive drug tacrolimus (FK506). *Blood*. 2009;114(23):4784-91.
 87. Brewin J, Mancao C, Straathof K, Karlsson H, Samarasinghe S, Amrolia PJ, et al. Generation of EBV-specific cytotoxic T cells that are resistant to calcineurin inhibitors for the treatment of posttransplantation lymphoproliferative disease. *Blood*. 2009;114(23):4792-803.
 88. Huye LE, Nakazawa Y, Patel MP, Yvon E, Sun J, Savoldo B, et al. Combining mTOR inhibitors with rapamycin-resistant T cells: a two-pronged approach to tumor elimination. *Mol Ther*. 2011;19(12):2239-48.