

Inducible Caspase 9 Suicide Gene to Improve the Safety of Allogeneic T Cells after Haploidentical Stem Cell Transplantation

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ABSTRACT

Addback of donor T cells following T cell-depleted stem cell transplantation (SCT) can accelerate immune reconstitution and be effective against relapsed malignancy. After haploidentical SCT, a high risk of graft-versus-host disease (GVHD) essentially precludes this option, unless the T cells are first depleted of alloreactive precursor cells. Even then, the risks of severe GVHD remain significant. To increase the safety of the approach and thereby permit administration of larger T cell doses, we used a suicide gene, inducible caspase 9 (iCasp9), to transduce allogeneic T cells, permitting their destruction should administration have adverse effects. We made a retroviral vector encoding iCasp9 and a selectable marker (truncated CD19). Even after allogeneic depletion (using anti-CD25 immunotoxin), donor T cells could be efficiently transduced, expanded, and subsequently enriched by CD19 immunomagnetic selection to >90% purity. These engineered cells retained antiviral specificity and functionality, and contained a subset with regulatory phenotype and function. Activating iCasp9 with a small-molecule dimerizer rapidly produced >90% apoptosis. Although transgene expression was downregulated in quiescent T cells, iCasp9 remained an efficient suicide gene, as expression was rapidly upregulated in activated (alloreactive) T cells. We have demonstrated the clinical feasibility of this approach after haploidentical transplantation by scaling up production using clinical grade materials.

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KEY WORDS

Hematopoietic stem cell transplantation • Graft-versus-host disease • Gene therapy • T lymphocyte • Immunotherapy • Haploidentical

INTRODUCTION

Donor T cell infusion is an effective strategy for conferring antiviral and antitumor immunity following allogeneic stem cell transplantation [1-3]. This can be particularly useful in T cell-depleted transplantation, where immune reconstitution is delayed. In haploidentical transplantation, the need to accelerate immune reconstitution is most pressing; here, profound immune deficiency as a consequence of vigorous T cell depletion and MHC-incompatibility, results in high rates of infectious complications and disease relapse [4,5]. Unfortunately, however, addback of unmanipulated donor T cells is unlikely to be feasible in the haploidentical setting because graft-versus-host disease (GVHD) can occur

after addback of as few as 3×10^4 CD3⁺ cells/kg [6]. This problem can be partially overcome by selective depletion of alloreactive cells, for example, by using immunotoxins directed to activation markers on alloreactive cells [7-9]. We, and others, have previously shown that addback of allogeneic T cells at doses between 1 to 8×10^5 cells/kg is associated with a low incidence of GVHD and significantly accelerates T cell recovery and reconstitutes antiviral immunity [7,8]. However, disease relapse remains high in these series, and because the estimated frequency of tumor-reactive precursors is 1 to 2 logs less than frequency of viral-reactive precursors [10,11], much greater dose escalation is likely required to reconstitute antitumor immunity.

Although dose escalation of allodepleted T cells may be desirable, it may not be safe. The risk of GVHD increases with increasing T cell dose [12], and the maximum dose that can be safely infused in any given individual cannot be predicted with certainty. Once established, severe GVHD unresponsive to frontline therapy has a poor prognosis. Hence, although severe GVHD occurs infrequently, the fact that it is unpredictable and may be fatal compromises dose intensity in all patients. Suicide gene-modification of T cells circumvents this biological uncertainty: effective T cell doses can be administered to all patients safe in the knowledge that any GVHD that develops can be effectively controlled by activation of the suicide gene mechanism.

One of the most widely used suicide genes is Herpes simplex virus thymidine kinase (HSVtk). This enzyme mediates the conversion of ganciclovir to ganciclovir triphosphate, which is toxic to dividing cells; administration of ganciclovir efficiently eliminates HSVtk-modified T cells and abrogates acute GVHD (aGVHD) [13-15]. Although providing proof of concept of suicide gene therapy, HSVtk has a number of drawbacks, the most important of which is immunogenicity: being a foreign protein, HSVtk is a target for CD4 and CD8 T cell-mediated immune response, which results in premature elimination of HSVtk-modified cells [16]. Other drawbacks of HSVtk include restriction of killing to dividing cells, the unintended elimination of gene-modified cells when ganciclovir is used for treatment of cytomegalovirus (CMV) reactivation, and ganciclovir resistance resulting from truncated HSVtk formed from cryptic splice donor and acceptor sites [17].

We investigated the suitability of an alternative suicide gene, inducible caspase 9 (iCasp9) [18]. iCasp9-mediated suicide is based on conditional dimerization of pro-apoptotic molecules [18,19] that are constructed from human proteins, and therefore, less likely to be immunogenic. The mechanism of killing allows the safe use of ganciclovir, and is independent of cell proliferation.

We now show the feasibility of engineering allodepleted T cells with an iCasp9 suicide gene transfer, and demonstrate the functionality of the modified T cells, and the scalability of the process.

MATERIALS AND METHODS

Generation of Allodepleted T Cells

Allodepleted cells were generated from healthy volunteers as previously described [7,20]. In brief, peripheral blood mononuclear cells (PBMCs) from healthy donors were cocultured with irradiated recipient Epstein Barr virus (EBV)-transformed lymphoblastoid cell lines (LCL) at a responder-to-stimulator

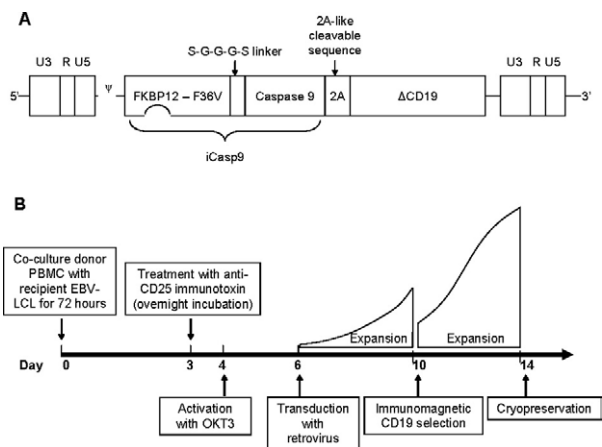


Figure 1. Generation of iCasp9 suicide gene-modified selectively allodepleted cells. A, Structure of SFG.iCasp9.2A.ΔCD19. The transgene consists of a suicide gene, inducible caspase 9 (iCasp9), and a selectable marker, truncated CD19 (ΔCD19), linked by a 2A-like sequence, which encodes a cleavable peptide. iCasp9 consists of a drug-binding domain (FKBP12-F36V) connected via a short linker (SGGGS) to human caspase 9. B, Overview of the production process. Selective allodepletion was performed by coculturing donor PBMC with recipient EBV-LCL to activate alloreactive cells: activated cells expressed CD25 and were subsequently eliminated by anti-CD25 immunotoxin. The allodepleted cells were activated by OKT3 and transduced with the retroviral vector 48 hours later. Immunomagnetic selection was performed on day 4 of transduction; the positive fraction was expanded for a further 4 days and cryopreserved.

ratio of 40:1 in serum-free medium (AIM V; Invitrogen, Carlsbad, CA). After 72 hours, activated T cells that expressed CD25 were depleted from the coculture by overnight incubation in RFT5-SMPT-dgA immunotoxin [21]. Allodepletion was considered adequate if the residual CD3⁺CD25⁺ population was <1% and residual proliferation by ³H-thymidine incorporation was <10% [7].

Plasmid and Retrovirus

SFG.iCasp9.2A.ΔCD19 consists of iCasp9 linked, via a cleavable 2A-like sequence, to truncated human CD19 (ΔCD19) (Figure 1A). iCasp9 consists of a human FK506-binding protein (FKBP12; GenBank AH002 818) with an F36V mutation, connected via a Ser-Gly-Gly-Gly-Ser linker to human caspase 9 (CASP9; GenBank NM 001229) [18]. The F36V mutation increases the binding affinity of FKBP12 to the synthetic homodimerizer, AP20187 or AP1903 [22]. The caspase recruitment domain (CARD) has been deleted from the CASP9 sequence because its physiologic function has been replaced by FKBP12, and its removal increases transgene expression and function [18]. The 2A-like sequence encodes a 20 amino acid peptide from *Thosea Asigna* insect virus, which mediates >99% cleavage between a glycine and terminal proline residue [23], resulting in 19 extra amino acids

in the C terminus of iCasp9, and 1 extra proline residue in the N-terminus of CD19. Δ CD19 consists of full-length CD19 (GenBank NM 001770) truncated at amino acid 333 (TDPTRRF), which shortens the intracytoplasmic domain from 242 to 19 amino acids, and removes all conserved tyrosine residues that are potential sites for phosphorylation [24,25].

A stable PG13 clone producing Gibbon ape leukemia virus (Gal-V) pseudotyped retrovirus was made by transiently transfecting Phoenix Eco cell line (ATCC product #SD3444; ATCC, Manassas, VA) with SFG.iCasp9.2A. Δ CD19. This produced Eco-pseudotyped retrovirus. The PG13 packaging cell line (ATCC) was transduced 3 times with Eco-pseudotyped retrovirus to generate a producer line that contained multiple SFG.iCasp9.2A. Δ CD19 proviral integrants per cell. Single-cell cloning was performed, and the PG13 clone that produced the highest titer was expanded and used for vector production.

Retroviral Transduction

Culture medium for T cell activation and expansion consisted of 45% RPMI 1640 (Hyclone, Logan, UT), 45% Clicks (Irvine Scientific, Santa Ana, CA) and 10% fetal bovine serum (FBS; Hyclone). Allodepleted cells were activated by immobilized anti-CD3 (OKT3; Ortho Biotech, Bridgewater, NJ) for 48 hours before transduction with retroviral vector (Figure 1B). In small-scale experiments, non-tissue culture-treated 24-well plates (Becton Dickinson, San Jose, CA) were coated with OKT3 1 μ g/mL for 2 to 4 hours at 37°C. Allodepleted cells were added at 1×10^6 cells per well. At 24 hours, 100 U/mL of recombinant human interleukin-2 (IL-2) (Proleukin; Chiron, Emeryville, CA) was added. Retroviral transduction was performed 48 hours after activation. Non-tissue culture-treated 24-well plates were coated with 3.5 μ g/cm² recombinant fibronectin fragment (CH-296; Retronectin; Takara Mirus Bio, Madison, WI) and the wells loaded twice with retroviral vector-containing supernatant at 0.5 mL per well for 30 minutes at 37°C, following which OKT3-activated cells were plated at 5×10^5 cells per well in fresh retroviral vector-containing supernatant and T cell culture medium at a ratio of 3:1, supplemented with 100 U/mL IL-2. Cells were harvested after 2 to 3 days and expanded in the presence of 50 U/ml IL-2.

Scaling-Up Production of Gene-Modified Allodepleted Cells

Scale-up of the transduction process for clinical application used non-tissue culture-treated T75 flasks (Nunc, Rochester, NY), which were coated with 10 mL of OKT3 1 μ g/mL or 10 mL of fibronectin 7 μ g/mL at 4°C overnight. We also used fluorinated ethylene pro-

pylene bags corona-treated for increased cell adherence (2PF-0072AC, American Fluoroseal Corporation, Gaithersburg, MD). Allodepleted cells were seeded in OKT3-coated flasks at 1×10^6 cells/mL; 100 U/ml IL-2 was added the next day. For retroviral transduction, retronectin-coated flasks or bags were loaded once with 10 mL of retroviral vector-containing supernatant for 2 to 3 hours. OKT3-activated T cells were seeded at 1×10^6 cells/mL in fresh retroviral vector-containing medium and T cell culture medium at a ratio of 3:1, supplemented with 100 U/ml IL-2. Cells were harvested the following morning and expanded in tissue culture-treated T75 or T175 flasks in culture medium supplemented with 50 to 100 U/ml IL-2 at a seeding density of 5 to 8×10^5 cells/mL.

CD19 Immunomagnetic Selection

Immunomagnetic selection for CD19 was performed 4 days after transduction. Cells were labeled with paramagnetic microbeads conjugated to monoclonal mouse antihuman CD19 antibodies (Miltenyi Biotech, Auburn, CA) and selected on MS or LS columns in small-scale experiments and on a Clinimacs Plus automated selection device in large-scale experiments. CD19-selected cells were expanded for a further 4 days and cryopreserved on day 8 post transduction. These cells were referred to as “gene-modified allodepleted cells.”

Immunophenotyping and Pentamer Analysis

Flow cytometric analysis (FACSCalibur and CellQuest software; Becton Dickinson) used the following antibodies: CD3, CD4, CD8, CD19, CD25, CD27, CD28, CD45RA, CD45RO, CD56, and CD62L. CD19-PE (Clone 4G7; Becton Dickinson) was found to give optimum staining and was used in all analyses. Nontransduced control was used to set the negative gate for CD19. An HLA-pentamer, HLA-B8-RAKFKQLL (Proimmune, Springfield, VA) was used to detect T cells recognizing an epitope from EBV lytic antigen (BZLF1). HLA-A2-NLVPM-VATV pentamer was used to detect T cells recognizing an epitope from CMV-pp65 antigen.

Interferon- γ ELISPOT Assay for Antiviral Response

Interferon- γ ELISPOT for assessment of responses to EBV, CMV, and adenovirus antigens was performed as previously described [20]. Gene-modified allodepleted cells cryopreserved at 8 days post transduction were thawed and rested overnight in complete medium without IL-2 prior to use as responder cells. Cryopreserved PBMCs from the same donor were used as comparators. Responder cells were plated in duplicate or triplicate in serial dilutions of 2×10^5 , 1×10^5 , 5×10^4 , and 2.5×10^4 cells per well.

Stimulator cells were plated at 1×10^5 per well. For response to EBV, donor-derived EBV-LCLs irradiated at 40 Gy were used as stimulators. For response to adenovirus, donor-derived activated monocytes infected with Ad5f35 adenovirus were used. In brief, donor PBMCs were plated in X-Vivo 15 (Cambrex, Walkersville, MD) in 24-well plates overnight, harvested the next morning, infected with Ad5f35 at multiplicities of infection (MOI) of 200 for 2 hours, washed, irradiated at 30 Gy, and used as stimulators. For anti-CMV response, a similar process using Ad5f35 adenovirus encoding the CMV pp65 transgene (Ad5f35-pp65) at an MOI of 5000 was used. Specific spot-forming units (SFU) were calculated by subtracting SFU from responder-alone and stimulator-alone wells from test wells. Response to CMV was the difference in SFU between Ad5f35-pp65 and Ad5f35 wells.

EBV-Specific Cytotoxicity

Gene-modified allodepleted cells were stimulated with 40 Gy-irradiated donor-derived EBV-LCL at a responder:stimulator ratio of 40:1. After 9 days, the cultures were restimulated at a responder:stimulator ratio of 4:1. Restimulation was performed weekly as indicated. After 2 or 3 rounds of stimulation, cytotoxicity was measured in a 4-hour ^{51}Cr -release assay, using donor EBV-LCL as target cells and donor OKT3 blasts as autologous controls. NK activity was inhibited by adding 30-fold excess of cold K562 cells.

Induction of Apoptosis with Chemical Inducer of Dimerization, AP20187

Suicide gene functionality was assessed by adding a small molecule synthetic homodimerizer, AP20187 (Ariad Pharmaceuticals; Cambridge, MA), at 10 nM final concentration the day following CD19 immunomagnetic selection. Cells were stained with annexin V and 7-amino-actinomycin (7-AAD) (BD Pharmingen, San Diego, CA) at 24 hours and analyzed by flow cytometry. Cells negative for both Annexin V and 7-AAD were considered viable, cells that were annexin V positive were apoptotic, and cells that were both annexin V and 7-AAD positive were necrotic. The percentage killing induced by dimerization was corrected for baseline viability as follows: Percentage killing = $100\% - (\% \text{Viability in AP20187-treated cells} \div \% \text{Viability in nontreated cells})$.

Assessment of Transgene Expression following Extended Culture and Reactivation

Cells were maintained in T cell medium containing 50 U/ml IL-2 until 22 days after transduction. A portion of cells was reactivated on 24-well plates coated with 1 $\mu\text{g}/\text{mL}$ OKT3 and 1 $\mu\text{g}/\text{mL}$ anti-CD28 (Clone CD28.2, BD Pharmingen) for 48 to 72 hours.

CD19 expression and suicide gene function in both reactivated and nonreactivated cells were measured on day 24 or 25 post transduction.

In some experiments we also cultured the cells for 3 weeks post transduction but simulated them with 30 Gy-irradiated allogeneic PBMC at responder:stimulator ratio of 1:1. After 4 days of coculture, a portion of cells was treated with 10 nM AP20187. Killing was measured by annexin V/7-AAD staining at 24 hours, and the effect of dimerizer on bystander virus-specific T cells was assessed by pentamer analysis on AP20187-treated and untreated cells.

Regulatory T Cells

We analyzed CD4, CD25, and Foxp3 expression in gene-modified allodepleted cells using flow cytometry. For human Foxp3 staining we used the eBioscience (San Diego, CA) staining set with an appropriate rat IgG2a isotype control. These cells were costained with surface CD25-FITC and CD4-PE. Functional analysis was performed by coculturing CD4⁺CD25⁺ cells selected after allodepletion and gene modification with autologous PBMC labeled with carboxyfluorescein diacetate N-succinimidyl ester (CFSE). CD4⁺CD25⁺ selection was performed by first depleting CD8⁺ cells using anti-CD8 microbeads (Miltenyi Biotec, Auburn, CA), followed by positive selection using anti-CD25 microbeads (Miltenyi Biotec). CFSE-labeling was performed by incubating autologous PBMC at $2 \times 10^7/\text{mL}$ in phosphate-buffered saline (PBS) containing 1.5 μM CFSE for 10 minutes. The reaction was stopped by adding an equivalent volume of FBS and incubating for 10 minutes at 37°C. Cells were washed twice before use. We stimulated CFSE-labeled PBMCs with OKT3 500 ng/mL and 40 Gy-irradiated allogeneic PBMC feeders at PBMC: allogeneic feeders ratio of 5:1. We cultured the cells with or without an equal number of autologous CD4⁺CD25⁺ gene-modified allodepleted cells. After 5 days of culture, cell division was analyzed by flow cytometry; CD19 was used to gate out non-CFSE-labeled CD4⁺CD25⁺ gene-modified T cells.

Statistical Analysis

Paired, 2-tailed Student's *t*-test was used to determine the statistical significance of differences between samples. All data are represented as mean \pm 1 standard deviation.

RESULTS

Selectively Allodepleted T Cells Could Be Efficiently Transduced with iCasp9 and Expanded

Selective allodepletion was performed in accordance with procedures established for our recently completed clinical protocol [7], by coculturing 3/6 to

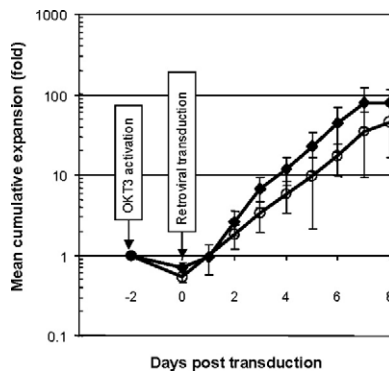


Figure 2. Allodepleted cells could be successfully expanded following transduction. Filled diamond denotes large-scale experiments performed in flasks and bags ($n = 3$), open circle denotes small-scale experiments performed in 24-well plates ($n = 5$). Error bars indicate standard deviation.

5/6 HLA-mismatched PBMC and LCL. RFT5-SMPT-dgA immunotoxin was applied after 72 hours of coculture and reliably produced allodepleted cells with $<10\%$ residual proliferation (mean: $4.5\% \pm 2.8\%$; range: $0.74\%-9.1\%$; 10 experiments) and containing $<1\%$ residual $CD3^+CD25^+$ cells (mean: $0.23\% \pm 0.20\%$; range: $0.06\%-0.73\%$; 10 experiments), thereby fulfilling the release criteria for selective allodepletion, and serving as starting materials for subsequent manipulation.

Allodepleted cells activated on immobilized OKT3 for 48 hours could be efficiently transduced with Gal-V pseudotyped retrovirus vector encoding SFG.iCasp9.2A. Δ CD19. Transduction efficiency assessed by FACS analysis for CD19 expression 2 to 4 days after transduction was $53\% \pm 8\%$, with comparable results for small-scale (24-well plates) and large-scale (T75 flasks) transduction ($55\% \pm 8\%$ versus $50\% \pm 10\%$ in 6 and 4 experiments, respectively). Cell numbers contracted in the first 2 days following OKT3 activation such that only $61\% \pm 12\%$ (range: $45\%-80\%$) of allodepleted cells were recovered on the day of transduction (Figure 2). Thereafter, the cells

showed significant expansion, with a mean 94 ± 46 -fold expansion (range: 40-153) over the subsequent 8 days, resulting in a net 58 ± 33 -fold expansion. Cell expansion in both small- and large-scale experiments was similar, with net expansion of 45 ± 29 -fold (range: 25-90) in 5 small-scale experiments and 79 ± 34 -fold (range: 50-116) in 3 large-scale experiments.

Δ CD19 Enables Efficient and Selective Enrichment of Transduced Cells on Immunomagnetic Columns

The efficiency of suicide gene activation will depend not only on the functionality of the suicide gene itself, but also on the selection system used to enrich for gene-modified cells. Because of limitations of selectable markers currently in use, we investigated whether Δ CD19 enabled the selection of gene-modified cells with sufficient purity and yield, and whether selection had any deleterious effects on subsequent cell growth. Small-scale selection was performed according to manufacturer's instruction; however, we found that large-scale selection was optimum when we used $10 \mu\text{L}$ of CD19 microbeads per 1.3×10^7 cells. FACS analysis was performed at 24 hours after immunomagnetic selection to minimize interference from anti-CD19 microbeads. The purity of the cells after immunomagnetic selection was consistently $>90\%$: mean percentage of $CD19^+$ cells was $98.3\% \pm 0.5\%$ ($n = 5$) in small-scale selections and $97.4\% \pm 0.9\%$ ($n = 3$) in large-scale CliniMacs selections (Figure 3). The absolute yield of small- and large-scale selections were $31\% \pm 11\%$ and $28\% \pm 6\%$, respectively; after correction for transduction efficiency, the mean recovery of transduced cells was $54\% \pm 14\%$ in small-scale and $72\% \pm 18\%$ in large-scale selections. The selection process did not have any discernable deleterious effect on subsequent cell expansion: in 4 experiments, the mean cell expansion over 3 days following CD19 immunomagnetic selection was 3.5-fold for the CD19 positive fraction versus 4.1-fold for nonselected

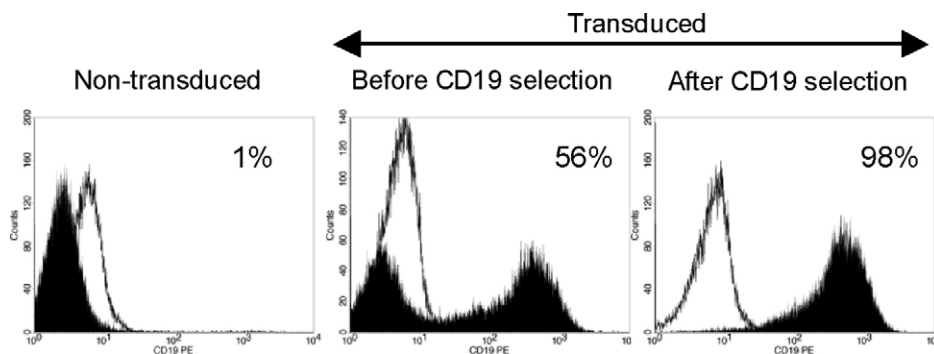


Figure 3. Suicide gene-modified cells could be enriched to high purity by CD19 immunomagnetic selection. CD19 immunomagnetic selection was performed on day 4 post transduction using CliniMacs Plus automated selection device. Shown here are FACS analyses performed 2 days after immunomagnetic selection. Filled lines indicate CD19-PE; unfilled lines indicate isotype control.

Table 1. Comparison of Immunophenotype of Gene-Modified Allodepleted Cells and Unmanipulated PBMC

	Unmanipulated PBMC (mean % \pm SD)	Gene-Modified Allodepleted Cells (mean % \pm SD)	P-Value
T cells: Total CD3⁺	82 \pm 6	95 \pm 6	NS
CD3 ⁺ 4 ⁺	54 \pm 5	23 \pm 8	<.01
CD3 ⁺ 8 ⁺	26 \pm 9	62 \pm 11	<.001
NK cells: CD3⁻ 56⁺	6 \pm 3	2 \pm 1	NS
Memory phenotype			
CD45RA ⁺	66 \pm 3	10 \pm 5	<.001
CD45RO ⁺	26 \pm 2	78 \pm 7	<.001
CD45RA ⁻ CD62L ⁺	19 \pm 1	24 \pm 7	NS
CD45RA ⁻ CD62L ⁻	9 \pm 1	64 \pm 7	<.001
CD27 ⁺ CD28 ⁺	67 \pm 7	19 \pm 9	<.001
CD27 ⁺ CD28 ⁻	7 \pm 3	9 \pm 4	NS
CD27 ⁻ CD28 ⁺	4 \pm 1	19 \pm 8	<.05
CD27 ⁻ CD28 ⁻	22 \pm 8	53 \pm 18	<.05

NS indicates not significant; PBMC, peripheral blood mononuclear cell; SD, standard deviation.

transduced cells ($p = .34$) and 3.7-fold for nontransduced cells ($P = .75$).

Immunophenotype of Gene-Modified Allodepleted Cells

We immunophenotyped the final cell product (gene-modified allodepleted cells that had been cryopreserved 8 days after transduction). This final cell product contained both CD4 and CD8 cells, with CD8 cells predominant (Table 1) at 62% \pm 11% CD8⁺ versus 23% \pm 8% CD4⁺. The majority of cells were CD45RO⁺ and had the surface immunophenotype of effector memory T cells. Expression of memory markers, including CD62L, CD27, and CD28, was heterogeneous. Approximately 24% of cells expressed CD62L, a lymph node-homing molecule predominantly expressed on central memory cells.

Gene-Modified Allodepleted Cells Retained Antiviral Repertoire and Functionality

The ability of end-product cells to mediate antiviral immunity was assessed by interferon- γ ELISPOT, cytotoxicity assay, and pentamer analysis. These cryopreserved gene-modified allodepleted cells were used in all analyses, because they were representative of the product we propose to administer in the clinical study. Interferon- γ secretion in response to adenovirus, CMV, or EBV antigens presented by donor cells was preserved, although there was a trend towards reduced anti-EBV response in gene-modified allodepleted cells versus unmanipulated PBMC (Figure 4A).

Cytotoxicity was assessed using donor-derived EBV-LCL as targets. Gene-modified allodepleted cells that had undergone 2 or 3 rounds of stimulation with donor-derived EBV-LCL could efficiently lyse virus-infected autologous target cells (Figure 4B). Because EBV-LCLs were used as antigen-presenting cells during selective allodepletion, it was possible that EBV-specific T cells could be significantly depleted

when the donor and recipient were haploidentical. We therefore included 3 experiments using unrelated HLA-haploidentical donor-recipient pairs, and showed that cytotoxicity against donor-derived EBV-LCL was retained. The results were corroborated by pentamer analysis for T cells recognizing HLA-B8-RAKFKQLL, an EBV lytic antigen (BZLF1) epitope, in 2 informative donors following allodepletion against HLA-B8 negative haploidentical recipients (Figure 4C). The RAK-pentamer positive population was retained and could expand following stimulation with donor EBV-LCL. Together, these results provided evidence that gene-modified allodepleted cells retained significant antiviral functionality.

Regulatory T Cells in the Gene-Modified Allodepleted Cell Population

We used flow cytometry and functional analysis to discover whether regulatory T cells were retained in our allodepleted, gene-modified, T cell product. We found a CD4⁺CD25⁺Foxp3⁺ population (Figure 5A), and following immunomagnetic separation, the CD4⁺CD25⁺ enriched fraction demonstrated suppressor function when cocultured with CFSE-labeled autologous PBMC in the presence of OKT3 and allogeneic feeders (Figure 5B). Hence, allodepleted T cells may reacquire regulatory phenotype even after exposure to a CD25-depleting immunotoxin.

Gene-Modified Allodepleted Cells Were Efficiently and Rapidly Eliminated by Addition of Chemical Inducer of Dimerization

The day following immunomagnetic selection, 10 nM of the chemical inducer of dimerization, AP20187, was added to induce apoptosis, which appeared within 24 hours. FACS analysis with annexin V and 7-AAD staining at 24 hours showed that only 5.5% \pm 2.5% of AP20187-treated cells remained viable, whereas 81.0% \pm 9.0% of untreated cells were

viable (Figure 6A). Killing efficiency after correction for baseline viability was $92.9\% \pm 3.8\%$. Large-scale CD19 selection produced cells that were killed with similar efficiency as small-scale selection: mean viability with and without AP20187, and percentage killing, in large and small scale were 3.9%, 84.0%, and 95.4% ($n = 3$), and 6.6%, 79.3%, and 91.4% ($n = 5$), respectively. AP20187 was nontoxic to nontransduced cells: viability with and without AP20187 were $86\% \pm 9\%$ and $87\% \pm 8\%$, respectively ($n = 6$).

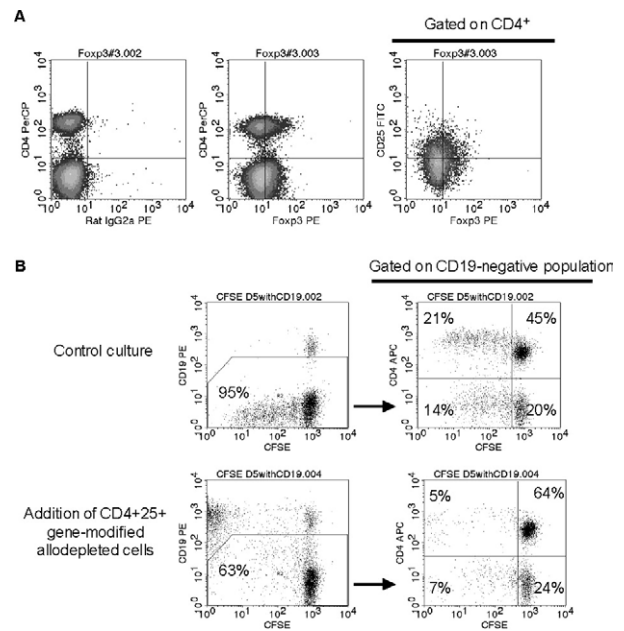
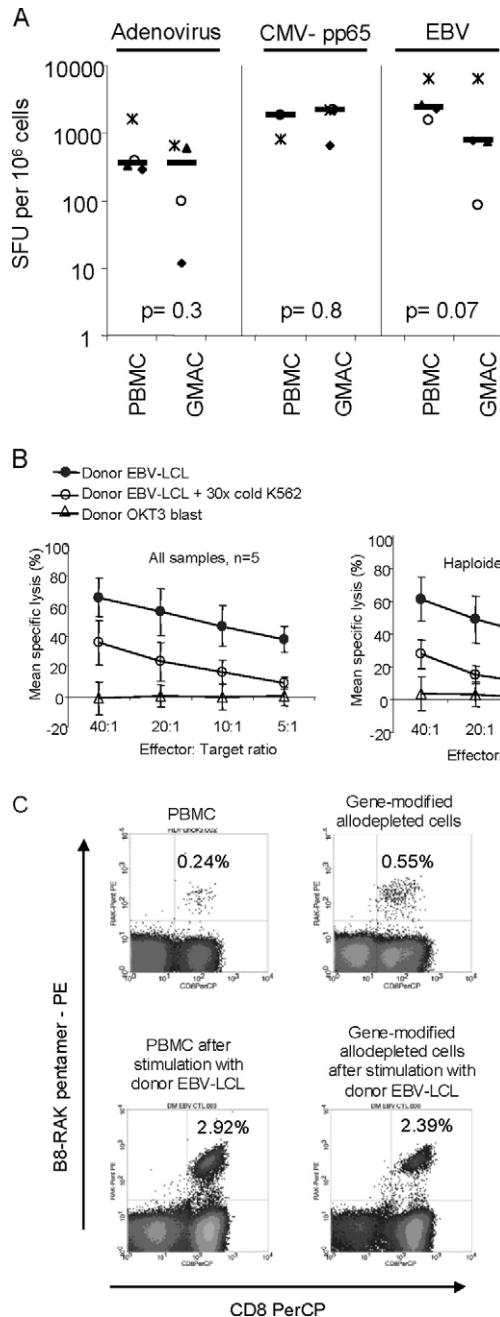


Figure 5. Regulatory T cells could be isolated from gene-modified end-product cells despite initial allopepletion using CD25-immunotoxin. A, FcγR3 expression. A small population of CD4⁺CD25⁺FcγR3⁺ cells was detectable. B, Functional assay. Donor-derived PBMC was labeled with CFSE and stimulated with OKT3 and allogeneic feeders. CD4⁺CD25⁺ cells were immunomagnetically selected from allopepleted gene-modified cell population and added at 1:1 ratio to test wells. Flow cytometry was performed after 5 days. Gene-modified T cells were gated out by CD19 expression. The addition of CD4⁺CD25⁺ gene-modified cells (bottom panel) significantly reduced cell proliferation.

Figure 4. Gene-modified allogeneic cells retained antiviral repertoire and functionality. A, Interferon-γ secretion in response to viral antigens was assessed by ELISPOT in 4 pairs of unmanipulated PBMC and gene-modified allogeneic cells (GMAC). Adenovirus and CMV antigens were presented by donor-derived activated monocytes through infection with Ad5f35 null vector and Ad5f35-pp65 vector, respectively. EBV antigens were presented by donor EBV-LCL. Number of spot-forming units (SFU) were corrected for stimulator- and responder-alone wells. Only 3 of 4 donors were evaluable for CMV response, 1 seronegative donor was excluded. Horizontal bars = median. B, Cytotoxicity assay. Gene-modified allogeneic cells were stimulated with donor EBV-LCL for 2 or 3 cycles. ⁵¹Cr release assay was performed using donor-derived EBV-LCL and donor OKT3 blasts as targets. NK activity was blocked with 30-fold excess cold K562. Left panel shows results from 5 independent experiments using totally or partially mismatched donor-recipient pairs. Right panel shows results from 3 experiments using unrelated HLA haploidentical donor-recipient pairs. Error bars indicate standard deviation. C, The frequency of T cells specific for HLA-B8- RAKFKQLL, an epitope from an EBV lytic antigen (BZLF1), was analyzed. Unmanipulated PBMC were used as comparators. The RAK-pentamer positive population was retained in gene-modified allogeneic cells and could be expanded following several rounds of in vitro stimulation with donor-derived EBV-LCL. Percentages indicate percentage of pentamer positive cells within CD8 population.

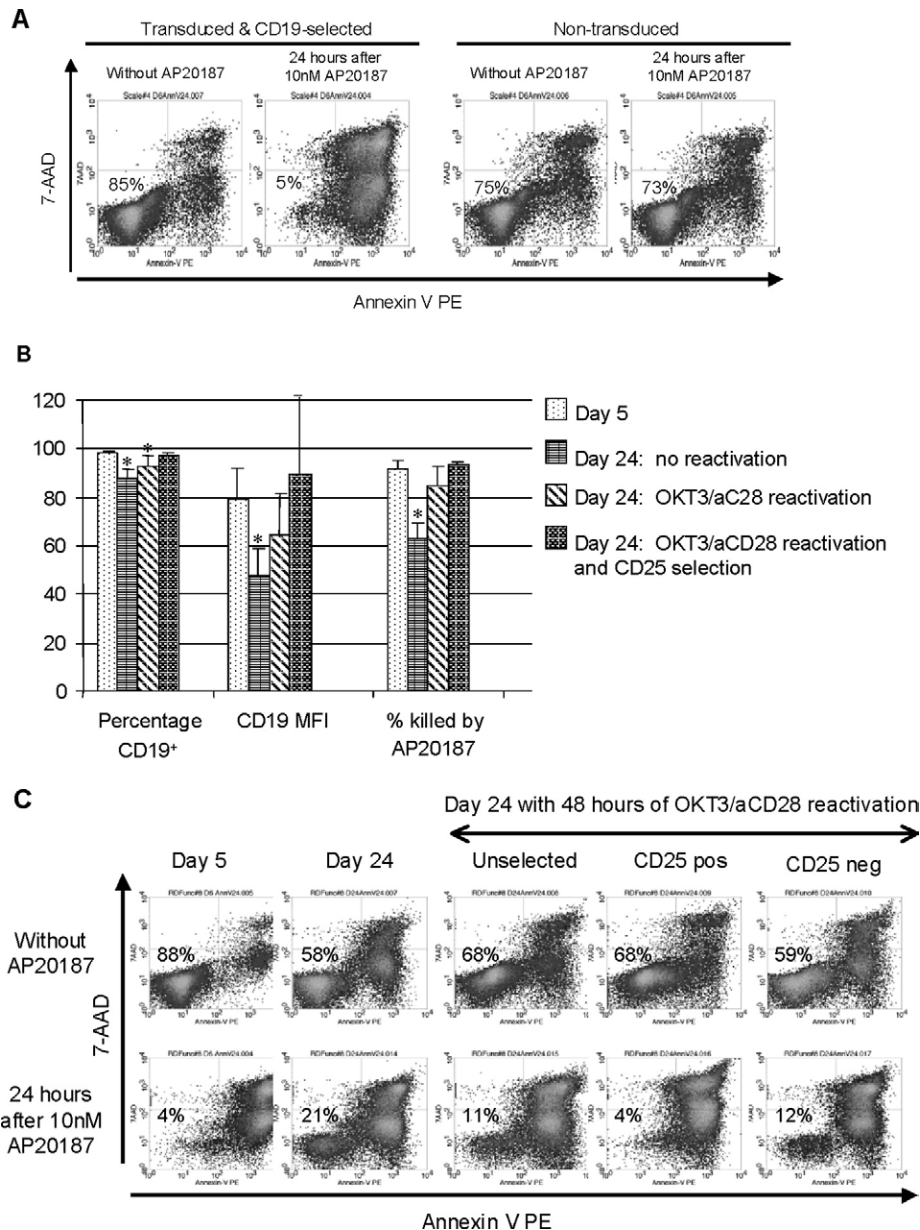


Figure 6. Gene-modified allodepleted cells were rapidly and efficiently eliminated by AP20187. Transgene expression and killing efficiency diminished with extended culture but were completely restored upon T cell reactivation. **A**, The day after immunomagnetic selection, cells were treated with 10 nM dimerizer (AP20187). FACS analysis for annexin V and 7-AAD was performed at 24 hours. The percentages of viable cells are indicated in the plots. AP20187 resulted in >90% killing of transduced cells but had no effect on nontransduced controls. Results are representative of 3 large-scale experiments. **B**, Transduced cells were maintained in culture for 22 days post transduction in low dose IL-2 (50 U/mL). A portion of cells was then reactivated with OKT3/anti-CD28. CD19 expression was analyzed by flow cytometry 48 to 72 hours later, and suicide gene function was assessed by treatment with 10 nM AP20187. Results shown are for cells from day 5 post transduction (ie, 1 day after CD19 selection) and day 24 post transduction, with or without 48-72 hours of reactivation (5 experiments). In 2 experiments, CD25 selection was performed after OKT3/aCD28 activation to further enrich activated cells. Error bars represent standard deviation. *Indicates $P < .05$ when compared to cells from day 5 post transduction. **C**, Representative FACS plots showing the effect of extended culture and T cell activation on suicide gene function. Greater than 90% of activated CD25 positive T cells were killed by AP20187.

Transgene Expression and Function Decreased with Extended Culture But Were Restored upon Cell Reactivation

To assess the stability of transgene expression and function, we maintained cells in T cell culture medium and low-dose IL-2 (50 U/mL) until 24 days after

transduction. By day 24, surface CD19 expression fell from $98\% \pm 1\%$ to $88\% \pm 4\%$ ($P < .05$) with a parallel decrease in mean fluorescence intensity (MFI) from 793 ± 128 to 478 ± 107 ($P < .05$) (Figure 6B). Similarly, there was a significant reduction in suicide gene function: residual viability was $19.6\% \pm 5.6\%$

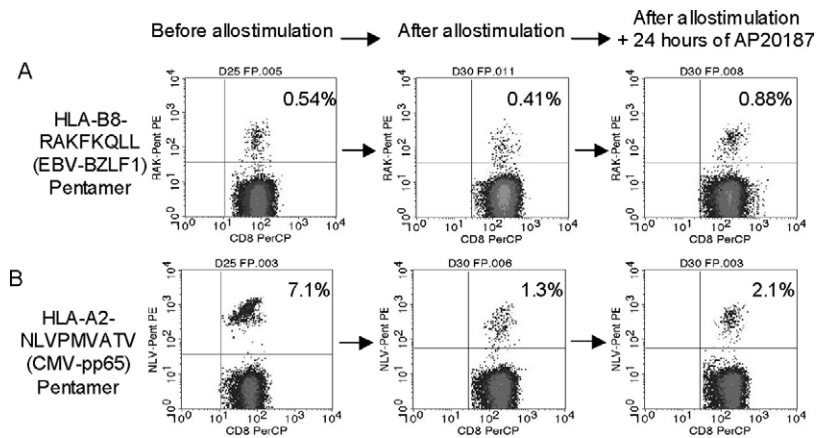


Figure 7. Virus-specific T cells were partially retained after treatment of allostimulated cells with dimerizer. Gene-modified allodepleted cells were maintained in culture for 3 weeks post transduction to allow transgene down-modulation. Cells were stimulated with allogeneic PBMC for 4 days, following which a portion was treated with 10nM AP20187. The frequency of A, EBV-specific T cells, and B, CMV-specific T cells were quantified by pentamer analysis before allostimulation, after allostimulation, and after treatment of allostimulated cells with dimerizer. The percentage of viral-specific T cells decreased after allostimulation. Following treatment with dimerizer, viral-specific T cells were partially and preferentially retained.

following treatment with AP20187; after correction for baseline viability of $54.8\% \pm 20.9\%$, this equated to a killing efficiency of only $63.1\% \pm 6.2\%$.

To discover whether the decrease in transgene expression with time resulted in reduced transcription following T cell quiescence or to elimination of transduced cells, we reactivated a portion of cells on day 22 post transduction with OKT3 and anti-CD28 antibody. At 48 to 72 hours (day 24 or 25 post transduction), OKT3/aCD28-reactivated cells had significantly higher transgene expression than nonreactivated cells. CD19 expression increased from $88\% \pm 4\%$ to $93\% \pm 4\%$ ($P < .01$) and CD19 MFI increased from 478 ± 107 to 643 ± 174 ($P < .01$). More importantly, suicide gene function also increased significantly from $63.1\% \pm 6.2\%$ killing efficiency to $84.6\% \pm 8.0\%$ ($P < .01$). Furthermore, killing efficiency was completely restored if the cells were immunomagnetically sorted for the activation marker CD25: killing efficiency of CD25 positive cells was $93.2\% \pm 1.2\%$, which was the same as killing efficiency on day 5 post transduction ($93.1\% \pm 3.5\%$) (Figure 6C). Killing of the CD25 negative fraction was $78.6\% \pm 9.1\%$.

Of note, virus-specific T cells are spared when dimerizer is used to deplete gene-modified cells that have been reactivated with allogeneic PBMC, rather than by nonspecific mitogenic stimuli. After 4 days reactivation with allogeneic cells, Figure 7A and B show that treatment with AP20187 spares (and thereby enriches) viral reactive subpopulations, as measured by the proportion of T cells reactive with HLA pentamers specific for peptides derived from EBV and CMV.

DISCUSSION

We have demonstrated the feasibility of engineering allogeneic T cells with two distinct safety mechanisms, selective allodepletion and suicide gene modification. In combination, these modifications should enable addback of substantial numbers of T cells with antiviral and antitumor activity, even after haploidentical transplantation. Our data show that the suicide gene, iCasp9, functions efficiently ($>90\%$ apoptosis after treatment with dimerizer) and that down-modulation of transgene expression that occurred with time was rapidly reversed upon T cell activation, as would occur when alloreactive T cells encountered their targets. We also showed that Δ CD19 was a suitable selectable marker that enabled efficient and selective enrichment of transduced cells to $>90\%$ purity, and that these manipulations had no discernable effects on the immunologic competence of the engineered T cells with retention of antiviral activity, and regeneration of a $CD4^+CD25^+Foxp3^+$ population with Treg activity.

Given that the overall functionality of suicide genes depends on both the suicide gene itself and the marker used to select the transduced cells, translation into clinical use requires optimization of both components, and of the method used to couple expression of the two genes. The two most widely used selectable markers in clinical practice each have drawbacks. Neomycin phosphotransferase (*neo*) encodes a potentially immunogenic foreign protein and requires a 7-day culture in selection medium, which not only increases the complexity of the system, but is also potentially damaging to virus-specific T cells [26]. The most widely used surface selection marker is

Δ LNGFR, but a recent report of leukemia in a mouse model raises concerns regarding its oncogenic potential [27], despite its apparent clinical safety [28]. Furthermore, Δ LNGFR selection is not widely available, because it is used almost exclusively in gene therapy. A number of alternative selectable markers have been suggested. CD34 has been well-studied in vitro [29,30], but the steps required to optimize a system designed primarily for selection of rare hematopoietic progenitors and, more critically, the potential for altered in vivo T cell homing [31], make CD34 suboptimal for our purpose. As an alternative marker, we used Δ CD19, because clinical grade CD19 selection is readily available as a method for B cell depletion of stem cell autografts. We demonstrated that Δ CD19 enrichment could be performed with high purity and yield and, more importantly, the selection process had no discernable effect on subsequent cell growth and functionality.

The effectiveness of suicide gene activation in Δ CD19-selected iCasp9 cells compared very favorably to that of *neo*- or Δ LNGFR-selected cells transduced to express the HSVtk gene. The earlier generations of HSVtk constructs provided 80%-90% suppression of 3 H-thymidine uptake [14,32,33] and showed similar reduction in killing efficiency upon extended in vitro culture [33], but were nonetheless clinically efficacious. Complete resolution of both aGVHD and chronic GVHD (cGVHD) has been reported with as little as 80% in vivo reduction in circulating gene-modified cells. These data supported the hypothesis that transgene down-modulation seen in vitro is unlikely to be an issue because activated T cells responsible for GVHD will upregulate suicide gene expression and will therefore be selectively eliminated in vivo [14]. Whether this effect is sufficient to allow retention of virus- and leukemia-specific T cells in vivo remains to be tested in a clinical setting. By combining in vitro selective allodepletion prior to suicide gene modification, we hope that the need to activate the suicide gene mechanism will be significantly reduced, thereby maximizing the benefits of addback T cell.

The high efficiency of iCasp9-mediated suicide seen in vitro has been replicated in vivo. In an SCID mouse-human xenograft model, >99% of iCasp9-modified T cells were eliminated after a single dose of dimerizer [18]. Safety testing has been performed on healthy human volunteers using AP1903, which has extremely close functional and chemical equivalence to AP20187, and would be used in a proposed clinical application. Maximal plasma level of 10 to 1275 ng/mL AP1903 (equivalent to 7 to 892 nM) was attained over a 0.01 mg/kg to 1.0 mg/kg dose range administered as a 2-hour intravenous infusion [34]. There were no significant adverse effects. After allowing for rapid plasma redistribution, the concentration

of dimerizer used in vitro remains readily achievable in vivo.

We believe iCasp9 will prove to have several advantages over HSVtk, including the ability to use ganciclovir and related drugs without causing inadvertent T cell destruction, and a reduced risk of destructive immune responses against transduced cells because the iCasp9 is almost entirely human derived. Although both our selectable marker (Δ CD19) and our suicide gene (iCasp9) are of human origin, one potential limitation of our approach is that linkage of expression of the selectable marker to the suicide gene uses a 2A-like cleavable peptide that is of nonhuman origin. However, this product is only 20 amino acids long, and is therefore less likely to be immunogenic than entirely nonhuman proteins such as *neo* or HSVtk.

It is essential to define the culture conditions that are optimal for maintaining the immunologic competence of suicide gene-modified T cells, because phenotype, repertoire, and functionality can all be affected by the stimulation used for polyclonal T cell activation [35,36], the method for selection of transduced cells [26], and duration of culture [37]. The addition of CD28 costimulation and the use of cell-sized paramagnetic beads to generate gene modified-cells that more closely resemble unmanipulated PBMC in terms of CD4:CD8 ratio, and expression of memory subset markers, including lymph node homing molecules CD62L and CCR7, have been proposed as methods for improving the in vivo functionality of gene-modified T cells [35,36]. CD28 costimulation has also been reported to increase the efficiency of retroviral transduction and expansion [35,36]. Interestingly, however, we found that the addition of CD28 costimulation had no impact on transduction of allodepleted cells (data not shown), and the degree of cell expansion seen in our study was higher compared to the anti-CD3 alone arm in these studies. Furthermore, iCasp9-modified allodepleted cells retained significant antiviral functionality, and approximately a quarter retained CD62L expression. There was also regeneration of CD4⁺CD25⁺Foxp3⁺ regulatory T cells. The allodepleted cells we used as starting material for T cell activation and transduction may have been less sensitive to the addition of anti-CD28 antibody as costimulation. CD25-depleted PBMC/EBV-LCL cocultures contained T cells and B cells that already express CD86 at significantly higher levels than unmanipulated PBMC (data not shown) and may themselves provide costimulation. In addition, it has been reported that depletion of CD25⁺ regulatory T cells prior to polyclonal T cell activation with anti-CD3 enhances the immunological competence of the final T cell product [37]. To minimize the effect of in vitro culture and expansion on functional competence, we used a relatively brief culture period whereby cells

were expanded for a total of 8 days post transduction with CD19-selection being performed on day 4.

Finally, we have demonstrated that we can scale up production sufficiently to treat adult patients at doses of up to 10^7 cells/kg: allodepleted cells can be activated and transduced at 4×10^7 cells per flask, and a minimum of 8-fold return of CD19-selected final cell product can be obtained on day 8 post transduction, to produce at least 3×10^8 allodepleted gene-modified cells per original flask. The increased culture volume is readily handled in additional flasks or bags.

The allodepletion and iCasp9-modification we describe should significantly improve the safety of adding back T cells, particularly after haploidentical stem cell allografts. This should, in turn, enable greater dose escalation, with a higher chance of producing a hoped for antileukemia effect. We will explore this possibility in a clinical study of our gene-modified allodepleted T cells.

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