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A foundation for “universal” T-cell based immunotherapy: T-cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR

Running title: CAR⁺ T-cells modified to eliminate TCR

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ABSTRACT

Clinical-grade T cells are genetically modified *ex vivo* to express a chimeric antigen receptor (CAR) to redirect specificity to a tumor associated antigen (TAA) thereby conferring anti-tumor activity *in vivo*. T cells expressing a CD19-specific CAR recognize B-cell malignancies in multiple recipients independent of MHC because the specificity domains are cloned from the variable chains of a CD19 monoclonal antibody. We now report a major step towards eliminating the need to generate patient-specific T cells by generating “universal” allogeneic TAA-specific T cells from one donor that might be administered to multiple recipients. This was achieved by genetically editing CD19-specific CAR⁺ T cells to eliminate expression of the endogenous $\alpha\beta$ T-cell receptor (TCR) to prevent a graft-versus-host response without compromising CAR-dependent effector functions. Genetically modified T cells were generated using the *Sleeping Beauty* system to stably introduce the CD19-specific CAR with subsequent permanent deletion of α or β TCR chains with designer zinc finger nucleases. We show that these engineered T-cells display the expected property of having redirected specificity for CD19 without responding to TCR stimulation. CAR⁺TCR^{neg} T cells of this type may potentially have efficacy as an off-the-shelf therapy for investigational treatment of B-lineage malignancies.

INTRODUCTION

Allogeneic hematopoietic stem-cell transplantation (HSCT) can cure some patients with high risk B-cell leukemia/lymphoma, but relapse remains a major cause of death. To improve the graft-versus-leukemia/lymphoma (GVL)-effect, donor-derived T cells can be genetically modified to express a tumor-specific chimeric antigen receptor (CAR) with specificity derived from the variable domains of a monoclonal antibody, thus focusing immunoreactivity towards the tumor in an MHC non-restricted manner.¹ However, the endogenous $\alpha\beta$ T-cell receptor (TCR) on infused allogeneic T cells may recognize major and minor histocompatibility antigens in the recipient leading to graft-versus-host-disease (GVHD). As a result, the majority of current clinical trials infuse autologous CAR⁺ T-cells relying on immune tolerance to prevent TCR-mediated deleterious recognition of normal tissues after adoptive transfer.² This approach has achieved initial clinical successes targeting CD19⁺ malignancies,³⁻⁷ but is limited by the time and expense to manufacture patient-specific T-cell products. Our goal is to generate off-the-shelf universal CAR⁺ T cells from allogeneic healthy donors which can be administered to any patient without causing GVHD.

CD19 is constitutively expressed on most acute and chronic B-cell malignancies. Therefore, to target malignant B cells, we have adapted the *Sleeping Beauty* (SB) transposon/transposase system for human application to stably express a CD19-specific CAR (designated CD19RCD28).⁸⁻¹¹ SB modified CAR⁺ T cells can be numerically expanded to clinically-sufficient numbers by the recursive addition of γ -irradiated artificial antigen presenting cells (aAPC) that co-express CD19 and desired T cell co-stimulatory molecules.^{12,13} These platforms have been adapted for human application as clinical trials based on the electroporation and propagation of CAR⁺ T cells have achieved institutional and

federal regulatory approvals for the adoptive transfer of patient-derived and allogeneic CD19RCD28⁺ T cells after autologous and allogeneic HSCT (INDs #14193, 14577, 14739).^{2,8,10,11}

To test the feasibility of using allogeneic CAR⁺ T cells we modified the culturing process for generating CAR⁺ T cells (**Supplement Figure 1**) to include the editing of the genome of CAR^{neg} and CAR⁺ T cells to irreversibly eliminate expression of the $\alpha\beta$ TCR. To knockout the $\alpha\beta$ TCR loci we employed zinc finger nucleases (ZFNs),¹⁴ comprised of zinc finger protein DNA binding domains fused to the DNA cleavage domain from the *Fok I* endonuclease, targeting genomic sequences in the constant regions of the endogenous α or β subunits of the TCR. ZFNs mediate genome editing by catalyzing the formation of a DNA double strand break (DSB) in the genome. Targeting a DSB to a predetermined site within the coding sequence of a gene has been previously shown to lead to permanent loss of functional target gene expression via repair by non-homologous end joining (NHEJ), an error-prone cellular repair pathway that results in the insertion or deletion of nucleotides at the cleaved site.^{15,16}

Here we demonstrate that ZFNs targeting either the α or β chains of endogenous TCRs in T cells resulted in the desired loss of TCR expression. As expected, these modified T cells did not respond to TCR stimulation, but maintained their CAR mediated re-directed specificity for CD19.

MATERIALS AND METHODS

Human Subjects

Peripheral blood mononuclear cells (PBMC) were obtained from healthy adult volunteer donors who had provided informed consent from Gulf Coast Regional Center (Houston, TX). Primary tumor cells were obtained after informed consent from patients at MD Anderson Cancer Center (MDACC) with chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and diffuse large B-cell lymphoma (DLBCL). Clinical research in accordance with the Declaration of Helsinki and approved by MDACC.

ZFNs targeting constant regions of α and β TCR

ZFNs containing 5 or 6 fingers were assembled from an established archive of pre-validated 2-finger and 1-finger modules as described.^{17,18} The ZFN pairs designed to bind either a sequence within exon 1 of the TCR α constant region (*TRAC*: NG_001332.2; ZFNs designated as TRAC-ZFN-1 and TRAC-ZFN-2) or a consensus sequence common to exon 1 of both TCR β constant regions 1 and 2 (*TRBC1* and *TRBC2*: NG_001333.2; ZFNs designated as TRBC-ZFN-1 and TRBC-ZFN-2), will be described in detail elsewhere.¹⁹ Genes encoding the ZFNs were assembled using PCR-based methodology and cloned into a DNA expression plasmid (pVAX; Invitrogen, Carlsbad, CA). These plasmids were linearized with *XhoI* and the RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI) with ARCA cap analog (Ambion, Austin, TX) was used to produce and cap mRNA. After *in vitro* transcription poly-adenines were added using a poly A tailing kit (Ambion), the integrity and size of the mRNA species was validated on a denaturing 1% agarose gel with 3-(N-morpholino) propanesulphonic acid (MOPS) buffer and concentration was measured using a spectrophotometer (BioRad, Hercules, CA) at OD₂₆₀. The mRNA was stored at -80°C in nuclease-free vials for single use.

Flow cytometry

The following monoclonal antibodies (mAbs) and reagents were used with indicated specificity and the appropriate isotype controls. From BD Biosciences (San Jose, CA): phycoerythrin (PE)-conjugated anti-CD3 ϵ (cat # 347347, clone SK7), PE-anti-CD19 (cat # 555413, clone HIB-19), PE-Cy5 CD45RA (cat# 552888, clone 5H9), PE-CD56 (cat # 555516, clone B159), PE-CD62L (cat # 555544, clone Dreg 56), PE-CD64 (cat # 558592, clone 10.1), PE-CD86 (cat # 555658, clone 2331), PE-CD137L (cat # 559446, clone C65-485), FITC-conjugated anti-CD4 (cat # 555346, clone RPA-T4), APC-conjugated anti-CD4 (cat # 340443, clone SK3), FITC-anti-CD8 (cat #555634, clone HIT8a), APC-conjugated anti-CD8 (cat # 340659, clone SK1), PE-anti-TCR $\alpha\beta$ (cat # 555548, clone T10B9.1A-31), APC-anti-TCR $\gamma\delta$ (cat# 555718, clone B1), PE-mouse IgG2b κ (cat # 555058), APC-mouse IgG1 (cat # 5555751), and FITC-mouse IgG1 (cat # 349041); From Jackson ImmunoResearch (West Grove, PA): PE-anti-mouse Fab (H+L) (cat # 115-116-146), Alexa 488-conjugated or Alexa 647-conjugated CAR-specific antibody (clone 136-20-1) that recognizes an epitope within scFv region of CD19RCD28 was generated in our laboratory. TCR V β usage was analyzed by a panel of anti-V β monoclonal antibody (IOTest $\text{\textcircled{R}}$ Beta Mark; Beckman Coulter, Brea, CA). We added propidium iodide (Sigma-Aldrich, St. Louis, MO) just before collecting cells on a flow-cytometer to exclude dead cells from analysis. Data was acquired on a FACS Calibur (BD Biosciences) using CellQuest version 3.3 (BD Biosciences) and analyzed by FCS Express version 3.00 (De Novo Software, Los Angeles, CA) or FlowJo version 7.6.1 (Tree Star, Inc. Ashland, OR).

Artificial antigen presenting cells

K562-derived aAPC were previously modified by lentiviral transduction to constitutively co-express CD19, CD64, CD86, CD137L, membrane-bound (MB) IL-15 and EGFP (the latter

encoded following the EMCV IRES element). A clone (#4) was obtained by limiting dilution and numerically expanded for use.²⁰ For some experiments CD3-specific antibody (OKT3; eBioscience, San Diego, CA) was used to activate T cells by pulsing the mAb onto the CD64⁺ (FcR) clone #4 (**Supplement Figure 2**). Expression of desired transgenes and bound OKT3 was validated weekly by flow cytometry before use in co-culture with T cells.

Propagation of primary T-cells

Healthy donor derived PBMC were isolated by density gradient separation using Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA). T cells were numerically expanded in the presence of 50 IU/mL of recombinant human interleukin-2 ([rhIL-2] added three times a week; Chiron, Emeryville, CA) on γ -irradiated (100 Gy) aAPC (clone #4, 1:2 T cell : aAPC ratio) that had been pre-loaded with OKT3. T cells with aAPC were cultured in complete medium (CM) defined as Hyclone-RPMI 1640 (Thermo Fisher Scientific, Waltham, MA) supplemented with 2 mmol/L L-glutamine (Glutamax-1: Invitrogen) and 10% heat-inactivated Hyclone-fetal bovine serum (Thermo Fisher Scientific).

Generation and Propagation of CAR⁺ T cells

DNA supercoiled plasmids (15 μ g of CD19RCD28/pSBSO and 5 μ g of pKan-CMV-SB11)¹³ encoding the SB transposon (to stably express CD19RCD28) and the SB transposase (to transiently express SB11) were electro-transferred using an Amaxa Nucleofector II device (Lonza, Basel, Switzerland) at 2×10^7 PBMC/cuvette as previously described (**Supplement Figure 1**).¹² T cells expressing CD19RCD28 were preferentially propagated in CM by recursive addition every 7 or 14 days of clone #4 (not loaded with OKT3) at 1:2 T-cell : aAPC (γ -irradiated to 100 Gy) ratio in the presence of rhIL-2 50 IU/mL, added three times a week.

Electro-transfer of messenger RNA species into primary or CAR⁺ T cells

Six days after stimulation of unmodified T cells with OKT3-loaded clone #4 or 2 to 4 days after the last stimulation of CD19RCD28⁺ T-cells with clone #4, 5x10⁶ T-cells were mixed with 2.5 to 10.0 µg of each ZFN mRNA in 100 µL of Human T-Cell Nucleofector solution (Cat #VPA-1002, Lonza) and electroporated using the Nucleofector II device with program T-20. Following electroporation, cells were immediately placed in 2 mL of pre-warmed CM and cultured at 37°C, 5% CO₂ for 4 to 6 hours and then 50 IU/mL of rhIL-2 was added with 2 mL of 20%FBS-RPMI. In some experiments to enhance ZFN-mediated enzymatic activity, after overnight culture, cells were transferred to 30°C, 5% CO₂ and cultured for 2 days then returned to 37°C-5% CO₂.

Enrichment of CD3^{neg} T cells

Cells washed with PBS supplemented with 2% FBS and 2mM EDTA, were incubated for 10 minutes with CD3 microbeads (Cat # 130-050-101, MilteneyiBiotec, Auburn, CA) at 4°C. After washing twice, cells were passed through an LD column (Cat # 130-042-901, MilteneyiBiotec), and the flow-through fraction was collected for further use.

Surveyor Nuclease assay

The levels of genomic disruption of *TRAC*, *TRBC1*, and *TRBC2* in T cells were determined by Surveyor Nuclease assay (Transgenomics, Omaha, NE) using CEL I nuclease.²¹ The percent target disruption was quantified by densitometry. The PCR primers used for the amplification of target locus are:

TRAC forward 5'-GGGCAAAGAGGGAAATGAGA-3'

TRAC reverse 5'-CAATGGATAAGGCCGAGACC-3'

TRBC1 forward, 5'-CTGAACAAGGTGTTCCCACCC-3'

TRBC1 reverse 5'-GTGTGCGCTGGTTCCTTTCTT-3'

TRBC2 forward, 5'-CCTGGCCACAGGCTTCTACC-3'

TRBC2 reverse 5'-CCACCTTGTCCACTCTGGCTT-3'

⁵¹ Chromium release assay

Target cells were labeled with 0.1 mCi of ⁵¹Cr (Perkin Elmer, Boston, MA) for 2 hours. After washing thrice with ice-cold CM, labeled cells were diluted and plated at 10³ cells/well in 100 μL CM in 96-well v-bottomed plates. T-cells were added in 100 μL/well at indicated effector target ratios and the plate was spun (180 x g for 3 minutes without brake) to facilitate cell-to-cell contact. After 4 hours or 6 hours (when using primary tumor cells as targets) incubation at 37°C, 5%CO₂, 50 μL of supernatants were counted on TopCount (Perkin Elmer, Shelton, CT). All assays were performed in triplicate. The percent specific lysis was calculated as follows: ((experimental cpm - spontaneous cpm) / (maximum cpm - spontaneous cpm)) × 100.

PKH-26 dilution assay

T-cells were incubated with 2.0 μM of the red-fluorescent lipophilic dye PKH-26 (Cat # PKH26GL, Sigma-Aldrich) for 5 minutes at room temperature according to the manufacturer's instructions. Cells, 100% labeled with PKH-26, were stimulated with either OKT3 loaded aAPC or CD19⁺ aAPC in CM supplemented with 50 IU/mL rhIL-2 (added every-other-day). PKH-26-derived fluorescence was measured by flow cytometry 10 days after stimulation and CD19⁺CD28⁺ T cells were revealed using anti-CAR mAb clone 136-20-1.

RESULTS

Disruption of the $\alpha\beta$ TCR-CD3 complex on T cells using ZFNs Two ZFN pairs targeting the constant regions of TCR α (TRAC-ZFN-1 and TRAC-ZFN-2) or TCR β (TRBC-ZFN-1 and TRBC-ZFN-2) (**Figure 1**) were developed and tested in primary human T-cells propagated *ex vivo* for 6 days on OKT3-loaded aAPC (clone #4). Since transient expression of ZFNs is sufficient to mediate gene knockout, we used a “hit-and-run” delivery strategy to transiently express the ZFNs utilizing electro-transfer of *in vitro* transcribed mRNA species coding for the ZFN pairs (**Figure 2a**). To measure TCR expression we used a mAb specific for CD3 ϵ , which is only present on the cell surface when TCR $\alpha\beta$ is expressed. Nine days after electro-transfer, flow cytometric analysis revealed that ZFN pairs targeting *TRAC* or *TRBC* eliminated CD3 ϵ expression on primary T cells at levels reaching 19.4% and 5.2% respectively. The efficiency of TCR knockout correlated with the amount of electro-transferred mRNA (**Figure 2b**, upper panel). Electro-transfer of mRNA to primary T cells was generally well-tolerated, though a slight reduction in cell viability was observed at higher doses. ZFN-mediated gene disruption has been reported to be more efficient when cells are transiently exposed to mild hypothermia.²² Thus, we cultured T cells for 2 days at 30°C after electro-transfer. ZFN-mediated disruption of CD3 ϵ was up to 2.4-fold higher when electroporated T cells were cultured at 30°C versus 37°C. Using this approach, 37% and 15% of electroporated T-cells lost expression of CD3 ϵ using the ZFN pair targeting *TRAC* and *TRBC*, respectively, (**Figure 2b**, lower panel) with no change in the levels of CD3 negative cells in the untransfected samples and without an appreciable decrease in viability (measured by Trypan blue).

To confirm that electroporated T cells had been genetically modified at the intended ZFN target sites (TCR α or β loci), a Surveyor Nuclease assay was performed using specific oligonucleotide primers flanking target sites within *TRAC*, *TRBC1*, or *TRBC2*. CEL I nuclease

digestion products, representative of genetic changes induced by the ZFNs, were present only after electro-transfer of the TCR-specific ZFN pairs and the percent disruption assessed by densitometry correlated with loss of cell surface CD3 ϵ expression (**Figure 2c**). These experiments in primary T cells confirmed that ZFNs designed to target *TRAC* or *TRBC* lead to permanent disruption of $\alpha\beta$ TCR expression, as assessed by the CEL I-mediated surveyor nuclease assay and confirmed by flow cytometry analysis of CD3 ϵ .

Enrichment of TCR^{neg} T cells

For future clinical applications, rapid and robust methods for isolating sources of a TCR-disrupted population will be needed. To address this issue, we enriched the TCR/CD3^{neg} population by negative selection using paramagnetic beads and a depletion column. With a single depletion step, we enhanced the CD3 ϵ ^{neg} population to over 93% (**Figure 3a**). A CD3 ϵ ^{neg} population could not be enriched from control T cells that were not genetically edited with ZFNs. Back-to-back CD3-depletion resulted in >99% enrichment without skewing the CD4⁺ or CD8⁺ T-cell subsets (**Figure 3b**). The depletion of CD3⁺ T cells will also deplete remaining $\gamma\delta$ TCR⁺ T cells. A flow cytometry analysis of TCR V β repertoire in enriched TCR^{neg} T cells validated the elimination of TCR β expression from the T-cell surface (**Figure 3c**). This degree of depletion is clinically appealing as the loss of TCR on donor-derived T cells will prevent GVHD in HLA-disparate recipients.

Generation of TCR^{neg}CAR⁺ T cells by ZFNs

To test the ability of ZFN pairs to knock out TCR $\alpha\beta$ expression from allogeneic CD19RCD28⁺ T cells, we initially genetically modified PBMC to stably express the CD19RCD28 CAR using the SB transposon/transposase system. The CD19RCD28⁺ T-cell population was specifically propagated by stimulating with γ -irradiated CD19⁺ aAPC (clone #4) every 7 days (**Supplementary Figure 1b**). After four rounds of stimulation, we observed

over 90% CAR expression in T cells similar to our previously published results.¹² Within 2 to 4 days after the fifth stimulation with CD19⁺ aAPC, when T cells were activated, we electroporated the cells with mRNA encoding the *TRAC* or *TRBC* ZFNs (**Figure 4a**). Flow cytometry analysis revealed that up to 30% and 26% of CD19⁺CD28⁺ T cells lost CD3 ϵ expression after transfection of the *TRAC* or *TRBC* ZFNs, respectively (**Figure 4b**). The CD3 ϵ ^{neg} population was again readily enriched by paramagnetic beads, and the Surveyor Nuclease assay confirmed that the CD3 ϵ ^{neg} population contained a high percentage of modified alleles at the intended ZFN target sites within the *TRAC* and *TRBC* loci (**Figure 4c**). The frequency of *TRBC1* and *TRBC2* disruption at the DNA level was approximately 20-25% and that of *TRAC* disruption was approximately 60%. These numbers fit with the observed frequencies of CD3 ϵ ^{neg}CD19⁺CD28⁺ T cells because in each cell only 1 out of 4 *TRBC* alleles (2 *TRBC1* and 2 *TRBC2*) is expressed. Similarly 1 of 2 *TRAC* alleles is expressed in each T cell. Therefore, disruption of the expressed allele is sufficient to achieve the CD3 negative phenotype.

TCR^{neg}CAR⁺ T cells do not respond to TCR stimulation, but do maintain CD19 specificity

We anticipated that TCR^{neg}CAR⁺ T cells could not respond to TCR stimulation. To test this, we measured the proliferative response of these cells after stimulation by cross-linking CD3 with OKT3 in comparison to activating CAR for sustained proliferation upon docking with CD19. TCR^{neg}CD19⁺CD28⁺ T cells proliferated in response to CD19, but not OKT3 (**Figure 5a**). Next, we assessed the ability of TCR^{neg}CD19⁺CD28⁺ T cells to lyse CD19⁺ target cells in a standard 4-hour ⁵¹Cr release assay (**Figure 5b**). The capacity of TCR^{neg}CAR⁺ T cells to specifically lyse CD19 target cells was similar to that observed for TCR⁺CD19⁺CD28⁺ T cells. Moreover, these TCR^{neg}CAR⁺ T cells maintain cytotoxicity against CD19⁺ primary tumors

(**Figure 5c**). Together, these data confirmed that the absence of a measurable TCR on TCR^{neg}CD19RCD28⁺ T cells corresponds with abrogation of TCR activity, but does not impact the ability of the CAR to activate genetically modified T cells for proliferation and target cell killing.

TCR^{neg} CD19RCD28⁺ T-cells can be propagated on CD19 expressing aAPCs

We validated that CD19RCD28⁺ T cells sustain their proliferative capacity to expand to the cell numbers required for clinical applications. Both the TCR^{neg}CD19RCD28⁺ and parental TCR⁺CD19RCD28⁺ T cells exhibited similar growth kinetics in response to stimulation with the CD19⁺ aAPC (**Figure 6a**). We did not observe any changes in CD3ε expression on TCR^{neg}CD19RCD28⁺ T cells after aAPC-mediated propagation (**Figure 6b**, top panel). As predicted, these T cells failed to express TCRαβ on their cell surface (**Figure 6b**, middle panel and **Figure 6c**). As expected, the ZFN-mediated disruption of αβTCR expression and depletion of CD3⁺ T cells led to loss of γδTCR⁺ T cells (**Figure 6b**, middle panel). After propagation on aAPC a subset of TCR^{neg}CD19RCD28⁺ T cells exhibited memory phenotype based on expression of CD62L and absence of CD45RA²³ (**Figure 6b**, bottom panel) which may benefit persistence and thus the therapeutic potential of our approach to “off-the-shelf” adoptive T-cell therapy. These data confirm that TCR^{neg}CAR⁺ T cells may be able to be propagated to achieve sufficient cell numbers from a single donor-derived modified T-cell pool for infusions into multiple recipients.

DISCUSSION

We have demonstrated that T cells and indeed CAR⁺ T cells can be genetically edited by ZFNs to eliminate expression of the endogenous $\alpha\beta$ TCR. This has therapeutic implications where donor-derived T cells are infused to achieve an anti-tumor effect. Therapeutic success after allogeneic HSCT is defined as achieving a GVL-effect without causing clinically-significant GVHD.²⁴ Thus, separation of GVL and GVHD is a crucial issue following engraftment of allogeneic hematopoietic stem cells and strategies to accomplish this are based on infusing desired T-cell effector populations predicted to reduce unwanted allogeneic effects. This includes the adoptive transfer of donor-derived memory T cells employing a narrowed TCR V β repertoire compared with naïve T cells^{25,26} or *in vitro* depletion of T-cells activated through allo-antigens^{27,28,29}. Adding to this approach, we have previously demonstrated that CAR⁺ T cells expressing alloreactive TCRs can be rendered anergic to disparate HLA while maintaining specificity for CD19.¹³ This was achieved by blockade of co-stimulatory molecules upon co-culture of genetically modified T cells with stimulator cells expressing disparate HLA. An alternative to pre-selection includes conditional ablation of infused allogeneic CAR⁺ T cells in the event that serious adverse events occur. This has been accomplished by genetic modification of allogeneic T cells to express “suicide genes” such as thymidine kinase (TK),³⁰ iCasp9,³¹ CD20,³² thymidylate kinase,³³ and a modified Fas³⁴ that can be triggered for conditional ablation via the administration of specific molecules (e.g., ganciclovir to TK⁺ expressing cells).

We recognized that approaches to selectively deplete T cells expressing undesired $\alpha\beta$ TCR may be incomplete and that complete knockout of the endogenous TCR might be advantageous to prevent GVHD. Therefore, we undertook a genetic approach using designer ZFNs to permanently disrupt the α and β constant region sequences in T cells thereby

eliminating TCR expression. Since TCR $\alpha\beta$ receptors need to form heterodimers to express a functional cell surface molecule, knocking out either *TRAC* or *TRBC* was sufficient to eliminate TCR $\alpha\beta$ expression. This is supported by a recent publication showing that a mutation in *TRAC* gene leads to the loss of TCR $\alpha\beta$ expression.³⁵

ZFNs have been demonstrated to disrupt target gene expression as a consequence of error-prone DNA DSB repair by NHEJ, which in most cases results in a frame shift mutation leading to a premature stop of translation.¹⁵ This technology is being evaluated in early stage clinical trials infusing HIV-resistant T cells generated by ZFN-mediated disruption of the *CCR5* co-receptor for HIV-1.^{16,36} ZFNs target and thus disrupt gene expression at the genomic level which is an advantage over techniques that involve transcriptional repression and require sustained expression of the inhibiting factor (e.g., enforced expression of shRNA to mediate TCR down regulation³⁷). That ZFNs can permanently disrupt gene expression after transient expression (without the inherent dangers of genomic integration) enabled our use of *in vitro* transcribed mRNA species in a “hit-and-run” manner for electro-transfer of ZFNs into T cells.

The human application of “universal” CAR⁺ T cells that have been genetically edited with ZNFs will depend on efficacy as well as safety. The genetically modified T cells specifically lysed primary targets and cell lines. Their therapeutic potential is also dependent on persistence after adoptive transfer. The 2nd generation CAR chosen for this study activates T cells through chimeric CD28 and CD3- ζ . It remains to be determined in side-by-side clinical trials if other CAR designs, such as signaling through CD137 and CD3- ζ are superior. Safety depends on selective elimination of endogenous TCR and minimizing ZFN-mediated enzymatic activity at off-target sites. We evaluated the most likely “off-target” sites using CEL-I nuclease and did not detect cleavage. We did observe that the efficiency of

enzymatic activity at TRAC or TRBC genomic loci is approximately 20-40% after a single electro-transfer of mRNA species coding for ZFN pairs. However, continued cell surface expression of TCR from HLA-disparate candidate T-cell donors may cause GVHD after adoptive immunotherapy. Therefore, to prevent GVHD after infusion we used CD3-specific paramagnetic beads to deplete and re-deplete T cells with residual expression of TCR. This is a clinically appealing strategy as this approach can readily be undertaken in compliance with current good manufacturing practice for Phase I/II trials. Our planned clinical trials will include release criteria of the manufactured T-cell product based on residual $\alpha\beta$ TCR expression and an assessment of the maximum number of genetically modified T cells that can be safely infused from an allogeneic donor into multiple recipients. In addition, the wellbeing of the patients can also be safeguarded by co-expressing CAR with a transgene capable of mediating conditional T-cell ablation.

Previous reports suggest that T-cell activation mediated through an endogenous TCR is required to obtain a fully functional CAR in a model system using Jurkat cell lines³⁸. In contrast, we observed that knocking out TCR $\alpha\beta$ expression from CD19⁺CD28⁺ T cells did not appreciably alter the ability of these cells to specifically kill CD19⁺ targets or proliferate in response to CD19. One reason for this discrepancy other than the difference in host cells may be the use of a 2nd generation CAR, which includes signaling not only through CD3 ζ (signal 1) but also CD28 (signal 2; co-stimulation).³⁹⁻⁴¹ A benefit to expressing a TCR with known specificity is that activation through the endogenous immunoreceptor can be used to propagate T cells to achieve an anti-tumor effect mediated by the CAR.^{42,43} It remains to be tested in humans whether coordinated co-stimulation achieved through multiple CAR signaling endodomains will be sufficient to sustain persistence *in vivo* or if triggering of T cells through TCR is needed. However, the propagation of CD19⁺CD28⁺ T cells on aAPC

modified to co-express CD19 along with co-stimulatory molecules results in the significant expansion of CAR⁺ memory T-cell subsets predicted to have prolonged *in vivo* survival.^{44,45} Therefore, any loss of persistence of TCR^{neg}CD19RCD28⁺ T cells may be off-set by co-stimulatory properties of aAPC and the encoded CD28 intra-cellular domain within the CAR.

Preparing antigen-specific T cells from a third-party donor is clinically appealing as these products can be generated, stored and validated before use and infused to multiple patients immediately as needed.⁴⁶ Indeed, third-party T cells have been successfully infused into patients with post-transplantation lymphoproliferative diseases.^{47,48} Despite the fact that a majority of the viral-antigen specific TCR $\alpha\beta$ chains demonstrate cross-reactivity to allo-HLA *in vivo*,⁴⁹ clinically significant GVHD was not observed. This may be in part due to the *ex vivo* repetitive antigen stimulation resulting in the emergence of either an oligoclonal or monoclonal TCR $\alpha\beta$ repertoire which decreases the chance of T-cell alloreactivity. On the contrary, when we numerically expand CD19RCD28⁺ T cells through *in vitro* CD19 stimulation on γ -irradiated aAPC independent of TCR stimulation we did not observe skewing of the TCR V β usage as assessed by a panel of V β -specific antibodies.

In conclusion, we demonstrate that TCR^{neg}CAR⁺ T cells can be generated using a genetic approach to remove (a) endogenous undesired TCR with ZFNs and (b) introduce a desired CAR with the SB system using a common electro-transfer platform. Our approach abolishes the danger of GVHD posed by adoptive transfer of large numbers of allogeneic T cells while maintaining desired effector functions mediated by CD19RCD28 CAR to target malignant B cells. This strategy provides an important step to developing a “universal” CAR⁺ T cell which can be manufactured from one donor and administered on demand to multiple patients. Subsequent studies are focusing on preventing rejection of the infused allogeneic TCR^{neg}CAR⁺ T cells by the recipient’s immune system recognizing disparate HLA. This may

be accomplished using genetic modifications including ZFN-mediated knockout of HLA and over-expression of conserved HLA homologues to inhibit NK-cell activity.

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AUTHORSHIP CONTRIBUTIONS

H.T., A.R. designed and performed experiments analyzed data and wrote the paper. P.L., Y.Z., L.Z., S.M., J.C.M. performed the experiments. H.H. supported experiments. P.K., B.R., D.A.L., R.E.C. contributed discussion and edited the paper. E.J.R., P.D.G, M.C.H. designed experiments, analyzed data, and edited the paper. C.B., L.N. tested the TRAC and TRBC target ZFNs developed by Sangamo BioSciences, and edited the paper. L.J.N.C. conceived the idea, coordinated the project, designed experiments, analyzed data, and wrote the paper.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1

ZFN pairs targeting sites within genomic loci of TCR- α and β constant region.

Each exon is shown by a block. Black blocks represent coding regions. Grey columns represent non-coding regions. One ZFN pair was designed to bind exon 1 of the TCR α constant region (*TRAC*) and another ZFN pair binds a conserved sequence on exon 1 of the TCR β constant regions 1 (*TRBC1*) and 2 (*TRBC2*). Underlined nucleotide sequences represent the intended binding sequence of each ZFN.

Figure 2

Disruption of the TCR $\alpha\beta$ -CD3 complex in primary T cells

a. Schematic presentation of ZFN transfer

A pair of ZFN-encoding mRNA was electro-transferred 6 days after stimulation of CAR^{neg} T cells. T cells were then cultured with 50 IU/mL of IL-2 and incubated at 30°C or 37°C-5%CO₂, as indicated. CD3 expression was analyzed by flow cytometry on Day 7 to 9 after electroporation.

b. Down regulation of CD3 after electro-transfer of mRNA encoding the TCR $\alpha\beta$ targeted ZFNs

Day 9 after electro-transfer of the indicated doses of mRNA coding for *TRAC* or *TRBC* targeted ZFN pairs, TCR $\alpha\beta$ -CD3 expression was analyzed by co-staining for CD4, CD8, and CD3 ϵ . Representative flow data at Day 9 after ZFN electro-transfer is shown. Flow cytometry data are gated on cells excluding propidium iodide. Numbers in the lower right quadrant represent the percentage of CD3 ϵ negative cells in T-cell populations.

Top panels shows CD3 ϵ expression in T cells cultured at 37°C after ZFN transfer and bottom panels shows CD3 ϵ expression in T cells transiently cultured at 30°C from Day 2 to 3 after ZFN transfer.

c. Surveyor Nuclease assay to detect ZFN-mediated modification of TCR target sites in T cells

Arrows indicate the fragments produced by a Surveyor Nuclease digest of amplicons bearing a mismatch at the intended site of ZFN cleavage in the TRAC or TRBC loci, respectively. Lane headings indicate both the mRNA dose, specific ZFN pair delivered via electro-transfer, and temperature of incubation for the different samples. Numbers beneath each lane indicate the percentage of modified alleles in each sample.

Figure 3

TCR^{neg} T cells can be enriched by depletion of CD3 ϵ ⁺ T cells

a. CD3 expression before and after depletion using CD3-specific paramagnetic beads.

Flow cytometry reveals expression of CD3 ϵ in CD4⁺ and CD8⁺ T cells 15 days after stimulation by OKT3-loaded aAPC (9 days after ZFN transfection). Numbers in the lower right quadrant represent the percentage of CD3 ϵ ^{neg} T cells. Representative results using *in vitro* numerically expanded T cells.

b. CD3 ϵ ^{neg} T cells can be further enriched by additional round of depletion with CD3-specific paramagnetic beads

Flow cytometry revealing expression of CD3 ϵ in CD4⁺ and CD8⁺ T-cells after two rounds of depletion of CD3 ϵ ^{pos} T-cells. Numbers in the lower right quadrant represents the percentage of CD3 ϵ negative cells in CD4⁺ and CD8⁺ T-cell populations.

c. V β repertoire analysis in T cells modified with ZFN.

The V β usage clonogram was analyzed by a panel of TCR-specific mAbs, co-stained with CD4 and CD8. Percentage of specific V β ⁺ T-cell fractions within CD4 and CD8 gating is shown. The nomenclatures of V β repertoire shown are based on Arden *et al.*⁵⁰ Representative data from 3 independent experiment is shown.

Figure 4

Elimination of TCR $\alpha\beta$ - CD3 complex from CD19-specific CAR⁺ T-cells

a. Schematic of electro-transfer of mRNA coding for ZFN pairs in CAR⁺ T cells

mRNA species encoding the indicated ZFN pairs were electro-transferred into CAR⁺ T cells two days after stimulation with CD19⁺ aAPC. After electroporation, cells were maintained with 50 IU/mL of IL-2 and incubated for two days at 30°C-5%CO₂. CD3 ϵ expression was analyzed 9 days after electroporation by flow cytometry.

b. Disruption of TCR $\alpha\beta$ - CD3 complex expression after electro-transfer of mRNA encoding the TCR-specific ZFNs

Flow cytometry analysis of CD3 ϵ expression in T cells 9 days after electro-transfer of mRNA species encoding the indicated ZFN pairs, gated on the propidium iodide negative population.

c. Surveyor Nuclease assay

Arrows indicate the fragments produced by a Surveyor Nuclease digest of amplicons bearing a mismatch at the intended site of ZFN cleavage in the TRAC or TRBC loci, respectively. Samples were analyzed 9 days after electroporation. The numbers at the bottom represent percentages of modified alleles in each sample.

Figure 5

Functional consequences of ZFN-mediated TCR knockout in CAR⁺ T cells

a. Loss of responsiveness of TCR^{neg} CAR⁺ T cells to TCR stimulation

Dilution of PKH26 was measured 10 days after stimulation with aAPC loaded with OKT3 (upper panel) or expressing CD19 (lower panel). Flow cytometry data was gated on CAR⁺ T cells. Parental: CAR⁺ T-cells without modification; no mRNA: mock electroporated CAR⁺ T cells ; TRAC CD3^{neg}: CAR⁺ T-cells electroporated with mRNA encoding ZFN pairs specific for *TRAC*, and depleted CD3^{pos} population; TRBC CD3^{neg}: CAR⁺ T cells electroporated with mRNA encoding ZFN pairs specific for *TRBC*, and depleted for CD3^{pos} population.

b. Redirected specificity of TCR^{neg} CAR⁺ T cells

Specific lysis by CAR⁺ T cells of an EL4 (mouse T-cell line) modified to express a truncated version of human CD19 (closed symbols) was measured by standard 4 hour ⁵¹Cr release assay. Specificity is shown by lack of lysis of CD19^{neg} (parental) EL4 cells (open symbols). CAR⁺ T-cells were modified by ZFNs (*TRAC* and *TRBC*) or unmodified CAR⁺ T-cells (parental and no mRNA). The error bars represent the standard deviation.

c. Cytotoxicity by TCR^{neg} CAR⁺ T cells against CD19⁺ primary B-cell tumors

Specific lysis by CAR⁺ T cells of B-cell malignancies derived from patients was measured by 6 hour ⁵¹Cr release assay (effector : target ratio = 30:1). DLBCL: diffuse large B-cell lymphoma, CLL: chronic lymphocytic lymphoma, and MCL: mantle cell lymphoma. The error bars represent the standard deviation.

Figure 6

Propagation of TCR^{neg} CAR⁺ T cells on CD19 expressing aAPC

a. Sustained proliferation of TCR^{neg} CAR⁺ T cells

CAR⁺ T cells with (*TRAC* and *TRBC*) or without (parental and no mRNA) TCR modification by ZFNs were stimulated with γ -irradiated CD19⁺ aAPC every 2 weeks. Viable T cells were enumerated every 7 days and inferred total numbers were calculated. Representative data from 3 independent experiments is shown.

b. Analysis of TCR^{neg} CAR⁺ T cells after propagation

Flow cytometry analysis of CD3 ϵ expression (top panel), $\alpha\beta$ TCR and $\gamma\delta$ TCR expression (middle panel), and subset analysis for memory pool (bottom panel) of TCR^{neg}CAR⁺ T cells after 28 days of propagation on aAPC. Numbers are percent expression for the quadrant.

c. V β repertoire analysis in TCR^{neg} CAR⁺ T cells after propagation on aAPC

The V β usage clonogram was analyzed by a panel of TCR-specific mAbs, co-stained with CD4 and CD8. Percentage of identified V β ⁺ T-cell fractions within CD4 and CD8 flow cytometry gates is shown. The nomenclatures of V β repertoire shown are based on Arden *et al.*⁵⁰ Representative data from 3 independent experiments is shown.

Fig.1

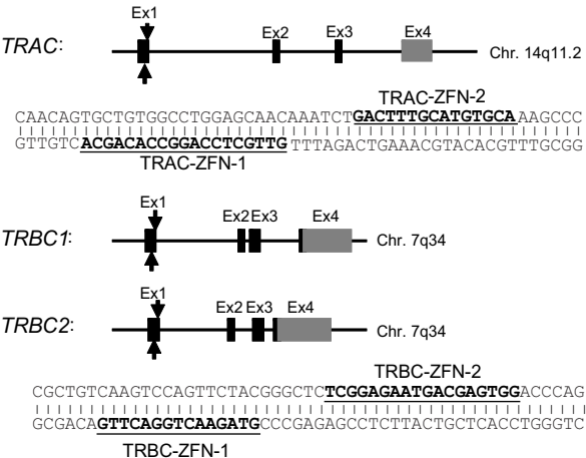
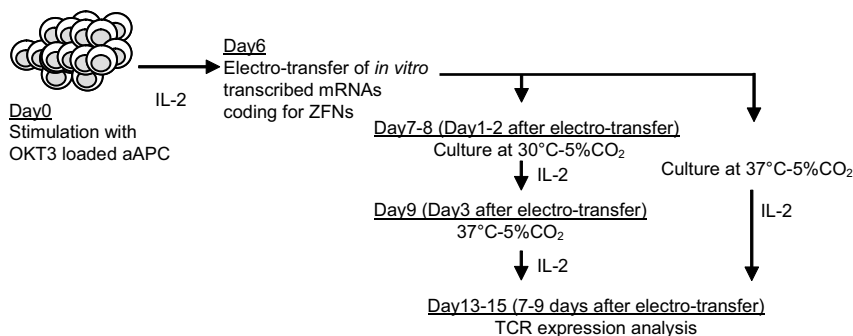
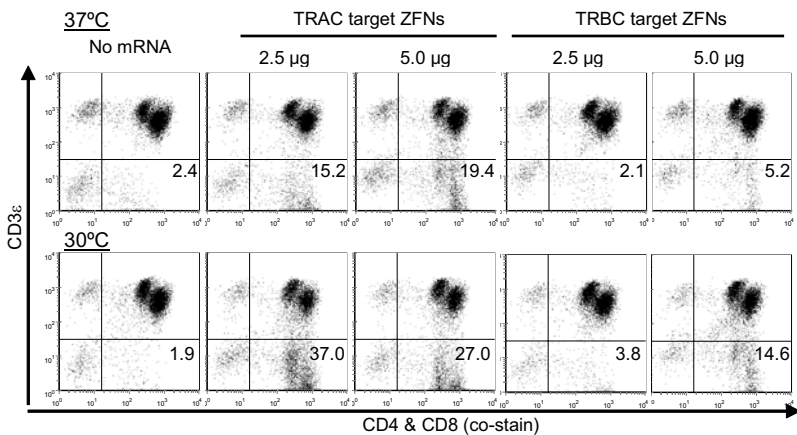


Fig. 2

a.



b.



c.

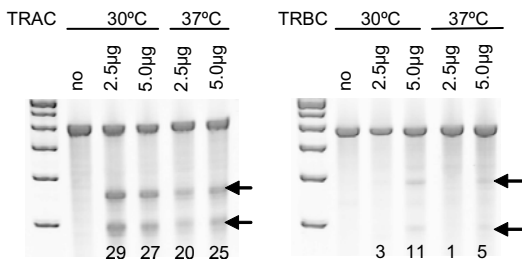


Fig. 3

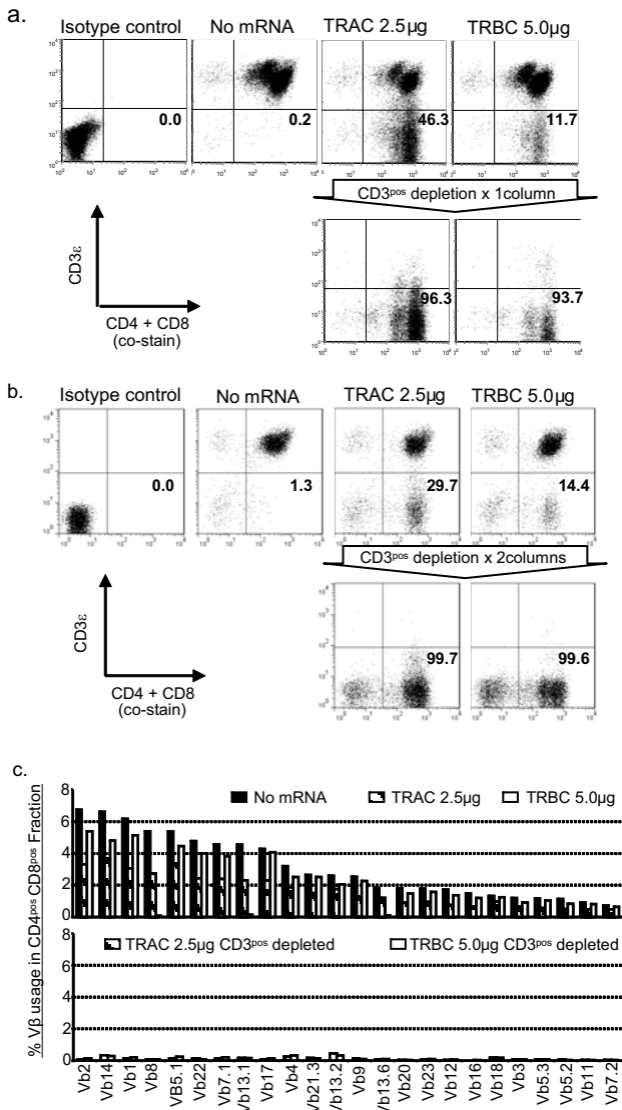


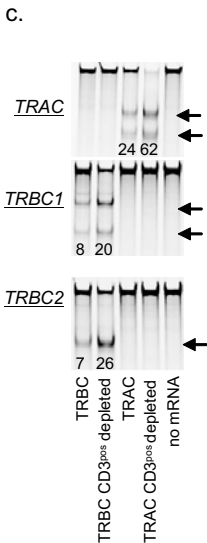
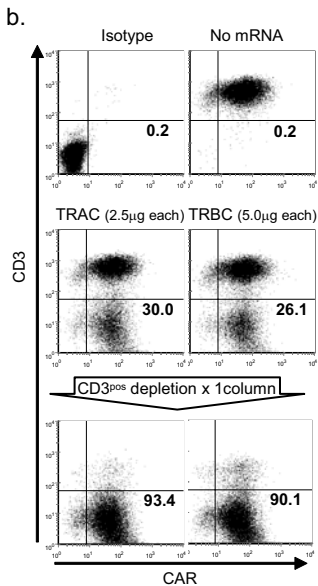
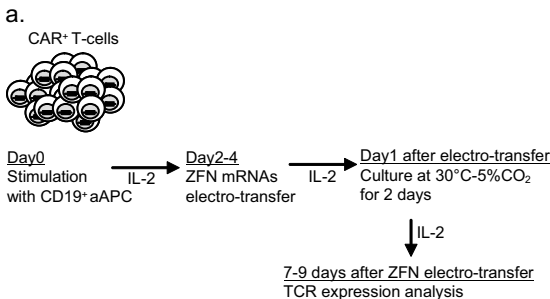
Fig. 4

Fig.5

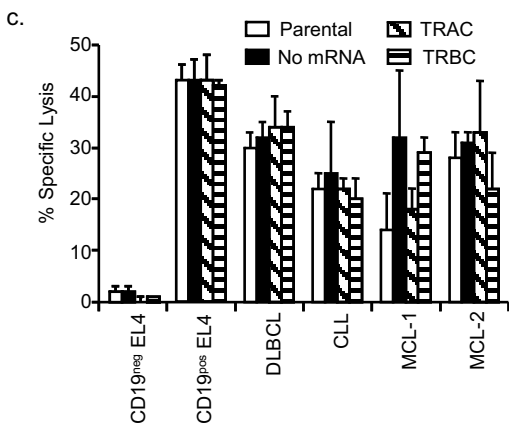
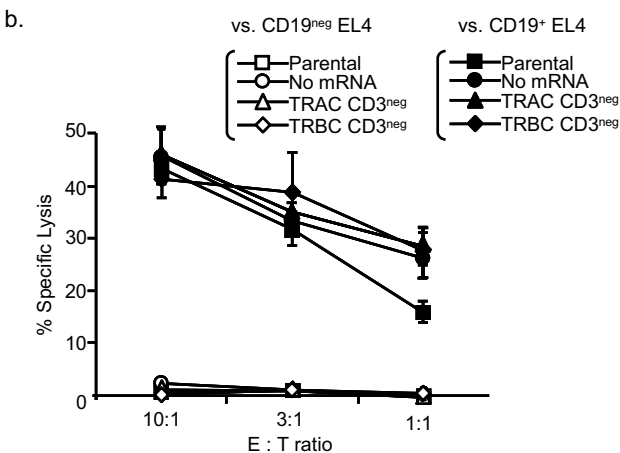
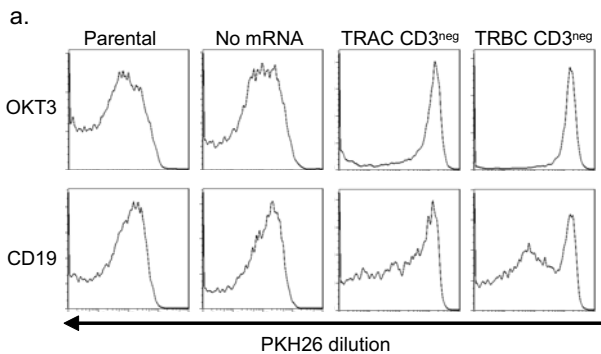
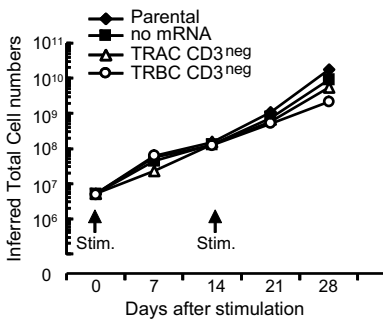
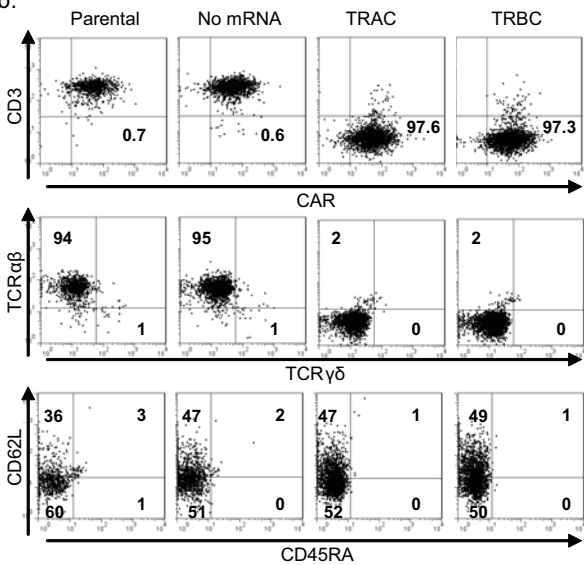


Fig.6

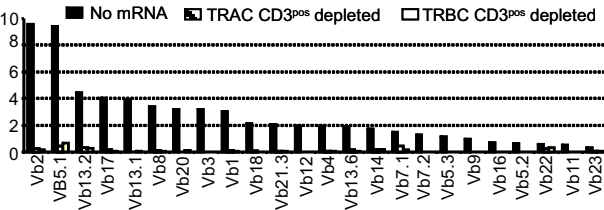
a.



b.

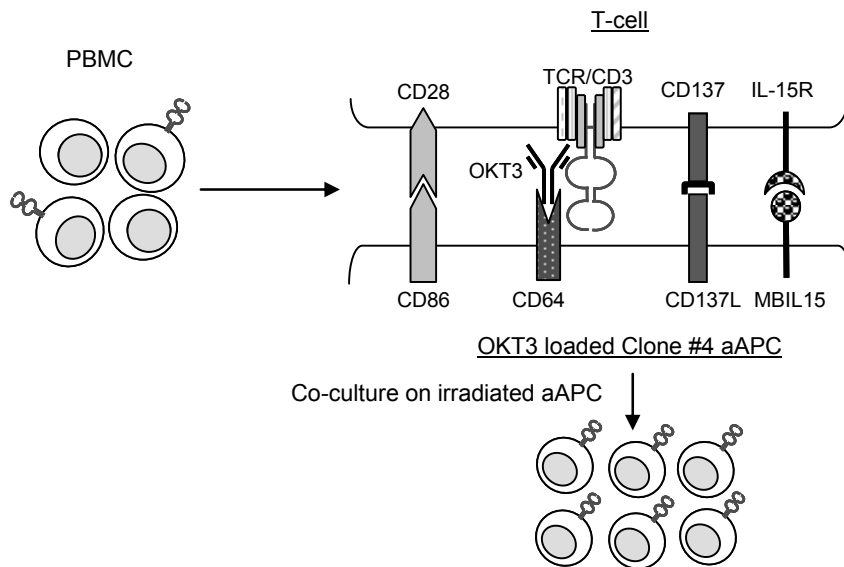


c.

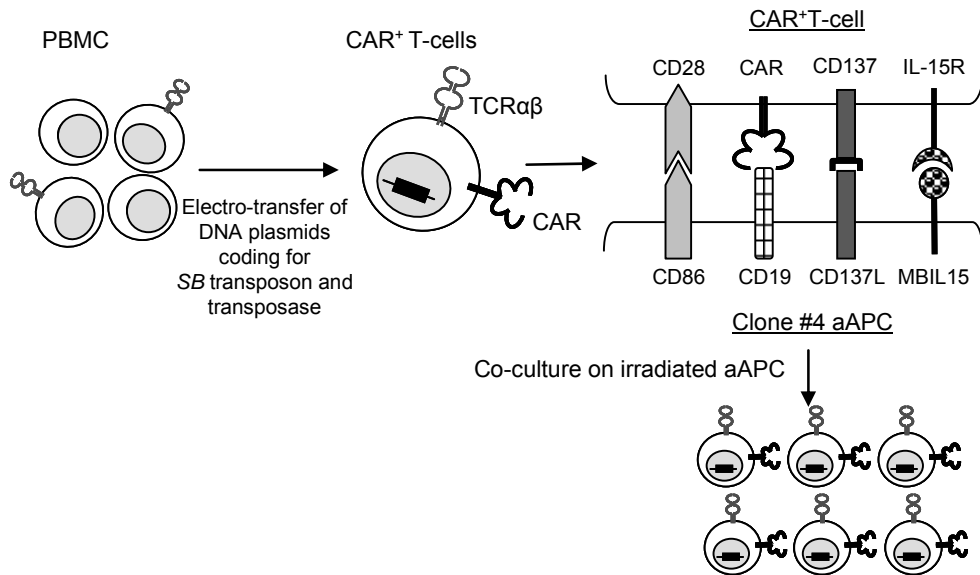


SUPPLEMENTAL DATA

a. CAR^{neg} T cells



b. CAR⁺ T cells

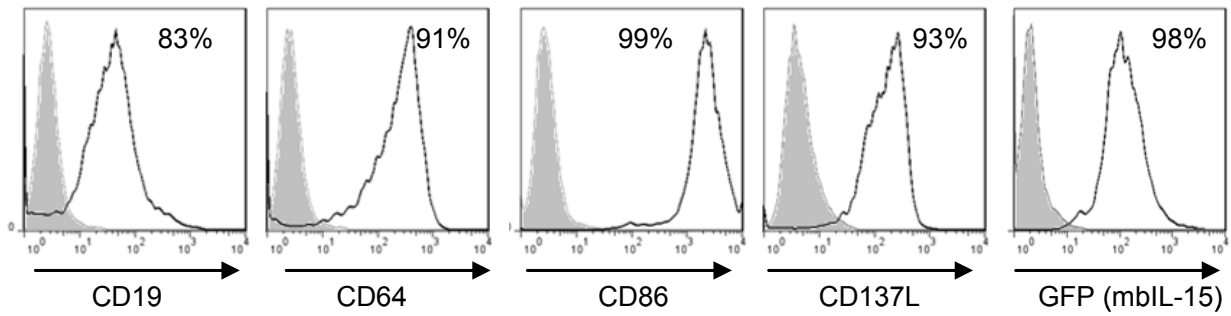


Supplemental Figure 1

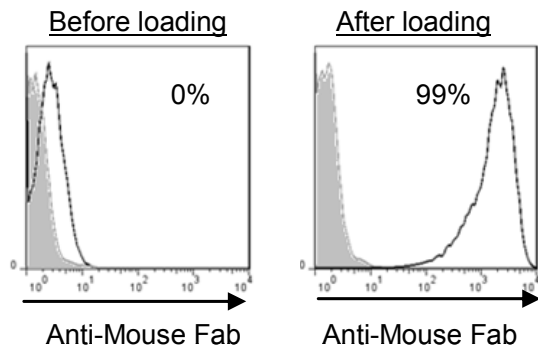
Schematic of the approach to genetically modify and propagate T-cells from PBMC.

(a) T-cells were propagated by stimulation with OKT3 loaded γ -irradiated aAPC (clone #4) in the presence of soluble IL-2 (b) DNA plasmids coding for SB transposon (CD19RCD28) and SB transposase (SB11) were electro-transferred into primary human T-cells. CAR⁺ T cells were selectively propagated by repeated additions of γ -irradiated aAPC (clone #4) in the presence of rhIL-2.

a.



b.



Supplemental Figure 2

Characterization of aAPC clone #4. (a) Flow cytometry was used to compare expression of CD19, CD64, CD86, CD137L and MBIL-15 between parental K562 (grey shaded histogram) and K562-aAPC clone #4 (black open histogram). MBIL-15 is composed of human IL-15 peptide fused to modified human IgG4 Fc region and CD4 transmembrane domain and was detected by presence of EGFP (co-expressed with MBIL-15 after IRES element). aAPC were used to co-culture T-cells if expression of introduced transgenes were $\geq 80\%$. (b) OKT3 was loaded onto aAPC clone #4 at 1 μg per 10^6 cells. Flow cytometry data before and after OKT3 loading detected by antibody specific for mouse Fab region. No-staining control: grey shaded histogram, stain with anti-mouse Fab: black open histogram.