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iCaspase 9 Suicide Gene System

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Abstract

Although cellular therapies may be effective in cancer treatment, their potential for expansion, damage of normal organs, and malignant transformation is a source of concern. The ability to conditionally eliminate aberrant cells in vivo would ameliorate these concerns and broaden the application of cellular therapy. We devised an inducible T-cell safety switch that can be stably and efficiently expressed in human T cells without impairing phenotype, function, or antigen specificity. This system is based on the fusion of human caspase 9 to a modified human FK-binding protein, allowing conditional dimerization using a small-molecule drug. When exposed to a synthetic dimerizing drug, the inducible caspase 9 (iC9) becomes activated and leads to the rapid apoptosis of cells expressing this construct. We have demonstrated the clinical feasibility and efficacy of this approach after haploidentical hematopoietic stem cell transplant (haplo-HSCT). A single dose of a small-molecule drug (AP1903) eliminated more than 90 % of the modified T cells within 30 min after administration and symptoms resolved without recurrence. This system has the potential to broaden the clinical applications of cellular therapy.

Keywords

Allodepleted T cells; Suicide gene; Safety switch; Dimerizer; AP1903; Inducible caspase 9; GvHD

1 Introduction

1.1 Development of Cellular Safety Switches

The advantage of using cellular therapies for the treatment of malignant diseases is the augmentation of immune response, particularly if the cells are genetically modified to alter or gain function [1–3]. On the other hand, significant toxicities from the cells themselves or from their transgene products hinder clinical investigation. There is considerable interest in developing a means by which infused cells could be ablated if problems arise from their use. The ability to conditionally eliminate engineered T cells through the activation of cellular safety switches, also known as “suicide genes,” will significantly enhance the safety and feasibility of T cell infusion.

The first studies on safety switch-modified donor T cells involved the *herpes simplex virus thymidine kinase* (HSV-TK) gene, the product of which will phosphorylate ganciclovir or acyclovir to the active moiety, which interferes with DNA synthesis. Adoptive transfer of donor-derived T cells engineered with HSV-TK gene can enhance immune recovery post-transplant, and resultant acute GvHD has been controlled by administration of the ganciclovir prodrug [4, 5]. Subsequent studies using HSV-TK-modified T cells given

prophylactically on day 0 of matched-sibling transplant or starting from day 28 of haplo-HSCT have demonstrated similar results [6, 7]. Although HSV-TK can be effective as a safety switch for acute GvHD, it has significant drawbacks. HSV-TK is potentially immunogenic and requires activation by a drug that remains a crucial pharmacological agent for the treatment of cytomegalovirus infection, leading to undesired elimination of the transduced cell population [4, 8]. HSV-TK-mediated killing also takes days to complete and ganciclovir-resistant truncated HSV-TK forms have been described [9].

1.2 Development of Inducible Caspase 9 Safety Switches

Two decades ago, Spencer and colleagues developed a method to control cellular signaling through ligand-mediated dimerization or oligomerization of intracellular proteins [10]. They used cell-permeable synthetic ligands that bind to FK506 binding protein 12 (FKBP12). FKBP12 belongs to the immunophilin family of receptors, a physiological function of which is to bind to and inactivate calcineurin [11]. Calcineurin inhibition leads to impaired T-cell receptor signaling and consequent immunosuppression [12]. In order to create a cellular control switch without the unwanted physiological and toxic effects of calcineurin inhibition, Clackson and colleagues redesigned the ligand-FKBP12 interface [13]. They created a specificity binding pocket in FKBP12 by substituting the bulky phenylalanine with the smaller valine residue (FKBP12-F36V). The redesigned ligand has high affinity and selectivity for FKBP12-F36V and interacts minimally with endogenous FKBP [13]. In 2001, a dimeric form of this ligand, so-called AP1903, underwent safety testing in healthy volunteers without significant adverse effects [14].

Based on these studies, we have devised a safety switch for T cells that exploits dimerization of a modified caspase 9 molecule, which is part of the intrinsic apoptotic pathway. Under physiological conditions, caspase 9 is activated by the release of cytochrome C from damaged mitochondria. Activated caspase 9 then activates caspase 3 and the other terminal effector molecules, leading to apoptosis. The optimized inducible caspase 9 molecule (iC9) consists of an FKBP12-F36V domain linked, via a flexible Ser-Gly-Gly-Gly-Ser linker, to caspase 9, which is caspase 9 without its physiological dimerization domain, caspase activation domain (CARD), followed by a selectable marker, truncated CD19 (CD19), linked by a 2A-like sequence, which encodes a cleavable peptide (Fig. 1). Inducible caspase 9 has low dimerizer-independent basal activity and can be stably expressed in human T cells without impairing their phenotype, function, and antigen specificity [15, 16]. A single 10 nM dose of AP1903, or the closely related AP20187, also referred to as chemical inducer of dimerization (CID), induces apoptosis in vitro and in vivo in 99 % of iCasp9-transduced cells selected for high transgene expression. The killing efficiency is significantly lower in cells with low or intermediate level of trans-gene expression and this has implication for iC9 clinical functionality [17].

1.3 Current Clinical Applications of iC9 Safety Switch

Haplo-identical hematopoietic stem cell transplant (haplo-HSCT) is limited by three major complications: graft rejection, acute graft-versus-host-disease (GvHD), and delayed immune reconstitution. Because the donor graft for haplo-HSCT has a high frequency of alloreactive T cells recognizing the non-shared HLA haplotype, extensive T-cell depletion can prevent

graft rejection and acute GvHD, but leads to an increased incidence of disease recurrence and opportunistic infections caused by delayed T-cell reconstitution. Activated T cells express a number of markers including CD25 (IL-2 receptor α), CD69, CD71, CD147, and HLA-DR. Preclinical and clinical studies indicated that allodepletion using anti-CD25 ricin A-chain (RFT5-SMPT-dgA) efficiently eliminated alloreactive cells [18, 19]. We have shown that the post-transplant infusion of small numbers of donor T lymphocytes that have been depleted of recipient-reactive T cells can improve immune reconstitution and antiviral immunity [20, 21]. The ability to conditionally eliminate T cells in the event of GvHD can greatly improve the safety of T-cell addback.

We started a phase 1/2 clinical trial using the iC9 system (CaspALLO study) to investigate the efficacy and specificity of this system, on the availability of a clinical-grade CD25-specific immunotoxin (RFT5-dgA) [17, 22]. Patients who had undergone CD34-selected haplo-HSCT were administered escalating doses (1×10^6 – 1×10^7 /kg) of iC9-modified allodepleted T cells from day 30 after transplant. The iC9-T cells expanded and were detected in the peripheral blood as early as 7 days after infusion and persisted for an average of 3.5 years in surviving patients. Four patients out of ten developed acute GvHD grade 1–2 of the liver and/or skin. When GvHD occurred, the iC9-T cells were >90 % eliminated within 2 h of dimerizer administration, and GvHD was rapidly (within 24 h) and permanently reversed. Remarkably, residual iC9-T cells were able to re-expand, contained pathogen-specific precursors, and persisted for a long term without recurrence of GvHD [17, 23]. Ongoing clinical studies will be critical for the future development of genetically modified T-cell therapy into routine clinical practice. Here we describe the manufacture practice for clinical products modified with the iCaspase 9-suicide gene system.

2 Materials

2.1 Specimens

1. Donor peripheral blood, buffy coat, or apheresis product.
2. Recipient EBV-LCL or LCL from designated donor as prescribed by protocol.

2.2 Common Materials and Reagents

1. Lymphoprep (Axis-Shield).
2. Therapeutic grade AIM V medium (GIBCO-Invitrogen).
3. 100 μ M Acyclovir final concentration.
4. T-cell medium: 45 % Advanced RPMI 1640, 45 % EHAA (Click's medium), 10 % FBS, 2 mM L-glutamine.
5. Human interleukin-2 (stock solution 200 U/ μ L).
6. 25 % Human serum albumin.
7. DPBS.
8. DMSO.

9. 3, 5, 10, 30, and 60 mL syringes.
10. 15, 50, and 250 mL conical tubes.
11. T25 and T75 tissue culture flasks with vented caps.
12. T150, T175, or T225 tissue culture flasks (vented caps).
13. Serological pipettes.
14. 96-Well round-bottom plates.
15. 24-Well plates.
16. Cryovials and Cryocyte bags.
17. 18G and 20G needles.
18. PALL acrodisc 0.2 μ m low protein binding syringe filter.
19. 0.22 μ m filter flask (250 mL).
20. Sterile bottles 125, 500 mL.
21. Fungal, aerobic, and anaerobic bacterial BACTEC bottles.

2.3 Allodepletion

1. 2 M Ammonium chloride stock, NH_4Cl .
2. 400 mM sodium HEPES stock.
3. 400 mM acid HEPES stock.
4. RFT5-dgA (U.T. Southwestern Medical Center).

2.4 Transduction

1. 1 mg/mL Anti-CD3 (Miltenyi Biotec).
2. 1 $\mu\text{g}/\mu\text{L}$ Retronectin (Takara).
3. Nonenzymatic cell dissociation solution (Sigma).
4. T75 non-tissue culture-treated flask.
5. Non-tissue culture-treated 24-well plate.
6. Retrovirus vector-SFG.iC9.2A. CD19 (CAGT).

2.5 CliniMACS Enrichment

1. CliniMACS tubing set (standard) (161-01) (Miltenyi Biotec).
2. CliniMACS CD19 reagent (193-01) (Miltenyi Biotec).
3. CliniMACS EDTA/PBS buffer (2 $\times$ 1 L bag) (Miltenyi Biotec).
4. Transfer bag with at least two spike couplers (300 mL Fenwal 4R2014 or equivalent).

5. Tubing (2" or 4") with pinch clamp, piercing pin, syringe adapter.
6. Luer caps.

2.6 In Vitro Evaluation of Genetically Modified T Cells

Dimerizer: AP1903 (Bellicum), AP20187 (Clontech).

3 Methods

3.1 General Guidelines

1. The start date for allodepletion co-culture is designated "Day 0." The main components of the protocol run from Day 0 to Day 10 or 14 (Fig. 2).
2. The average time for generation LCL takes 6–8 weeks; the recipient LCL should be initiated as soon as possible once the recipient is identified (*see Note 1*).
3. Determine the number of donor PBMC required. Aim for 6×10^5 donor PBMC for every 1×10^6 final product cell required (for setting up co-culture), plus 5×10^7 cells for follow-up and controls. On average, every 1×10^6 donor PBMC co-cultured returns around 2×10^6 final product cells (*see Note 2*).

3.2 Co-culture Donor PBMC with Recipient LCL (Day 0)

1. Collect donor peripheral blood or unstimulated leukapheresis sample from donor for mononuclear cell preparation (*see Note 3*) [24].
2. Dilute heparinized peripheral blood in an equal volume of D-PBS or RPMI 1640 at ambient temperature (*see Note 4*).
3. In a 50 mL centrifuge tube, carefully overlay approximately 10–15 mL lymphoprep with approximately 20–30 mL of diluted blood. Adjust as necessary to utilize all the available cells.
4. Centrifuge at $400 \times g$ for 40 min at ambient temperature. Make sure that the brake is turned off.
5. Harvest PBMC interface into at least an equal volume of D-PBS or RPMI 1640.
6. Centrifuge at $450 \times g$ for 10 min at room temperature. Aspirate supernatant.
7. Loosen pellet by "finger-flicking" and resuspend in D-PBS or RPMI 1640.
8. Centrifuge at $400 \times g$ for 5 min at room temperature. Aspirate supernatant.
9. Loosen pellet by "finger-flicking" and resuspend in D-PBS or RPMI 1640. Use approximately 10 mL buffer per 20 mL starting blood volume. Count cells using a hemacytometer.
10. Determine the amount of PBMCs to be used for co-culture, and centrifuge at $400 \times g$ for 5 min at room temperature. Aspirate supernatant (*see Note 5*).
11. Resuspend PBMCs in AIM V at 2×10^7 /mL, and keep cells in the incubator while you prepare recipient's LCL (*see Note 6*).

12. Harvest 40–80 mL of acyclovir-treated recipient LCL into 50 mL centrifuge tube(s) (*see Note 7*).
13. Irradiate recipient LCL at 70Gy (*see Note 8*).
14. Centrifuge at $400 \times g$ for 5 min. Resuspend all cells in 5–10 mL AIM V and count.
15. Transfer 2×10^7 PBMC into labeled T25. Label as “PBMC alone.”
16. To the remaining donor PBMC, add irradiated recipient LCL at PBMC:LCL ratio of 40:1. Mix well by pipetting. Aliquot the mixture of PBMC + LCL evenly into labeled T75 or T175 flask(s). Add AIM V to adjust the donor PBMC concentration to 2×10^6 cells/mL. Label as “Co-culture.” Let it stand vertically.
17. Incubate all flasks (“Co-culture” and “PBMC alone”) at 37 °C, 5 % CO₂, for 3 days. Flasks should stand vertically (*see Note 9*).

3.3 Proliferation Assay (Day 0)

1. Use a 96-well U-bottom plate. Plate the following three conditions, each in triplicate:
 - a. 100 μ L per well $\times$ 3 wells of “LCL alone” (from Subheading 3.2, **step 11**) as irradiation control at 5×10^4 cells/mL, that is, 5×10^3 cells per well. This is obtained by diluting 5×10^4 irradiated LCL in 1 mL of AIM V.
 - b. 100 μ L per well $\times$ 3 wells of “PBMC alone,” at 2×10^5 cells/well (from Subheading 3.2, **step 15**). Do not pipette directly from T25. Transfer a small aliquot (approximately 0.4 mL) into a cryovial. Pipette 100 μ L per well.
 - c. 100 μ L per well $\times$ 3 wells of “Co-culture” (from Subheading 3.2, **step 16**). Do not pipette directly from flasks. Instead, thoroughly resuspend the cells in one of the flasks and transfer a small aliquot (approximately 0.4 mL) into a 50 mL conical tube. Pipette 100 μ L per well.
 - d. Add 100 μ L AIM V to each of the 9 wells.
 - e. Fill empty wells with 200 μ L sterile water per well.
 - f. Incubate 96-well U-bottom plate at 37 °C, 5 % CO₂ (then proceed to Subheading 3.7).

3.4 Treatment with Immunotoxin (Day 3)

1. Bring AIM V to room temperature (pH is temperature dependent). In BSC, add 1.5 mL 2 M NH₄Cl to 150 mL AIM V medium in a sterile container (the final concentration is 20 mM).
2. Aliquot 25 mL into a suitable container. Using a pH meter outside the BSC, adjust pH to 7.75 using 400 mM NaHEPES and/or 400 mM acid HEPES. Record the volume of Hepes used.

3. In the BSC, add proportionate volume of sterile (i.e., fresh aliquots) 400 mM NaHEPES and/or 400 mM acid HEPES to the remaining 125 mL “Aim V + 20 mM NH₄Cl.”
4. Remove a small volume and recheck pH. Adjust until pH of sterile AIM V = 7.75 ± 0.02. Record final pH.
5. Sterile filter using 0.20 or 0.22 µm syringe or bottle filter. This will be referred to as “Immunodepletion medium.” Warm immunodepletion medium in 37 °C water bath (*see Note 10*).
6. Harvest cells from “PBMC alone” and “Co-culture” flasks and dispense into 50 mL centrifuge tubes. Scrape gently with pipette tip to ensure thorough cell removal. Rinse flask with AIM V and add to the harvest.
7. Centrifuge at 450 × *g* for 10 min.
8. Resuspend “Donor PBMC alone” in 0.7 mL immunodepletion medium (*see Note 11*).
9. Resuspend “Co-culture” in immunodepletion medium at about 1/15th the volume of co-culture. Where applicable, pool into single 50 mL conical tube. Be gentle. Since the resuspension volume will be small, pipette 1 mL of immunodepletion medium into each conical tube, resuspend gently using a 1 mL Eppendorf pipettor, and pool the pellets; then rinse all tubes once with another 1 mL immunodepletion medium and pool.
10. Count cells using 1–10 dilutions. Expect 40–50 % recovery of starting cell dose with greater than 80 % viable cells.
11. Adjust cell concentration to 1 × 10⁷ cells/mL with immuno-depletion medium.
12. Split “PBMC alone” into two equal aliquots in 15 mL tubes (A and B).
13. Aliquot 600 µL of “Co-culture” into a 15 mL tube (C).
14. Remainder of “Co-culture” in 50 mL tube is now referred to as (D).
15. Put all four tubes (A, B, C, and D) into the incubator while preparing RFT5-dgA.
16. Obtain one vial of RFT5-SMPT-dgA antibody from the -80 °C freezer. Gently swirl (*do not create bubbles*) to hasten thawing and filter with 0.2 µm low protein binding PALL syringe filter. This will take 10–20 min. Do not thaw more than 4 h before intended use; if not used immediately, keep filtered RFT5-SMPT-dgA on ice (*see Note 12*).
17. Transfer contents of tube D to T25 or T75 flask(s). Suggested volumes are 3–15 mL for T25 and 20–25 mL for T75.
18. Cap tubes A, B, and C with air-permeable caps from T25 flasks.
19. Incubate tubes A, B, and C (rest in horizontally tilted position) and flask D (stand vertically) at 37 °C, 5 % CO₂.
20. Be ready to wash out RFT5 after 15–18 h before proceeding to Subheading 3.6.

3.5 Coat Flasks with Anti-CD3 Antibody (OKT3) (Day 3)

1. Prepare at least one T75 flask for every 5×10^7 (D) cells.
2. Into a 50 mL conical tube, pipette 10 mL of PBS for each T75 flask. Add 10 μ L OKT3 stock solution (1 mg/mL) for each flask, that is, 1 μ L per 1 mL sterile PBS. The final concentration should be 1 μ g/mL.
3. Pipette 10 mL PBS into each flask. Gently rock the flasks. The PBS should form an even film over the flasks. Let flasks rest horizontally in BSC for a few minutes to ensure that the film is stable. Aspirate PBS.
4. Pipette 10 mL of OKT3 solution into each flask. Tap and rock gently so that the OKT3 solution forms an even film over the entire surface.
5. Put flask horizontally in a clean container, e.g., sealed specimen bag. Keep at 4 °C overnight. Make sure that the OKT3 solution forms an even film over the flask.

3.6 Wash Off RFT5-dgA After 15–18 h (Day 4)

1. Pipette 5–10 mL AIM V into flask D and transfer cells into 50 mL centrifuge tube(s). Rinse flask(s) twice with 5–10 mL AIM V and pool these into the tube(s).
2. Add AIM V to tubes A, and C to final volume of 10 mL. Mix gently by pipetting.
3. Centrifuge all tubes at $450 \times g$ for 10 min.
4. Pipette supernatant from each tube D into a labeled sterile container. Aspirate the remaining supernatant from the other tubes (A to C). Finger-flick to loosen pellet. Using a 1 mL pipette, gently resuspend each pellet in 1 mL AIM V. Gently break up clumps. Where there is more than one tube D, pool pellets, rinse tubes with small volume of Aim V, and add this to the pool. Bring final volume of tube D to at least 20 mL with AIM V. Pipette up and down a few times to mix. Bring final volume of tubes A, B, and C to 10 mL with AIM V. Pipette up and down a few times to mix.
5. Centrifuge all tubes at $450 \times g$ for 10 min.
6. Resuspend pellet in 5 mL AIM V; gently break up clumps by pipetting. Bring the volume up to approximately three times the Day 3 volume, but to a maximum of 40 mL. Perform cell count and calculate the total number of cells. Pipette 3×10^6 cells into a 15 mL tube and add AIM V to bring volume to 1.5 mL (final concentration 2×10^6 cells/mL). This aliquot of D will be used for proliferation assay and FACS analysis. Return the remaining D cells to the incubator.
7. Aspirate supernatant from tubes A, B, and C. Resuspend pellets in 1 mL AIM V; gently break up clumps. Perform cell count and adjust concentration to 2×10^6 cells/mL.

3.7 Sampling for Proliferation Assay and FACS Analysis (Day 4)

1. Plate A, B, C, and D cells (all resuspended in AIM V at 2×10^6 /mL) in the 96-well U-bottom plate from day 0. Plate 100 μ L cells per well $\times 3$ wells each, that is, $2 \times$

10^5 cells per well. Add 100 μ L of AIM V to each of the 12 wells. Return plate to incubator.

2. Send the remaining A, B, C, and aliquot of D cells to QA/QC for FACS analysis. Expect less than 1 % CD3⁺CD25⁺ cells in D.
3. Transfer the 96-well plate to an incubator in the research laboratory for ³H-thymidine pulsing the next day (see **Note 13**).
4. Pulse 6-well plate with ³H-thymidine according to standard operation protocol and harvest the following day at 16–24 h (on day 5) (see **Note 14**).

3.8 Activation of Allodepleted Cells with OKT3 (Day 4)

1. OKT3-coated T75 flask as described in Subheading 3.5. Aspirate OKT3 from flask(s). Pipette 10 mL T-cell medium into each flask. Incubate (horizontally) for 15–30 min at 37 °C, 5 % CO₂.
2. Centrifuge tube D (allodepleted cells) at $450 \times g$ for 10 min.
3. Resuspend in an appropriate volume of T-cell medium (for example, 5 mL for every flask).
4. Aspirate T-cell medium from flasks and aliquot cell suspension into flasks.
5. Add additional T-cell medium to bring the final volume to 40 mL per flask. Incubate at 37 °C, 5 % CO₂, resting horizontally.

3.9 Feed OKT3- Activated T Cells with 100 U/mL IL-2 (Day 5)

1. In a cryovial or 50 mL tube, make a 1:10 dilution of IL-2 working solution (200 U/ μ L) in T-cell medium to obtain diluted IL-2 at 20 U/ μ L. For example: 100 μ L of IL-2 (200 U/ μ L)+900 μ L T-cell medium = 1,000 μ L of diluted IL-2 (20 U/ μ L). Each flask requires 20 μ L of neat IL-2 (200 U/ μ L), i.e., 200 μ L of diluted IL-2 (20 U/ μ L).
2. Pipette 200 μ L of diluted IL-2 (20 U/ μ L) into each flask. Because each flask has 40 mL of medium, the final concentration of IL-2 will be 100 U/mL.

3.10 Pre-coat T75 Flasks with Retronectin (Day 5)

1. Prepare at least one T75 flask for every 2×10^7 cells plated onto OKT3-coated flasks. Use non-tissue culture-treated flasks.
2. Into a 50 mL conical tube, pipette 10 mL of PBS for each T75 flask. Add 70 μ L retronectin (1 mg/mL) for every flask. The final concentration is 7 μ g/mL.
3. Pretreat the flask with PBS. Pipette 10 mL PBS into each flask. Gently tap and rock flasks. The PBS should form an even film over the flasks. Rest flasks horizontally in BSC for a few minutes to ensure that the film is stable. Aspirate PBS.
4. Pipette 10 mL of retronectin solution into each flask. Tap and rock gently such that the retronectin solution forms an even film over the entire surface.

5. Put flask in a sealable bag. Keep at 4 °C overnight, resting horizontally. Make sure that the retronectin solution forms an even film over the flask.

3.11 Transduction with SFG.iC9.2A. dCD19 Retrovirus (Day 6)

1. Preload retronectin-coated flasks with retrovirus. Let retronectin-coated T75 flasks rest in the BSC to bring it to room temperature (approximately 10 min). Aspirate retronectin. Pipette 20 mL T-cell medium into each flask. Incubate for 10–30 min at 37 °C, 5% CO₂, resting horizontally.
2. Each flask should be preloaded with 10 mL of retrovirus supernatant. In a 37 °C water bath, thaw retrovirus aliquots required for preloading only. Once thawed, keep on ice.
3. At the end of the 10–30-min incubation with T-cell medium, aspirate the T-cell medium from the flasks. Pipette 10 mL of retrovirus supernatant into each flask. Incubate for 1.5–3 h at 37 °C, 5 % CO₂. Keep flasks horizontally (*see Note 15*).
4. Harvest OKT3-activated T cells from flasks into 50 mL conical tubes (*see Note 16*).
5. Centrifuge at $400 \times g \times 5$ min (*see Note 17*).
6. Pool cell pellets in 10–40 mL T-cell medium. Count viable cells and calculate the number of flasks required. Each flask can transduce up to 4×10^7 cells, with optimum numbers being around 1.5 to 3×10^7 cells/flask. In order to minimize the amount of retroviral supernatant required, the minimum number of flasks should be used.
7. Adjust the volume of cell suspension to 10 mL T-cell medium per flask with IL-2 (final concentration is 100 U/mL).
8. Return cells to incubator until ready for transduction.
9. Thaw the required retroviral supernatant in water bath (30 mL per flask). Keep thawed supernatant on ice. The aim is to transduce cells in 10 mL T-cell medium + 30 mL retroviral supernatant per flask, supplemented with IL-2 100 U/mL final. Up to 4×10^7 cells can be transduced in each flask.
10. Set aside “non-transduced” control (*see Note 18*).
11. Aspirate retrovirus supernatant from T75 flasks.
12. Aliquot cell suspension into T75 flasks at 10 mL per flask.
13. Add 30 mL retrovirus supernatant to each flask. Use 10 mL of this to rinse the conical tube.
14. Transfer to incubator with flasks resting horizontally.

3.12 Transfer Cells into Tissue Culture-Treated Flasks (Day 7)

1. Warm T-cell medium and cell-dissociation solution in water bath. Approximately 2 mL of T-cell medium is required for every 1×10^6 cells transduced; 10 mL cell-dissociation solution is required for each T75 flask.
2. Harvest cells from retrovirus-coated flasks into 50 mL tubes. Pipette 10 mL cell-dissociation medium into each flask and return to the incubator for 5–10 min. Add these to the 50 mL tubes.
3. Centrifuge at $400 \times g$ for 5 min.
4. Resuspend cells and count viable cells.
5. The cells are to be seeded at 5×10^5 cells/mL in tissue culture- treated flasks (resting horizontally). Suggested volumes are as follows: T75: 25–50 mL; T150: 50–100 mL; T175: 60–120 mL; and T225: 75–150 mL.
6. Adjust cell suspension to an appropriate working volume.
7. Calculate the total amount of IL-2 required given the final concentration of 200 U/mL.
8. Add IL-2 to cell suspension, mix well, and aliquot cells into flasks.
9. Add T-cell medium directly to flasks to bring up to final volume.
10. Incubate flasks at 37 °C, 5 % CO₂, resting horizontally.

3.13 Feed Non- transduced Cells (Day 7)

Calculate the total amount of IL-2 required given the final concentration of 100 U/mL for non-transduced cells.

3.14 Split Cells (Optional) (Day 9)

If on Day 9 the cells look crowded and the cell concentration is greater than 2×10^6 cells/mL, split 1:1 and add additional T-cell medium containing IL-2 (final concentration 50–100 U/mL).

3.15 Preparation of CD19 Selection on CliniMACS (Day 10)

1. Gather all the materials required (*see* Subheading 2.2) (*see* **Note 19**).
2. Prepare two transfer bags (one for “positive fraction,” another for “cell preparation bag”).
3. The “cell preparation bag” must have at least two spike ports. The “positive fraction” bag should be at least 150 mL in size.
4. Make at least two seals using a heat sealer. Cut off excess tubing. Ensure that there are at least two seals between the bag and the cut end of the tubing.
5. Prepare CliniMACS buffer with 0.5 % human serum albumin. Using a syringe and 16G needle or similar, add 20 mL of 25 % human serum albumin to a 1 l bag of

buffer via the blue port. Mix well and label as “+0.5 % HSA” (“MACS buffer”) (see **Note 20**).

6. Using a syringe and 16G needle or similar, draw out 120–150 mL of the MACS buffer via the blue port. Transfer into labeled sterile container. This will be used to label cells.
7. Harvest cells from flasks and dispense into 50 mL tubes.
8. Pipette 2 mL transduced cells into T25 flask. Label “Unselected.” Bring up to 6 mL with T-cell medium and IL-2; final concentration is 100 U/mL.
9. Centrifuge tubes at $400 \times g$ for 5 min.
10. Save supernatant from each centrifuge tube, labeled “Pre- selection” for aerobic sterility testing and for RCR testing if performing the cryopreservation on the same day.
11. Resuspend cell pellets in DPBS (room temperature). Pool into one conical tube.
12. Count cells. Calculate the required volume of MACS buffer and anti-CD19 microbead reagent. For every 1×10^6 cells, use 2 μ L of microbeads and 20 μ L of MACS buffer.
13. Centrifuge tube at $400 \times g$ for 5 min.
14. Resuspend cell pellet in MACS buffer (room temperature) as calculated above. Ensure that you resuspend the pellet thoroughly by gentle pipetting.
15. Add anti-CD19 microbeads. If the final volume exceeds 8 mL, split this into two or more 50 mL conical tubes.
16. Incubate for 30 min at room temperature on a rocking platform.
17. At the end of the 30-min incubation, wash cells with MACS buffer, using at least five times the labeling volume.
18. Centrifuge at $300 \times g$ for 15 min with “1” acceleration and “0” deceleration, 25 °C.
19. Aspirate supernatant. Finger-flick to loosen the pellet.
20. Attach a luer-spike adaptor to the “cell preparation” bag. Remove the plunger from a 30 mL syringe. Attach the syringe to the luer. Open clamp.
21. Transfer the cells to the cell preparation bag, final volume 60 mL.
22. Clamp the adaptor tubing. Remove syringe and cap with luer cap.

3.16 CliniMACS Selection (Day 10)

1. Open a CliniMACS tubing set (161-01) in the BSC (see **Note 21**).
2. Setting up CliniMACS Instrument as described in the manual.
3. Select “Enrichment 5.1” and “CliniMACS Tubing Set REF 161-01.”

4. The labeled cells will be resuspended in 60 mL. Frequency of labeled cells = 60 %. Use this to calculate the cell concentration, where cell concentration = total cell number/60 mL. The minimum cell concentration accepted by the program is “ 20×10^6 cells/mL.” Enter the actual cell concentration or “ 20×10^6 cells/mL,” whichever is *greater*.
5. Connect “cell preparation” bag to the assembled tubing (*see Note 22*).
6. Start selection. The total time required for selection will be displayed on the screen (usually around 20–30 min).
7. At the end of the selection, seal the positive and the negative fraction bag three times with heat sealer. Cut the tubing to the positive fraction bag.
8. Take the positive fraction bag to the BSC and save the negative fraction in incubator at 37 °C 5 % CO₂ temporarily (*see Note 23*).
9. Close clamp on the luer-spike adaptor. Disconnect the tubing at the luer connector and attach a 30 or 60 mL syringe.
10. Transfer the positive fraction cells to 50 mL conical tubes with the 30 or 60 mL syringes. As you detach the syringe, attach a fresh syringe to the bag. Repeat the process until all the cells have been transferred.
11. Centrifuge at $400 \times g$ for 5 min.
12. Resuspend all cells in T-cell medium and count viable cell number.
13. Calculate the total volume of medium and IL-2 required for selected cell suspension at a concentration of 5×10^5 cells/ mL. Final concentration for IL-2 is 100 U/mL.
14. Aliquot cell suspension into flasks and add the appropriate volume of T-cell medium directly to the flasks to bring up to final volume.
15. Incubate at 37 °C, 5 % CO₂.

3.17 Feed Non- transduced Cells (Day 10)

Feed non-transduced cells by adding equal amount of medium with IL-2 as described in Subheading 3.14.

3.18 Killing Assay (Day 11)

1. Label a 24-well plate with the following (one well each).
 - NT no CID
 - NT+CID
 - Pos no CID
 - Pos+CID
2. Harvest 2 mL of non-transduced cells.

3. Pipette 2 mL non-transduced (NT) cells into a 15 mL conical tube.
4. Resuspend positively selected T cells and pipette 2 mL into a 15 mL conical tube.
5. Using a 1 mL pipettor, pipette 1 mL of cells into the appropriate marked wells.
6. Pipette 7 mL T-cell medium into a 50 mL tube. Add 2 μ L IL-2 working solution (200 U/ μ L).
7. Pipette 1 mL of T-cell medium + IL-2 into each of the “no CID” wells.
8. Dilute AP1903 (CID) stock solution to 50 μ M by adding 2 μ L of AP1903 (3.5 mM; 5 mg/mL) stock to 138 μ L of T-cell medium. Ensure that AP1903 stock was thawed less than 6 months previously. Otherwise, use AP20187 from Clontech (check expiration date).
9. If using AP20187 (CID), dilute stock solution to 50 μ M by adding 2 μ L of AP20187 (0.5 mM) stock to 18 μ L of T-cell medium.
10. Add 2 μ L of diluted AP1903 (50 μ M) or 2 μ L of diluted AP20187 (50 μ M) (if AP1903 is not applicable) to the remaining 5 mL medium μ 20 nM CID solution.
11. Pipette 1 mL 20 nM CID solution into each of the “+CID” wells. Final concentration = 10 nM CID.
12. Fill surrounding wells with sterile water.
13. Write down the time on the lid.
14. Transfer plate to incubator at 37 °C, 5 % CO₂.
15. Harvest cells from 24-well plate into labeled 15 mL tubes (22–30 h from plating). Perform FACS analysis.

3.19 iC9 Activity Evaluated by Flow Cytometry (Day 12)

Expected results for FACS analysis:

1. 90 % CD19⁺ in the pooled CD19-selected population (Fig. 3).
2. 90 % killing by AP1903 or AP20187 with killing calculated as follows:

$$\%Killing = (1 - (Viability\ with\ CID \div Viability\ without\ CID)) \times 100\ %$$
 (Fig. 4) [16, 17].

3.20 Release Criteria for Clinical Product

1. Clinical product will be cryopreserved by controlled-rate freezer according to requirement of cGMP and FDA regulations.
2. Less than 1 % residual CD3⁺CD25⁺ positive cells in donor PBMC + recipient LCL co-cultures treated with immunotoxin (results from day 4).
3. Less than 10 % residual proliferation in primary mixed lymphocyte reaction (MLR).

4. >90 % CD19 positive on FACS analysis performed the day after CliniMACS selection (day 12).
5. >90 % killing with CID (*see* Subheading 3.19, **step 2**).
6. Viability >70 % at the time of cryopreservation.
7. Negative culture for bacteria and fungi after 7 days.
8. Negative results for mycoplasma.
9. Endotoxin testing 5 EU/mL.
10. HLA Class I identical to bone marrow/hematopoietic stem cell donor.
11. Final product submitted for RCR testing if frozen more than 96 h from transduction.

4 Notes

1. Obtain 10–30 mL of peripheral blood from patient for ID testing and generation of LCL. LCL should have been grown in acyclovir for more than 14 days before use. Sufficient recipient LCL available: at least 1.5×10^4 , preferably 2.5×10^4 LCL for every 1×10^6 final cell product required. Excess LCL is always useful for setting up larger co-cultures if more PBMC than anticipated was obtained.
2. This varies from 1×10^6 to 5×10^6 cells; and the return is less in smaller cultures because of proportionately larger losses to controls and testing.
3. If the volume of peripheral blood collected is large (400–500 mL), it can be collected into a bag for processing into buffy coat. This reduces the volume to around 100 mL per unit. Allow 15 % cell loss in the process.
4. For buffy coats, dilute 1:1 in RPMI1640, or to a final volume of 200 mL, whichever is greater. For leukapheresis products, if density centrifugation is required (discuss with PI) dilute leukapheresis product to 3×10^7 white cells/mL for layering onto Ficoll.
5. Cryopreserve PBMC if co-culture will be performed on another day, or there are excess PBMCs.
6. If using cryopreserved PBMC, thaw cells at 37 °C in water bath and wash with at least four times the volume of AIM V and centrifuge at $400 \times g$ for 5 min (first wash), and resuspend in 10–40 mL AIM V and centrifuge $400 \times g$ for 5 min (second wash). Suggest no more than 1 mL AIM V for every 4×10^6 thawed, so that the final cell concentration can be adjusted to 2×10^6 /mL.
7. LCL concentration ranges from 0.3 to 1×10^6 /mL and 2.5×10^5 LCL is required for every 1×10^7 PBMC. Therefore, assuming that LCL concentration is 0.3×10^6 /mL, then 0.8 mL of LCL is required for every 1×10^7 PBMC for co-culture.
8. For Fanconi anemia patients, LCL should be irradiated at only 40Gy.

9. This procedure should be performed in the afternoon. RFT5- dgA antibody should be added at 65–75 h from start of co- culture and washed out the following morning after 15–18 h. Prolonged exposure to RFT-dgA may be toxic to bystander cells.
10. Do sterility testing when necessary: Inoculate 1 mL into aerobic BACTEC bottle and 1 mL into fungal BACTEC bottle.
11. Cells are fragile in immunodepletion medium, so be gentle.
12. If the filter becomes clogged, discard the entire filtered and unfiltered antibody and start with a freshly thawed vial of antibody. Add the filtered RFT5-SMPTdgA antibody to tubes B and D to a final concentration of 3 µg/mL, that is, 6 µl antibody per mL. Mix gently by tapping tubes. Remaining RFT5-SMPT-dgA may not be used for clinical purposes.
13. Do sterility testing when necessary. Inoculate aerobic and fungal BACTEC bottle with 2 mL supernatant from tube “D.”
14. This procedure is performed outside the GMP.
15. Excess thawed supernatant can be kept on ice for later use.
16. Perform this step 1–2 h after starting retrovirus preloading.
17. Rinse flasks with small volume of T-cell medium and add this to the harvest. Make sure that you wash down the edges and bottom of the flasks where cells tend to adhere. Inspect flasks under microscope. Cell dissociation medium can be used if cells are very adherent.
18. The non-transduced cells are needed as control for FACS analysis, transduction efficiency by PCR, and control for autonomous growth.
19. Perform an instrument check if CliniMACS instrument has not been used for more than 4 months or if there are other concerns.
20. If the total number of cells for selection is more than 1.8×10^9 , then µl L of MACS buffer will be required.
21. If the CliniMACS is operated in a non-clean room environment an alternative tubing setting is possible, where buffer port and cell preparation bag are connected inside the BSC and a clamp is applied below the buffer port and below the pre-system filter, respectively.
22. Ensure that the pathway to the positive fraction bag is open and there are no kinks in tubing. The clamp must be open. You will lose all the positive fraction cells if there are any obstructions.
23. Cells can be cryopreserved if they have sufficient cell numbers after CliniMACS selection. In this case, negative fraction can be used for QC testing. Otherwise, discard negatively selected cells.

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Fig. 1.

The structure of the *iCaspase9.2A-ΔCD19* transgene. The transgene consists of a suicide gene, inducible caspase 9 (iCasp9), and a selectable marker, truncated CD19 (Δ CD19), 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 (SGGS) to human caspase 9. The sequence cassette is then incorporated into the SFG retroviral vector

Fig. 2.

Overview of the manufacturing process. Selective allodepletion was performed by co-culturing 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 h later. Immunomagnetic selection was performed on day 4 of transduction; the positive fraction could be expanded for a further 4 days and cryopreserved. The *arrows* indicate the times of adding recombinant human interleukin-2 to the cultures

Fig. 3.

Detection of allodepleted T cells expressing iC9 by CD19 after transduction and selection. Suicide gene-modified cells could be enriched to high purity by CD19 immunomagnetic selection. CD19 immuno-magnetic 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 for non-transduced, transduced, and CD19-selected T cells. Region analyzed on viable cells

Fig. 4.

Gene-modified allodepleted T cells were rapidly and efficiently eliminated by CID. The day after immu-nomagnetic selection, cells were treated with 10 nM dimerizer (AP20187). FACS analysis for annexin V and 7-AAD was performed at 22–30 h. The percentages of viable cells are indicated in the *plots*. AP20187 resulted in 90 % killing of CD19-selected cells but had no effect on non-transduced controls. **(a)** Non-transduced cells without CID; **(b)** non-transduced cells treated with CID; **(c)** CD19-selected cells without CID; and **(d)** CD19-selected cells treated with CID