

Gene-corrected regulatory T cell therapy for IL2RA deficiency

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Bi-allelic germline deficiency of IL2RA causes a rare autoimmune disease with impaired regulatory T cell (Treg) function and interleukin-2 (IL-2) signaling. Definitive treatment is currently limited to allogeneic hematopoietic stem cell transplantation, which carries significant morbidity and mortality risks. We previously identified a family with three siblings affected by compound heterozygous mutations in their IL2RA gene, resulting in dysfunctional Tregs. Here, we introduce a novel therapeutic approach involving *ex vivo* generation of gene-corrected autologous regulatory T cells (gcTregs). One of the two disease-causing mutations in patient-derived Tregs was corrected with CRISPR-Cas9-mediated homology-directed repair, restoring IL2RA expression. The resulting gcTregs demonstrated robust suppressive activity *in vitro*. Clinical-scale manufacturing from a patient with IL2RA deficiency showed efficient gene correction, restored IL2RA expression, and functional equivalence to healthy donor Tregs. This work establishes a Good Manufacturing Practice-compatible manufacturing process for personalized gcTreg therapies, potentially providing a safer treatment option for patients with IL2RA deficiency as well as a framework for treating other inborn errors of immunity.

INTRODUCTION

Regulatory T cells (Tregs) are a subset of CD4⁺ T cells that play a critical role in preventing autoimmunity and excessive inflammation. Tregs are characterized by constitutive surface expression of IL2RA (also called CD25), the alpha subunit of the high-affinity interleukin-2 (IL-2) receptor. Expression of IL2RA is frequently used in combination with CD4⁺CD127^{lo/-} to identify Treg populations.¹ Both IL2RA and IL-2 play important roles in the development of Tregs in the thymus; the regulation, proliferation, and maintenance of Tregs in peripheral tissues; and the transcriptional program required for Treg function.²

The importance of Tregs is exemplified by the early-onset and progressive autoimmune syndromes that result from genetic deficiencies affecting Treg development, maintenance, and function. These diseases, called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) or related IPEX-like syndromes, are characterized by widespread multi-organ autoimmunity leading to severe disability and death, often in early childhood. IPEX syndrome is caused by X-linked mutations in the transcription factor *FOXP3*, which is highly expressed in Tregs and required for their specification and stability.^{3,4} IPEX-like syndromes have a similar clinical presentation with pathogenic mutations in a variety of additional genes critical for Treg function. Several IPEX-like syndromes have been linked to genes involved in the IL-2 signaling pathway.⁵⁻⁸

We previously identified a family that included three siblings who carry compound heterozygous loss-of-function mutations in *IL2RA*,⁹ resulting in an IPEX-like syndrome. Exome sequencing in the trio of proband and parents revealed two causative mutations in the *IL2RA* gene. The mother has a single heterozygous mutation (c.530G>A) in exon 4 of *IL2RA*, resulting in a premature stop codon. The father has a single heterozygous mutation (c.800delA) in exon 8 of *IL2RA*, resulting in a frameshift mutation that generates a 95-amino acid run-on sequence. Importantly, it was found that while the affected individuals carrying compound heterozygous mutations display normal to increased numbers of FOXP3⁺CD4⁺CD127^{lo} Tregs, these cells exhibit minimal to absent surface expression of IL2RA and were unable to respond normally to exogenous IL-2

Received 2 June 2025; accepted 3 December 2025;
<https://doi.org/10.1016/j.ymthe.2025.12.004>

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stimulation as measured by downstream STAT5 phosphorylation.⁹ Additionally, these Treg cells were completely deficient in their capacity to inhibit effector T (Teff) cell proliferation *in vitro*. The oldest affected sibling is an adult female with autoimmune pancytopenia including Coombs positive hemolytic anemia, anti-neutrophil antibodies, and anti-platelet antibodies. Current treatment options are limited to long-term immunosuppression or allogeneic hematopoietic stem cell transplant (HSCT), which carries a significant risk of morbidity and mortality.¹⁰

While advances in CRISPR-Cas9 technology have enabled efficient and precise DNA modification in primary human T cells in a research setting, to date no subject has been dosed on a clinical trial for a Mendelian disease using this approach. Here, we have developed an approach for *ex vivo* correction of the c.530 allele in *IL2RA* using CRISPR-Cas9-stimulated homology-directed repair (HDR) to generate an autologous gene-corrected regulatory T cell (gcTreg) therapy.

We hypothesize that adoptive cell therapy with gcTregs may ameliorate a patient's autoimmune syndrome. This therapy could prevent or delay the need for HSCT. Here, we established a clinical-scale *ex vivo* manufacturing process to isolate, correct, purify, and expand gcTregs using Good Manufacturing Practice (GMP)-grade reagents and equipment. We optimized gene-editing parameters to maximize *IL2RA* correction in Tregs, identifying optimal concentrations of gene-correction reagents and timing of critical correction and expansion processes. Furthermore, we investigated the immunophenotype of the patient's cells to identify key differences in her Tregs compared to those from healthy individuals, guiding optimization of the manufacturing process. Finally, we demonstrated the feasibility and effectiveness of the proposed gcTreg manufacturing process through full clinical-scale manufacturing runs using source material from a healthy donor and from the affected individual. Post-correction, the healthy donor and affected individual gcTregs demonstrate identity markers, expansion, and function consistent with a bona-fide Treg population.

RESULTS

Development of a GMP-compatible process for *IL2RA* gene correction

Previous work at research scale has established methods for efficient targeted non-viral gene correction or gene insertion by electroporation of Cas9 ribonucleoproteins (RNPs) and HDR templates (HDRTs) in primary human T cells.^{11–13} However, these methods have not yet been optimized for clinical-scale gene correction of Tregs. We developed a GMP-compatible process to enrich and gene correct Tregs from a patient lacking expression of *IL2RA* due to compound heterozygous mutations in *IL2RA* genes (Figure 1A).

To optimize gene correction, we evaluated gene-editing efficiency on the MaxCyte GTx instrument. Bulk T cells were electroporated with an RNP that contained a guide RNA (gRNA) targeting the human *CD5* gene and a short HDRT encoding an in-frame hemagglutinin

(HA) tag to enable a flow cytometry-based measurement of HDR efficiency. We tested three different pulse codes on the MaxCyte GTx with varied energy settings (labeled low, med, high). Knockin (KI) efficiency was then evaluated in comparison to cells electroporated on the Lonza 4D Nucleofactor using a previously optimized pulse code (EH-115). Cells transfected with MaxCyte pulse codes performed comparably or better than the Lonza instrument, with KI efficiencies ranging from 5% to 20% ($n = 2$; Figure 1B). While increasing pulse code strength and HDRT concentration led to higher KI efficiencies, we also observed a proportional decrease in cell viability. We determined that electroporation of 200 pmol HDRT (final concentration of 3.85 μ M) using the medium energy “expanded T cell 4” pulse code was optimal for efficient gene correction in this system.

Using these optimized parameters, we next evaluated an RNP and two HDRTs with different length homology arms (127 and 172 nt) targeting correction of the c.530 mutation within the *IL2RA* locus. These HDRTs also encode a protospacer-adjacent motif (PAM) mutation to prevent re-cutting of the locus after productive repair by HDR. Concurrently, we evaluated the editing efficiency of a standard wild-type (WT) Cas9 and a high-fidelity SpyFi Cas9 variant (Aldevron). Both Cas9 formats demonstrated ~90% knockout (KO) of *IL2RA* in the absence of an HDRT consistent with disruption of exon 4 through insertions or deletions (indels) (Figure 1C). For all combinations of Cas9 species and HDRT length, the expression of *IL2RA* was efficiently restored in ~50%–70% of cells (Figure 1C). As all combinations performed comparably, we proceeded with the high-fidelity SpyFi Cas9 and the shorter 127-nt HDRT.

To identify potential off-target editing events with the chosen gRNA, we employed a combination of *in silico* nomination using the COSMID algorithm and *in vitro* nomination in primary human T cells using the GUIDE-seq (Genome-Wide Unbiased Identification of Double-Stranded Breaks Enabled by Sequencing) methodology.^{14–16} Allowing for up to 3 mismatches and 1 insertion or deletion, COSMID nominated 15 potential off-target sites. GUIDE-seq analysis performed using the representative electroporation protocol with primary human T cells from 2 healthy donors nominated 38 additional sites and 2 shared with the COSMID analysis (Figures 1D and 1E; Table S1). We next designed a pooled next-generation sequencing (NGS) amplicon panel for assessment of the combined 54 nominated sites using the rhAmpSeq methodology (1 on-target, 53 off-target).¹⁶ Primary human T cells were electroporated using the representative manufacturing process, and genomic DNA was then analyzed for indels at each nominated off-target site (Figure 1D). Indels at the on-target site were identified in 83.9% and 84.1% of NGS reads for donors 1 and 2, respectively, confirming efficient editing (Figure 1E), while none of the putative off-target sites demonstrated indels greater than a threshold of 0.1%. These data demonstrate that the chosen gRNA is highly efficient and specific for targeted gene correction at the *IL2RA* c.530 locus.

Characterization of patient tregs by high-dimensional CyTOF

Patients with IL2RA deficiency maintain a population of dysfunctional Tregs in the peripheral blood despite the absence of IL2RA.^{7,9,17,18} To better understand the phenotypic and functional differences of these cells, we evaluated regulatory and conventional CD4⁺ T cell (Tconv) populations from the patient by high-dimensional cytometry by time of flight (CyTOF). The patient had a substantially higher percentage of FOXP3⁺HELIOS⁺ Tregs in her peripheral blood in comparison with an unaffected sibling, as described for other families with IL2RA deficiency (8.66% vs. 2.3%; Figure 2A).¹⁸ HELIOS expression is a marker of Treg stability.¹⁹ These Tregs remained CD4⁺CD127^{lo} but completely lacked expression of IL2RA. We next evaluated the Treg and conventional CD4 populations for markers of T cell lineage and function. While there were minor differences in expression of conventional Treg lineage markers between patient and sibling cells (CD3, CD4, FOXP3, and HELIOS), naive CD45RA⁺CCR7⁺ populations were nearly absent from the patient, and her cells expressed significantly higher levels of functional markers consistent with a memory, activated, or “exhausted” phenotype, including CD45RO, TIGIT, PD-1, ICOS, CTLA4, and CXCR3 (Figures 2B and 2C). These differences were particularly evident in the Treg populations compared to non-Treg CD4s, showing an ~20- to 25-fold increase. Notably, the patient’s Tregs had significantly higher levels of T-bet (T-box transcription factor expressed in T cells) expression, suggesting a polarization toward a T helper 1 (Th1) Treg lineage, which may reflect chronic Th1-driven inflammation associated with the patient’s disease²⁰ (Figures 2B and S1). These data suggest the patient maintained an expanded Treg population in the peripheral blood, with notable phenotypic changes likely driven by the loss of IL2RA and/or her underlying immune syndrome.

Developing a clinical-scale manufacturing process for IL2RA gene correction

To restore expression of IL2RA in patient cells, we next developed and optimized a GMP-compatible manufacturing process for *ex vivo* gene correction of the c.530 IL2RA mutation in autologous Treg populations. We have previous experience isolating polyclonal Tregs for clinical manufacturing using droplet-based flow sorting.²¹ Droplet-based sorting produces a nearly pure population of CD4⁺/CD127^{lo}/IL2RA⁺ Tregs before *ex vivo* expansion, which reduces the risk of non-Treg outgrowth during culture.^{21,22} Due to the patient’s lack of IL2RA expression, it was not possible to initially isolate a pure Treg population using this approach.²³ We therefore started with a CD4⁺ bead-based enrichment of an apheresis product using a CliniMACS Prodigy, followed by two distinct sorting steps: Tregs are first enriched by sorting for CD4⁺/CD127^{lo} population before correction, followed by a second CD4⁺/CD127^{lo}/IL2RA⁺ sort after IL2RA correction to isolate a pure Treg population.

IL2RA is transiently upregulated and CD127 is transiently downregulated in Tconv following the activation steps that are required for efficient Treg expansion and gene correction. We therefore sought to determine the time point post-activation at which these

markers are restored to basal levels in Tconv populations, such that a pure population of IL2RA⁺CD127^{lo} gcTregs can be efficiently separated. Tregs and Tconv from a healthy donor were isolated and evaluated for IL2RA and CD127 expression for 12 days following activation (Figure 3A). As expected, IL2RA and CD127 levels were initially indistinguishable in Tconv and Treg populations following activation. We observed downregulation of IL2RA and upregulation of CD127 in Tconv cells beginning on day 9, with expression returning to resting levels by day 12 (Figure 3A). We therefore determined that day 12 post-activation was the ideal time point to allow for high-purity sorting of an IL2RA⁺CD127^{lo} Treg population.

To further maintain Treg purity in culture, we next evaluated the optimal concentration of IL-2 necessary for *ex vivo* Treg expansion. Due to their constitutive expression of IL2RA, Tregs should have a proliferative advantage over Teff cells at lower concentrations of IL-2, allowing us to selectively expand and maintain a Treg population following IL2RA correction. To identify the optimal concentration of IL-2, we evaluated expansion, purity, and editing efficiency in Tregs cultured in 0–5,000 IU/mL of IL-2 (Figure 3B). Gene-editing efficiency was similar at all IL-2 concentrations ≥10 IU/mL as measured by TIDER (Tracking of Insertions, Deletions and Recombination events) analysis.²⁴ The purity of FOXP3⁺ or FOXP3⁺HELIOS⁺ populations was maintained between 10 and 200 IU/mL IL-2 and began to decrease at concentrations ≥500 IU/mL, likely due to higher concentrations of IL-2 being sufficient for binding to the IL2RB and IL2RG receptor expressed on Tconv independent of IL2RA. Treg populations demonstrated the greatest fold expansion (~80-fold) at 200 IU/mL IL-2. Altogether, we determined that 200 IU/mL IL-2 was ideal to correct, expand, and maintain gcTregs in culture.

Finally, we evaluated the effectiveness of these expansion and sorting processes for isolation of a pure Treg population at clinical scale. Starting from a healthy donor leukapheresis collection, CD4⁺ T cells were enriched on the CliniMACS Prodigy and then sorted for the bottom 15% of CD127^{lo} cells, which maximized Treg purity in the starting culture (26.7% FOXP3⁺) (Figure 3C). Following 12 days of expansion, we sorted the top ~15% of IL2RA⁺ cells, increased Treg purity to 89.5% FOXP3⁺. These results demonstrate an effective strategy for enriching, expanding, and purifying gcTreg populations at clinical scale.

Clinical-scale IL2RA correction and manufacturing of gcTregs

We combined optimizations for gene correction, expansion, and sorting to develop a clinical-grade manufacturing process with GMP-compatible reagents, equipment, and processes (Figure 4A). Two full-scale runs were performed in an academic GMP facility at the University of California, San Francisco (UCSF) with source material obtained from a healthy donor and also from the proposed female patient with IPEX-like syndrome due to IL2RA deficiency. The patient’s cells were collected by apheresis under an institutional review board (IRB)-approved clinical protocol at Yale University. Approximately 5 × 10⁸ CD4⁺ T cells were enriched from each leukapheresis product using bead-based magnetic selection. These cells

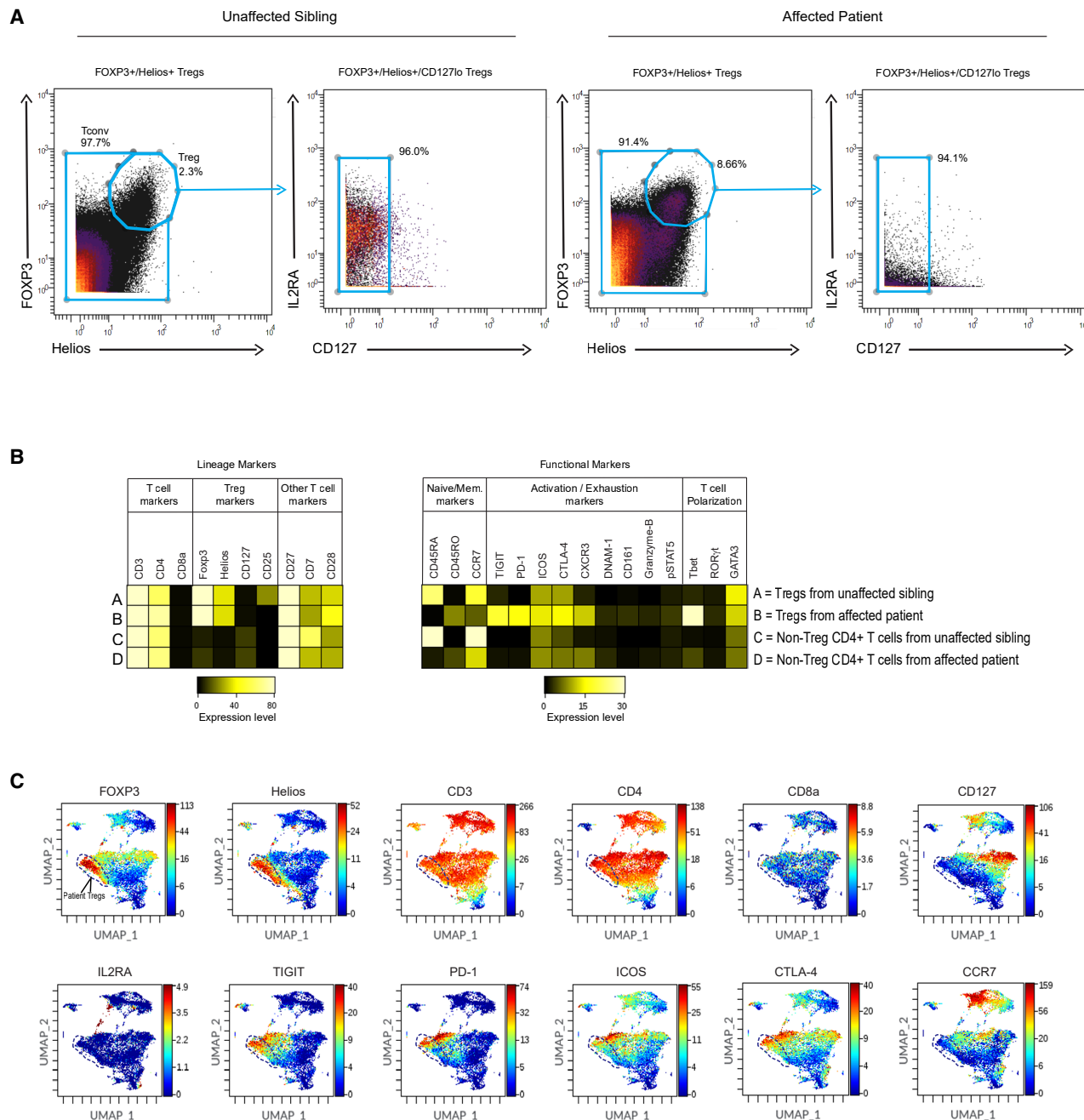


Figure 2. Analysis of functional and lineage marker expression in patient T cells by mass cytometry

(A) Flow cytometry plots showing Tregs (FOXP3⁺/HELIOS⁺) and conventional CD4⁺ T cells (Tconv) from an unaffected sibling (left) and the affected patient (right). Tregs were then further gated for low CD127 expression for subsequent analysis. (B) Heatmaps showing expression of lineage and functional markers in Tregs and Tconv from an unaffected sibling and the proposed patient. (C) UMAP dimensionality reduction of mass cytometry data for selected functional and lineage markers in patient Tregs (circled regions).

were then sorted, gene corrected, and sorted again into a pure population of gcTreg following the previously described protocol (Figure 1A).

On harvest day, gcTregs from both the healthy donor and patient were evaluated by flow cytometry. Both gcTregs were highly pure by FOXP3 expression (92%–95%) with no detectable CD8⁺ effector

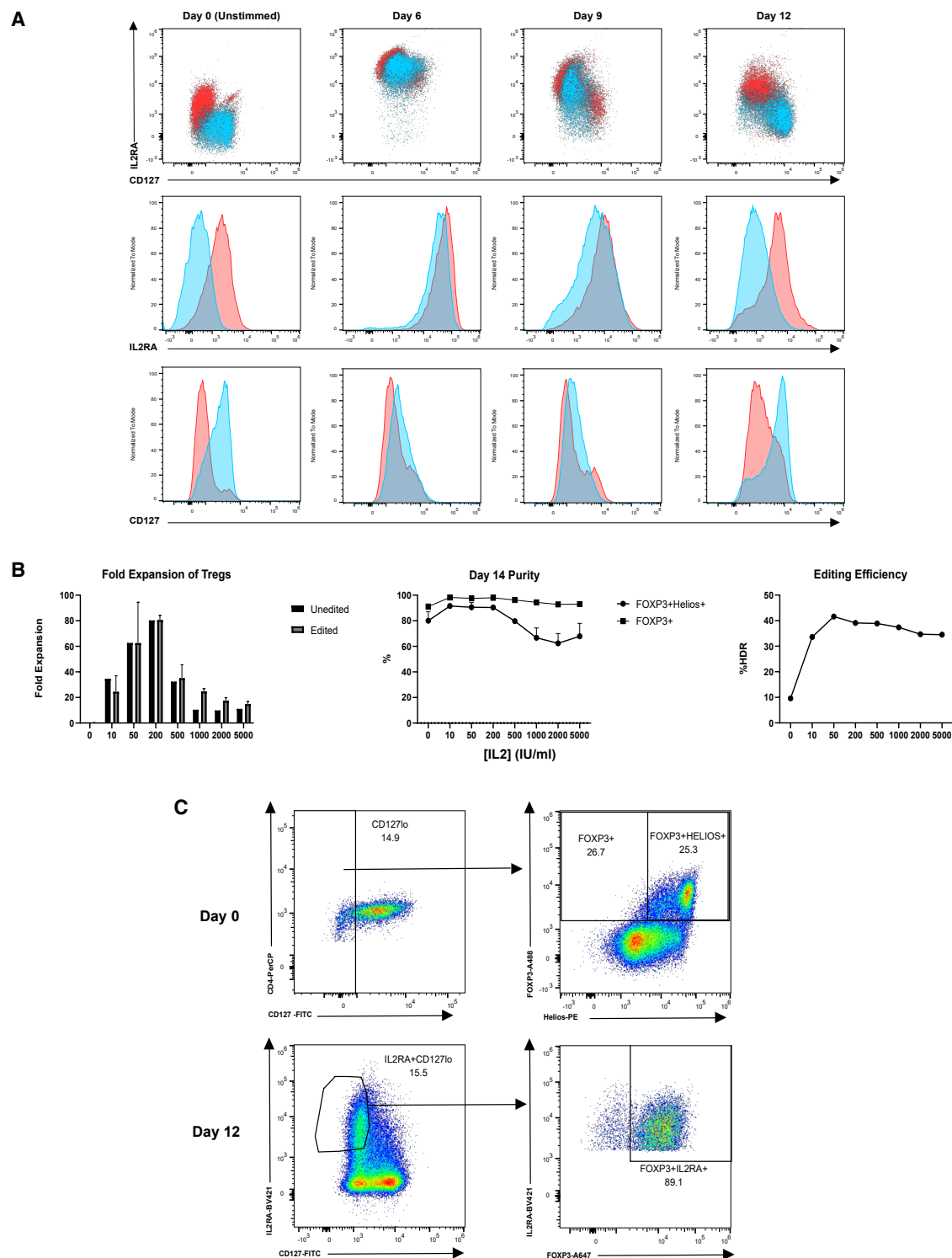
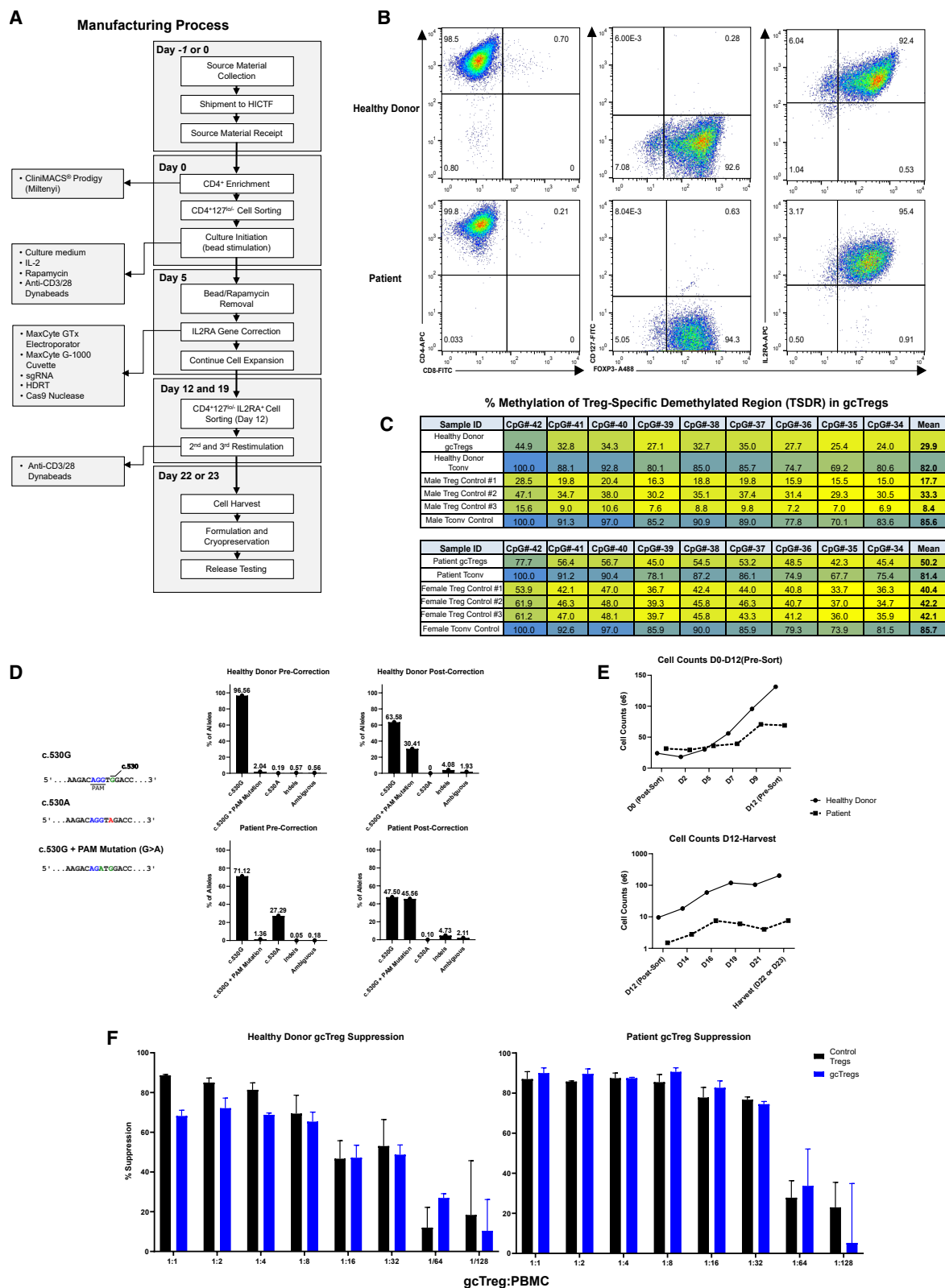


Figure 3. Optimization and evaluation of gcTreg manufacturing process

(A) Time course of IL2RA and CD127 expression in Tconv (blue) and Tregs (red). Histogram plots were normalized to the mode. Tconv and Tregs used in these experiments were isolated from the same donor. (B) Evaluation of Treg expansion (left), purity (center), and editing efficiency (right) at IL-2 concentrations ranging from 0 to 5,000 IU/mL. Fold expansion and purity were evaluated on day 14 of culture. Editing efficiency was measured by TIDER analysis (<http://shinyapps.datacurators.nl/tider/>). $n = 2$ independent healthy donors. (C) Demonstration of two-sort Treg isolation process. Sort gates were set (left) to maximize FOXP3 and IL2RA expression post-sort (right) on each respective sort day.



(legend on next page)

cell contamination (Figure 4B). Critically, IL2RA expression in patient gcTregs was similar to the healthy donor (95.4%), demonstrating successful correction and enrichment of IL2RA⁺ Tregs in the final product (Figure 4B). To further demonstrate Treg identity, bisulfite sequencing of the Treg-specific demethylated region (TSDR, CNS2) in the *FOXP3* gene was performed. This region is selectively demethylated in normal Tregs and is considered the gold standard marker of Treg identity.²⁵ When compared to sex-matched Treg controls (because *FOXP3* is an X-linked gene), we observed that gcTregs had a similar methylation pattern at both individual CpG sites and across the TSDR as a whole (Figure 4C). Uncorrected CD4⁺ cells remained highly methylated. In combination with high *FOXP3* expression, these data show that harvested gcTregs are comparable to normal Tregs in cell surface phenotype and epigenetic signature at the *FOXP3* locus.

In addition to surface IL2RA expression, we evaluated editing efficiency by targeted NGS at the c.530 *IL2RA* locus (Figure 4D). In the final drug product, we observed the normal c.530G sequence in ~94% of healthy donor gcTreg alleles and ~93% of patient gcTreg alleles, with the remainder demonstrating a mixture of indels (4%–5%) or ambiguous reads (~2%). The number of alleles also incorporating the PAM mutation introduced by the HDRT was 30.41% and 45.56%, respectively, suggesting a mixture of WT alleles and/or incomplete HDR outcomes. Most important, virtually no sequences contained the pathogenic c.530A mutation in patient gcTregs (0.10%), demonstrating highly efficient gene correction and enrichment of IL2RA-expressing gcTregs.

The final gcTreg yield is determined by the number of starting cells, expansion of Tregs pre- and post-correction, and the losses associated with the multiple sorting steps (Figure 4E). Healthy donor cells expanded ~5-fold and ~20-fold before and after the second sort, respectively. In contrast, the patient cells showed almost no expansion prior to gene correction and then, intriguingly, demonstrated improved rates of expansion following gene correction. This suggests an initial proliferative defect may be largely resolved by the restoration of IL2RA expression. Combined with substantial reductions during each sort to enhance Treg purity, this limited the number of gcTregs in the final drug product: 1.95×10^8 for healthy donor gcTregs and 7.6×10^6 for the patient gcTregs. Although neither the *in vivo* proliferative capacity of IL2RA⁺ gcTregs nor the total

number of gcTregs required for clinical efficacy has been determined, increasing the number of input cells or additional expansion of gcTregs post-correction may be required to further increase these numbers.

We evaluated the immunosuppressive function of gcTregs using an *in vitro* suppression assay established for the functional evaluation of polyclonal Tregs. The final gcTreg drug products from both the patient and healthy donor manufacturing runs were co-cultured with peripheral blood mononuclear cells (PBMCs) and assessed for their ability to suppress CD8⁺ T cell proliferation at varied gcTreg:PBMC ratios. Both gcTreg products demonstrated efficient suppression of CD8⁺ T cells and had equivalent potency when compared to previously manufactured polyclonal Treg products (Figure 4F). These results demonstrate that correction of IL2RA deficiency in patient Tregs restores their functional capacity to suppress conventional T cell proliferation.

Finally, we performed high-dimensional spectral flow cytometry followed by uniform manifold approximation and projection (UMAP) analysis of the gcTreg from the individual affected with *IL2RA* mutations and compared the expression of 21 cell-surface and intracellular markers to gcTreg from a healthy individual, expanded polyclonal Treg, and expanded Tconv (Figure 5A). The Treg samples are clearly separated from expanded Tconv on UMAP analysis, and the two gcTreg samples show very similar phenotypes (Figure 5B). The gcTreg from the affected individual have higher CD39 expression compared with the gcTreg from a healthy donor (Figure 5C). Otherwise, unsupervised clustering of the gcTreg samples shows very similar distribution of Treg subtypes and specific markers (Figures 5C and S5). The cells mostly have a central memory phenotype (CCR7⁺CD45RO⁺BCL-2^{hi}, TCF1⁺), and there is no evidence of excessive activation or terminal differentiation (KLRG1⁺, PD1^{lo}, LAG3^{lo}) (Figure S6). In conclusion, the gcTreg profile from the individual with *IL2RA* mutations is consistent with cells having a Treg identity and normal IL2RA expression.

Here, we demonstrate a GMP-compatible manufacturing process to generate a high-purity gene-corrected IL2RA⁺FOXP3⁺ Treg therapy for patients with IL2RA deficiency. Full-scale healthy donor and patient gcTreg drug products demonstrated high-efficiency gene correction, immunophenotypic and epigenetic markers consistent

Figure 4. Clinical-scale manufacturing of gcTregs

(A) Proposed clinical-scale manufacturing process for gcTregs with key manufacturing days, processes, and reagents (see methods). The second and third re-stimulations occurred on days 12 (post-sort) and 19, respectively. (B) Flow cytometry plots of harvested gcTregs manufactured at clinical scale stained for CD4/CD8 (left) and FOXP3/CD127/IL2RA (right). (C) DNA pyrosequencing for methylation of individual CpG sites in the *FOXP3* TSDR of harvested gcTregs manufactured from a healthy donor (top) and the proposed patient (bottom). Uncorrected CD4⁺ cells from the respective donors were included as uncorrected controls. Because *FOXP3* is an X-linked gene, gender-matched polyclonal Treg and effector T cell (Teff) controls were included. (blue = more methylated, yellow = less methylated). (D) Expected pre- and post-correction sequences (left) and gene-editing outcomes in manufactured gcTregs (right) by NGS. Pre-correction gDNA was isolated from naive CD4⁺ T cells from each respective donor, while post-correction gDNA was isolated from gcTregs at harvest. Ambiguous reads refer to alleles that were unable to be aligned to the donor reference or post-correction amplicons. (E) Cell counts of gcTregs from the healthy donor and patient over the course of the manufacturing process. Pre-sort counts refer to cell counts immediately prior to sorting, while post-sort counts were obtained immediately following sorting. (F) *In vitro* suppression assay of gcTregs at various ratios of gcTregs (blue) to PBMCs compared with previously manufactured and validated polyclonal Tregs (black). Suppression was evaluated by inhibition of CD8⁺ T cell proliferation after 4 days in culture compared to cultures without Tregs. *n* = 3 replicates for healthy donor gcTregs, *n* = 2 replicates for patient gcTregs.

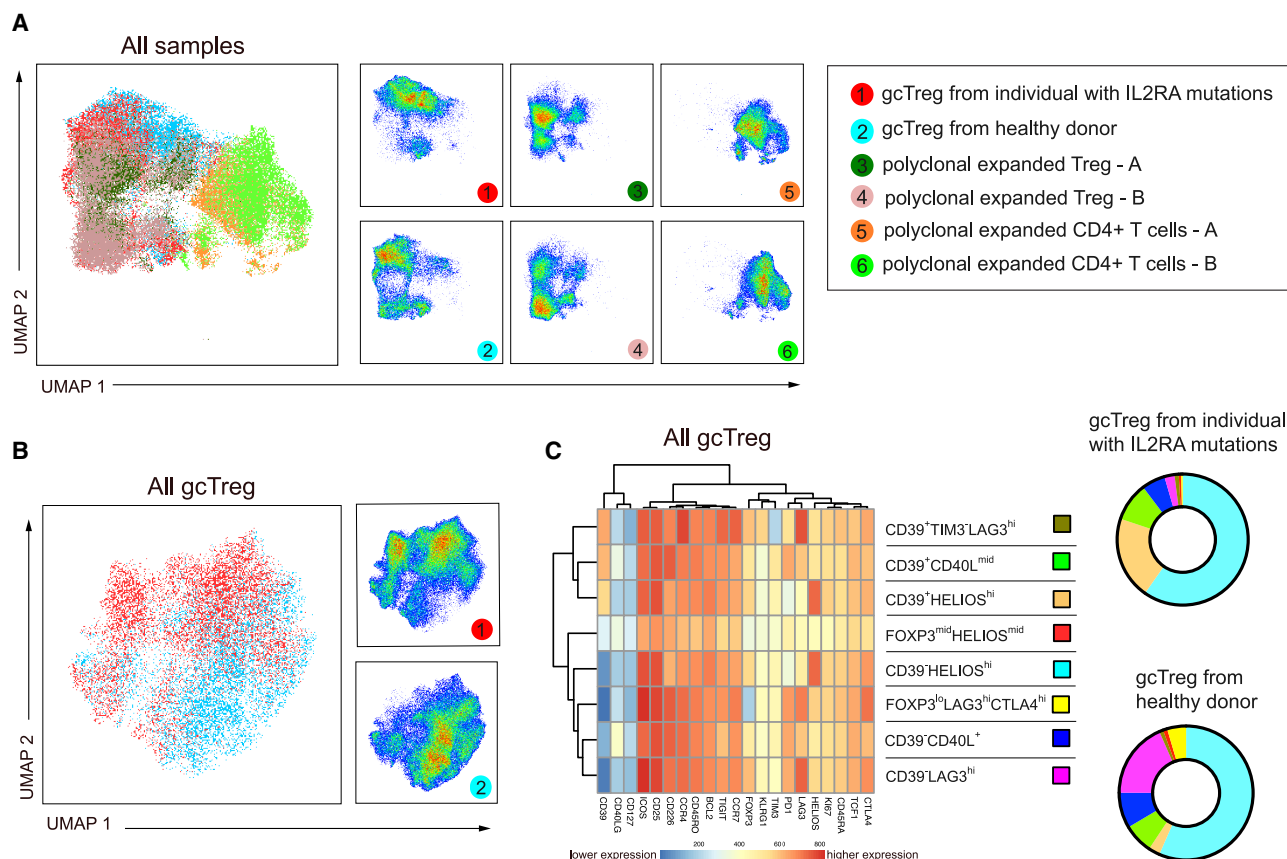


Figure 5. High-dimensional spectral flow cytometry analysis of gcTreg products shows limited heterogeneity

(A) Two samples each of gcTreg, polyclonal expanded Treg, and polyclonal expanded CD4⁺ effector cells were analyzed by high-dimensional spectral flow cytometry and UMAP analysis. Left, combined analysis of all samples. Right, individual panels for each sample are color-coded according to the boxed legend. (B) UMAP analysis of the two gcTreg samples analyzed together (left) and separately (right). (C) Left, unsupervised clustering of subsets among two gcTreg samples shows limited heterogeneity among the gcTreg both within and between samples. Right, each of the subsets is color-coded and labeled according to its relevant phenotypes. The proportion of each subset out of the total population is indicated for each gcTreg sample, according to the legend.

with Treg identity, and restoration of suppressive function and proliferative defects, and they met all established criteria for product release based on prior polyclonal Treg therapies and US Food and Drug Administration (FDA) guidance (Table 1). This approach could be applied to generate an autologous therapy for patients with loss-of-function c.530A mutation in the *IL2RA* gene or adapted for alternative disease-causing mutations affecting Treg function.

DISCUSSION

IL2RA deficiency is a rare immune disorder caused by loss-of-function mutations in the *IL2RA* gene. IL2RA plays a critical role in Treg function due to its reliance on IL-2 and role in balancing immune cell activation, in part as a sink for the IL-2 reservoir. Patients with IL2RA deficiency demonstrate elevation of serum IL-2 among other inflammatory cytokines and an expansion of CD8⁺ T cell populations with extensive tissue infiltration, lymphadenopathy, and splenomegaly.^{7,9,17,18} Symptoms are similar to IPEX or other IPEX-like syndromes affecting Treg function, with case reports

demonstrating a variety of autoimmune manifestations that range from mild to life-threatening, including dermatologic, hematologic, gastrointestinal, endocrine, and lung disease. A subset of patients also shows increased susceptibility to common viral, bacterial, and fungal infections. Current treatment options are limited to long-term immunosuppression or allogeneic HSCT, a therapy associated with significant morbidity and mortality.¹⁰

Here, we develop an autologous gcTreg cellular therapy for a single patient with autoimmune hemolytic anemia, neutropenia, and thrombocytopenia that has required multiple lines of therapy, including varied immunosuppressive regimens, intermittent transfusion, and regular granulocyte-colony-stimulating factor administration. Using CRISPR-based genome editing, we demonstrate high-efficiency correction of the pathogenic c.530A *IL2RA* mutation and isolation of a nearly pure population of IL2RA⁺FOXP3⁺ gcTregs at clinical scale. The corrected patient cells demonstrate an immunophenotype, epigenetic identity, and immunosuppressive function

Table 1. Release criteria for gcTreg manufacturing

Test	Release criteria	Method	Healthy donor gcTregs	Patient gcTregs
Percentage FOXP3 ⁺	≥70.0	flow cytometry	92.9	96.3
Percentage IL2RA ⁺	≥80.0	flow cytometry	98.4	98.6
Percentage CD4 ⁺ CD8 [−]	≥90.0	flow cytometry	98.5	99.8
Percentage CD4 [−] CD8 ⁺	≤5.00	flow cytometry	0.00	0.00
Recovery (post-thaw percentage)	≥50.0	automated cell counter or hemacytometer	66.0	63.3
Residual beads	<100 beads per 3 × 10 ⁶ cells	flow cytometry	2.54 beads/3 × 10 ⁶ cells	60.0 beads/3 × 10 ⁶ cells
Cell viability (post-thaw percentage)	≥70.0	automated cell counter or hemacytometer	75.8	85.9
Endotoxin	≤3.50 EU/mL	limulus amebocyte lysate	≤1.00 EU/mL	≤1.00 EU/mL
Mycoplasma	negative	MycoAlert	negative	negative
Anaerobic culture	no growth at 14 days	BacT/ALERT	no growth at 14 days	no growth at 14 days
Aerobic culture	no growth at 14 days	BacT/ALERT	no growth at 14 days	no growth at 14 days
Fungal culture	no growth at 14 days	BacT/ALERT	no growth at 14 days	no growth at 14 days
Visual inspection	no cell aggregates observed by visual inspection	visual inspection	no cell aggregates observed by visual inspection	no cell aggregates observed by visual inspection

All proposed release assays for gcTreg manufacturing with respective criteria for release and method of analysis. Assay results for gcTregs manufactured from the healthy donor and patient are included and meet all release criteria. Release criteria established based on previously manufactured polyclonal Treg products and established US Food and Drug Administration guidelines.

consistent with a true Treg population. Compared with a recent study demonstrating CRISPR-KO in Tregs, gcTregs have a significantly higher purity but a lower overall yield.²⁶ This represents one of the first clinical-scale demonstrations of targeted CRISPR gene editing to correct an inborn error of immunity (IEI) and confirms that *IL2RA* correction can restore endogenous Treg function in patients with *IL2RA* deficiency.

This gcTreg therapy could be administered as an autologous treatment for this patient and two other siblings in the family who have developed autoimmune manifestations of less severity. We observed no off-targets for the chosen gRNA, no cytogenetic abnormalities in the gcTreg drug products (Supplemental text [Cytogenetic analysis of gcTregs manufactured from healthy donor cells], [Figures S2 and S3](#)), and polyclonal Treg therapies have demonstrated safety in more than a decade of clinical trials with infusions of up to 2.6 × 10⁹ Tregs being tolerated with minimal adverse effects.^{21,22,27,28} Product heterogeneity is an important consideration for clinical products. Criteria for Treg product identity is a complex unresolved issue, and it is likely that different criteria would be needed for different Treg products and disease indications. High-dimensional spectral flow cytometry analysis of gcTreg products shows limited heterogeneity and a normal Treg central memory phenotype ([Figures 5, S5, and S6](#)). Ultimately, the issue of heterogeneity can only be addressed in the context of a carefully designed clinical trial and mechanistic studies of patient samples collected from the trial.

Mouse models of *IL2RA* deficiency exhibit an aggressive autoimmune syndrome similar to patients who are completely rescued by

the infusion of 1 × 10⁶ *IL2RA*⁺ Tregs within 4 weeks of treatment.^{3,29,30} A consensus conference held between clinical specialists at UCSF and Yale University concluded that gcTreg treatment was likely to be safe, with potential clinical benefit and favorable risk-benefit in comparison to alternative treatment options such as allogeneic HSCT. However, final gcTreg yields were limited by losses at each sorting step and initial proliferative defects in patient cells that appear to be rescued by *IL2RA* correction. Patients with *IL2RA* deficiency demonstrate significant elevation of serum IL-2, and gcTregs may continue to expand *in vivo* to fill this niche.¹⁸ However, the ultimate therapeutic dose, *in vivo* proliferative capacity, and long-term persistence of gcTregs are unknown. In initial conversations, the FDA has accepted a proposed initial dose range of between 5 × 10⁶ and 1 × 10⁹ gcTreg cells for the phase 1 trial. If a higher dose or multiple infusions were required, then the current protocol could be modified to increase the amount of starting material and/or include additional expansion post-*IL2RA* correction. We plan to conduct *in vivo* studies in NSG models to evaluate the suppressive activity and persistence of gcTregs, which will be included in the data package for a future Investigational New Drug (IND) submission. *In vitro* culture of gcTreg from the affected individual shows that, as expected, the gcTregs require IL-2 for survival ([Figure S4](#)). The affected individual has persistently elevated serum IL-2 levels, indicating the likely presence of a supportive niche for durable gcTreg engraftment ([Table S7](#)).

As a potential alternative approach, HSC-based gene correction could be considered. A recent report demonstrated CRISPR-based gene correction in HSCs for the treatment of sickle cell disease

(nula-cel).³¹ However, the first patient treated experienced prolonged cytopenias, leading to discontinuation of clinical development by the original sponsor. A similar approach may be explored for *IL2RA* correction in future studies. In this study, we chose a more conservative Treg-based correction that avoids the risks of autologous gene-corrected HSC transplantation.

This targeted gene correction strategy could be easily extended to other families with *IL2RA* deficiency and numerous other IEs affecting Treg or conventional T cell function. Despite technical feasibility, several logistical challenges prevent such widespread application under the current manufacturing and regulatory frameworks.³² The cost and lead time for the production of critical genome-editing reagents is a significant barrier, with custom GMP-grade reagents such as single-guide RNA (sgRNA) and HDRT requiring ~12 months for production and exceeding costs of \$600,000 for early-phase clinical trials at minimum scale. As this is the only family identified with the c.530 mutation, extending this approach to other mutations would require designing new gRNAs and HDRTs, incurring comparable production costs for each new patient. Compounding these issues is that any change in reagents or engineering strategy is treated as an entirely new drug product under the current regulatory framework. This requires submission of new regulatory filings and repeating extensive pre-clinical studies to demonstrate product safety and efficacy, typically including Good Laboratory Practice-compliant animal models. Recent FDA guidance allowing Biologics License Application (BLA) holders to apply for platform designation, leveraging prior Chemistry, Manufacturing, and Controls (CMC) and pre-clinical studies, offers a pathway to streamline this process.³³ Extending this platform-based approach to IND holders without a BLA could enable broader treatment of rare diseases. By allowing the reuse of prior CMC and pre-clinical studies within a given platform and focusing on risks associated with specific changes (e.g., off-target analysis and efficacy studies for a new gRNA), this framework could significantly reduce regulatory burden and foster wider accessibility of these advanced therapies.

Alternative strategies, such as inserting the corrected cDNA near the N terminus of the endogenous gene or into a safe harbor locus using an exogenous promoter, could offer more universal solutions independent of specific mutations.¹¹ However, these approaches can be limited by gene size and challenges in achieving appropriate expression levels or dynamic regulation. Emerging technologies such as base editing and prime editing also provide promising alternatives for targeted gene correction but currently face significant target limitations, restricting their application to a subset of mutations or cell types. Given the diversity of genetic defects, spanning more than 500 IEs, each with numerous unique mutations across the gene body, it is likely that a variety of correction methods will be needed.³⁴ The scalability of all such approaches to address ultra-rare immune disorders hinges on reducing the cost of critical genome editing reagents and addressing current regulatory challenges.

In summary, this study demonstrates the successful correction of a pathogenic *IL2RA* mutation in cells from a patient with a severe IPEX-like syndrome using CRISPR-Cas9-based gene editing, establishing a GMP-compatible manufacturing process to generate autologous gene-corrected T cells. This work lays a foundation for the use of precision gene editing in the treatment of ultra-rare immune disorders and represents a pivotal step toward accessible, personalized cellular therapies. Addressing challenges related to the cost and availability of critical starting materials, as well as streamlining regulatory frameworks, will be essential to enable the widespread implementation of these approaches and scale this therapy to broader patient populations.

MATERIALS AND METHODS

Isolation and culture of primary human T cells

For all non-GMP experiments, fresh primary human T cells were isolated from either whole blood (Vitalant) or a leukapheresis product (STEMCELL, StemExpress) collected from volunteer donors under IRB-approved protocols. When using whole blood, mononuclear cells were first separated by density gradient centrifugation using Lymphoprep (STEMCELL, catalog no. 07811). Bulk T cells were then isolated using EasySep Human T Cell Isolation Kits (STEMCELL, catalog no. 17951), while Tregs were isolated using the EasySep Human CD4⁺/CD127^{low}/CD25⁺ Regulatory T Cell Isolation Kit (STEMCELL, catalog no. 18063) according to supplier protocols. Isolated cells were then activated with either CD3/CD28 CTS Dynabeads (Gibco, catalog no. 40203D) for bulk T cells or CTS Dynabeads Treg Xpander (Gibco, catalog no. 46000D) for Tregs at a bead:cell ratio of 1:1 in addition to 5 ng/mL recombinant human IL-7 (R&D Systems, catalog no. 207-IL-200), 5 ng/mL recombinant human IL-15 (R&D Systems, catalog no. 207-ILB-25), and 500 IU/mL IL-2 (Proleukin, Clinigen, National Drug Code 76310-022-01) in X-Vivo 15 Culture Media (Lonza, catalog no. 04-418Q) supplemented with 2-mercaptoethanol (Gibco, catalog no. 21985023) to a final concentration of 50 μ M and *N*-acetyl-L-cysteine (Sigma-Aldrich, catalog no. A9165-100G) to a final concentration of 10 mM. Cells were cultured and maintained at $\sim 1 \times 10^6$ cells/mL at 37°C and 5% CO₂. Media and IL-2 were refreshed every 2–3 days.

For the healthy donor clinical-scale manufacturing run, a fresh leukapheresis product was collected by StemExpress, adhering to GMP-compliant processes from an IRB-approved and consented healthy donor at an FDA-registered collection center. For the patient cell manufacturing run, a leukapheresis product was collected by Yale-New Haven Medical Center's Apheresis Unit from the proposed patient following consent and following an IRB-approved protocol. It was then shipped to the UCSF Human Islet and Cellular Transplantation Facility (HICTF) in a temperature-controlled container maintained at 4°C and processed immediately upon receipt.

The leukapheresis products were enriched for CD4⁺ T cells using the CliniMACS Prodigy (Miltenyi) and CliniMACS GMP CD4 Microbeads (Miltenyi, catalog no. 170-076-702). Enriched CD4⁺ T cells were then sorted for CD127^{lo} cells by fluorescence-activated cell sorting

(FACS) using GMP-grade monoclonal antibodies for CD4-PerCP (Miltenyi, catalog no. 170-076-506) and CD127-fluorescein isothiocyanate (FITC) (Miltenyi, catalog no. 176-076-512). Isolated cells were then activated with CTS Dynabeads Treg Xpander at a bead:cell ratio of 1:1 in X-VIVO 15 Culture media supplemented with 10% Human Male AB Serum (Access Biologicals, catalog no. 516-100-HI GI), 500 IU/mL IL-2, and 100 nM rapamycin (Miltenyi, catalog no. 170-076-308). Cells were cultured at a density of 5×10^5 cells/cm² at 37°C and 5% CO₂. Media, IL-2, and rapamycin were refreshed 2 days after activation. Dynabeads were removed by magnetic separation on day 5 prior to electroporation. Following IL2RA correction, gcTregs were cultured for an additional 7 days in culture media with 200 IU/mL IL-2 only, refreshed every 2–3 days. On day 12, gcTregs were sorted by FACS using the above CD4 and CD127 monoclonal antibodies with the addition of a GMP monoclonal antibody targeting CD25-BV421 (Miltenyi, catalog no. 170-076-503). Sorted CD25⁺ gcTregs were then reactivated with dynabeads and cultured as described above until harvest.

RNP preparation

For non-GMP experiments, RNPs were prepared by complexing gRNA to Cas9. gRNA was synthesized either by complexing synthetic CRISPR RNA (crRNA, Dharmacon Horizon) and transactivating crRNA (tracrRNA, Dharmacon Horizon) or as a chemically synthesized sgRNA (Synthego). All RNA was prepared lyophilized, resuspended in IDT Nuclease-Free Duplex Buffer (Integrated DNA Technologies, catalog no. 11-05-01-12) at 160 μM and stored in aliquots at –80°C to minimize freeze-thaw cycles.

To prepare RNPs for Lonza experiments, aliquots of crRNA and tracrRNA were thawed, mixed 1:1 v/v, and annealed at 37°C for 30 min to form an 80-μM gRNA solution. We combined 40 μM Cas9-NLS (Berkeley QB3 MacroLab) with the gRNA solution to generate a complexed RNP solution at a 2:1 molar ratio of gRNA-Cas9. For MaxCyte experiments RNPs were prepared by complexing aliquots of 160 μM sgRNA with a 62.5-μM *spCas9* or *SpyFi* Cas9 (Aldevron, catalog nos. 9212-0.25MG, 9214-0.25MG) at a 1.56:1 v/v ratio to generate a complexed RNP solution at a 4:1 molar ratio of sgRNA-Cas9. RNPs were then briefly mixed by pipette and incubated at 37°C for 15–30 min.

For clinical-scale experiments, the MaxCyte process described above was used, except that all RNP components were chemically synthesized at GMP or GMP-like quality and sgRNA was prepared fresh from a pre-aliquoted lyophilized stock before each use.

Non-viral CRISPR gene editing

Except for clinical-scale manufacturing runs, activated cells were debeaded, counted on day 2 of culture, and electroporated either using the Lonza 4D 96-well electroporation system or the MaxCyte ExPERT GTx GMP Electroporation System. Immediately before electroporation, cells were centrifuged at $90 \times g$ for 10 min and then resuspended at $2.5\text{--}5.0 \times 10^7$ cells/mL in Lonza P3 electroporation buffer or at $\sim 1\text{--}2 \times 10^8$ cells/mL in MaxCyte electroporation

buffer. HDRT and RNP formulations were mixed and incubated for at least 5 min, combined with cells, and transferred either into the Lonza 96-well electroporation shuttle or into a MaxCyte OC-100 electroporation cuvette to a final volume of 25 or 52 μL, respectively.

For Lonza experiments, cells were electroporated using pulse code EH-115. Following electroporation, cells were rescued with 90 μL prewarmed culture media and incubated for at least 30 min. For non-clinical MaxCyte electroporations, pulse code “expanded T cell 4” was used for all experiments unless otherwise noted. Following electroporation, cells were rescued with 40 μL prewarmed culture media and incubated for at least 30 min. Electroporated cells were then transferred to culture plates/flasks at a concentration of $\sim 1 \times 10^6$ cells/mL with culture media and cytokines as described above.

For clinical-scale electroporations, activated cells were debeaded and counted on day 5 of culture and electroporated using the MaxCyte ExPERT GTx GMP Electroporation System. Cells were resuspended and electroporated following the same MaxCyte process described above, with the following changes: (1) an OC-1000 electroporation cuvette was used instead of the OC-100 to accommodate the increased number of cells and (2) the final electroporation volume was increased to 600 μL/cuvette per the manufacturer’s recommendation. All volumes of gene-editing reagents were scaled accordingly to account for the change in electroporation volume. Cells were then rescued with 400 μL prewarmed culture media and incubated for at least 30 min. They were transferred to culture flasks at a density of 5×10^5 cells/cm² with culture media and cytokines as described previously.

Flow cytometry

Flow cytometry was performed on an Attune NxT flow cytometer with a 96-well autosampler (Thermo Fisher Scientific), with the exception of the *in vitro* suppression assay described below. Cells were resuspended in FACS buffer (2% fetal calf serum [FCS] in PBS) and stained with either GhostDye Red 780 (Tonbo) or Fixable Viability Dye eFluor 780 (Invitrogen) and indicated cell-surface and intracellular markers (Table S4). For intracellular staining, cells were stained for surface markers and fixed and permeabilized using the True-Nuclear Transcription Factor Buffer Set (BioLegend). Events were recorded from an equivalent fixed volume for all samples to obtain comparable live cell counts between conditions. Analysis was done using FlowJo versions 10.8.1 and 10.9.

CytoF

Sample preparation and data collection for CyTOF analysis were performed as previously described.³⁵ In brief, mass cytometry was performed on the Helios mass cytometer (Fluidigm). Reagents and tagged antibodies are listed in Tables S2 and S3. Debarcoded FCS files were uploaded to Cytobank for data analysis. Within the CD4 T cell gate, Tregs were gated sequentially as Foxp3⁺ Helios⁺ and

CD127^{lo}. Heatmap illustrations and UMAP plots were constructed in Cytobank.

Spectral flow cytometry

Spectral flow cytometry was performed on a 5-laser Cytex Aurora. 1×10^6 cells from each sample (gcTreg, polyclonal regulatory T cell [polyTreg], or previously expanded Tconv) were thawed in FACS buffer and stained with Zombie UV Fixable Viability Dye (BioLegend) and then with the indicated cell-surface and intracellular markers (Table S7). Cells were fixed and permeabilized using the Foxp3 Transcription Factor Buffer Kit (Invitrogen) prior to intracellular staining. Events were recorded from an equivalent fixed volume for all samples. Analysis was completed using FlowJo version 10.10.0. Samples were gated on forward scatter/side scatter, single cells, viability, and CD4⁺. Then, an equal number of cells was randomly subsetted from each sample, tagged with SampleID, and concatenated, for a total of 2×10^5 cells in the concatenated sample. UMAP was generated using the UMAP-R plugin, and unsupervised hierarchical clustering was performed using the FlowSOM plugin³⁶ to define eight clusters. Analysis was performed on all six samples (gcTreg from an individual with IL2RA mutations, healthy donor gcTreg, *ex vivo* expanded polyTreg, and expanded CD4⁺ Tconv) combined or on only the two gcTreg samples combined.

NGS

Approximately 1×10^6 cells per condition were used to collect genomic DNA using the QIAmp DNA Mini Kit (Qiagen). We used 50 ng genomic DNA as a template in a 100- μ L PCR reaction using KAPA 2 $\times$ HiFi Master Mix (Roche) to generate a 215-bp amplicon with adapter sequences. A 2 $\times$ solid-phase reversible immobilization (SPRI) purification was performed and the resulting amplicon was normalized to 20 ng/ μ L. Samples were then sent to the Innovative Genomics Institute (IGI) NGS Core, where P5 and P7 Illumina sequencing adapters were annealed by PCR and purified by 0.8 $\times$ SPRI. Amplicons were sequenced using the iSeq100 Sequencing System (Illumina) and 2 $\times$ 150-bp reads mode according to manufacturer protocols. Sequencing data were processed using CRISPResso2, with paired-end reads referencing either the WT or mutated IL2RA c.530 sequence to quantify editing outcomes.

In vitro suppression assay

gcTregs were co-cultured with effector PBMCs at different ratios to evaluate suppressive activity. Frozen PBMCs were thawed and labeled with CellTrace Violet (CTV, Invitrogen, catalog no. C34557) by diluting 1 μ L of a 5-mM stock solution in 4 mL of warm Dulbecco's PBS (1:4,000). We added 1.5 mL of the working solution to 6×10^6 PBMCs and incubated for 20 min in a 37°C water bath. After incubation 13.5 mL of warm cRPMI (RPMI 1640 medium, 1 mM non-essential amino acids, 1 $\times$ sodium pyruvate, 5% human serum, 100 μ g/mL penicillin/streptomycin) was added and incubated for 5 min at room temperature. Cells were centrifuged at $470 \times g$ for 5 min, and the supernatant was removed. PBMCs were then resuspended to a final concentration of 1.5×10^6 cells/mL. gcTregs and control Tregs were thawed and then resuspended

to a final concentration of 1.5×10^6 cells/mL in cRPMI. We plated 50 μ L of either gcTregs or control Tregs (7.5×10^4 cells) duplicate or triplicate, depending on the availability of cells. A 1:1 serial dilution was performed until the lowest dilution of 1:128 was achieved. We then added 50 μ L of effector PBMCs to all wells along with 50 μ L cRPMI containing 5 μ g/mL anti-CD3 (BD, catalog no. 300331) and 2.5 μ g/mL anti-CD28 (BD, catalog no. 302914) monoclonal antibodies for cell stimulation. All wells and controls were brought to a final volume of 250 μ L with cRPMI and cultured for 72–96 h at 37°C and 5% CO₂.

Suppression of CD8⁺ effectors was then evaluated by flow cytometry. Briefly, cells were stained with CD4 APC (BD, catalog no. 340443), CD8 FITC (BD, catalog no. 347313), and 7AAD viability dye (BD, catalog no. 559925). Absolute Count Standard Beads (Bangs Laboratories, catalog no. 580) were added to each well and run at the same time. Live CD8⁺ cells were gated (lymphocytes > 7AAD[−] > CD8⁺) and evaluated for CTV expression to obtain counts of the proliferating CD8⁺ population. To obtain the absolute count of CD8⁺ cells, the following formula was used: (CD8⁺ count $\times$ input bead number)/beads count. Absolute values were averaged between all like replicates. To calculate the percentage of suppression, the following formula was used: $(100 - (\text{absolute CD8 count of dilution/absolute CD8 count of PBMC only})) \times 100$. Data were collected using the BD LSRFortessa with the HTS 96-well sampler. All analysis was performed using FlowJo, and plots were prepared using GraphPad Prism.

Off-target analysis and validation

To evaluate off-target editing by the RNP, we performed GUIDE-seq¹⁴ in primary T cells (two donors) and HEK-Cas9 cells (three technical replicates), as described previously.¹⁶ For *in silico* off-target prediction, we used the COSMID tool (<https://crispr.bme.gatech.edu/>), permitting up to two mismatches, one insertion, and one deletion. The hg38 reference genome was used for the analysis.

To validate hits identified by GUIDE-seq and COSMID, primary human Tregs were isolated from a leukapheresis product collected from two separate healthy donors. Tregs were activated and edited as described previously with or without the IL2RA RNP. Edited cells were then cultured for 2 additional days in culture media containing 1,000 IU/mL IL-2. IL2RA KO was confirmed by flow cytometry prior to gDNA isolation using the QIAamp DNA Mini Kit (Qiagen). Isolated gDNA was then used for rhAmpSeq analysis.

The panel for the rhAmpSeq assay was designed using the IDT rhAmpSeq designer tool (<https://www.idtdna.com/pages/tools/rhampseq-design-tool>). The panel included off-target sites identified by GUIDE-seq in T cells, predicted by COSMID, or present in the intersection of these datasets with HEK-Cas9 GUIDE-seq results (see Table S1). On-target and off-target CRISPR editing activity was then assessed through rhAmpSeq multiplex PCR following the protocol outlined previously.¹⁶ Sequencing of the rhAmpSeq libraries was conducted in a 2 $\times$ 150-bp paired-end format using

the Illumina MiSeq sequencer. Editing activity was quantified using CRISPECTOR (version 1.0.7) with default parameters.³⁷

DATA AND CODE AVAILABILITY

Raw data for sequencing, flow cytometry, and off-target analysis experiments will be made available upon reasonable request.

ACKNOWLEDGMENTS

We thank Dr. Jennifer Doudna for helpful discussions. Funding was provided by the IGI, the UCSF Department of Laboratory Medicine, the UCSF Diabetes Center, the Sandler Program for Breakthrough Biomedical Research at UCSF, and the UCSF Living Therapeutics Initiative. We acknowledge the UCSF Parnassus Flow Cytometry Core (PFC RRID: SCR_018206) supported in part by NIH P30 DK063720 and by the NIH S10 Instrumentation Grant (nos. S10 1S10OD021822-01, S10 1S10OD018040-01, and S10 1S10OD026940-01). Graphical abstract and schematic figures were created in BioRender.

AUTHOR CONTRIBUTIONS

A.S.H., B.R.S., and J.H.E. designed the study. A.S.H. and B.R.S. performed optimization experiments for non-viral Treg gene correction. N. Kalter, M.R., and A. Hendel designed and performed off-target analysis and validation experiments. W.L., B.R.S., S.P., and M.S. performed mass cytometry experiments and data analysis. A.S.H. performed optimization experiments for gcTreg manufacturing. A.S.H., L.A.A., B.L., and V.N., performed clinical-scale manufacturing of gcTregs. W.L. and S.P. performed *in vitro* suppression assays and flow cytometry on manufactured gcTregs. N. Krishnappa, A.S.H., and B.R.S. performed and analyzed NGS studies. L.A.A., B.L., W.L., S.P., and A.S.H. performed the release assays. Z.Q., J.S., and J.Y. performed the cytogenetic analysis. A.V. performed the high-dimensional flow cytometry analysis of the gcTreg product. A.S.H., B.R.S., and J.H.E. wrote the manuscript, with input from all authors.

DECLARATION OF INTERESTS

A.M. is a cofounder of Site Tx, Arsenal Biosciences, and Survey Genomics; serves on the boards of directors at Site Tx and Survey Genomics; is a member of the scientific advisory boards of Site Tx, Arsenal Biosciences, Cellanome, Survey Genomics, NewLimit, Network.bio, Amgen, and Tenaya; owns stock in Arsenal Biosciences, Site Tx, Cellanome, NewLimit, Survey Genomics, Network.bio, Tenaya and Lightcast; and has received fees from Site Tx, Arsenal Biosciences, Cellanome, Spotlight Therapeutics, NewLimit, Network.bio, Amgen, Abbvie, Gilead, Pfizer, 23andMe, PACT Pharma, Juno Therapeutics, Tenaya, Lightcast, Trizell, Vertex, Merck, Genentech, GLG, ClearView Healthcare, AlphaSights, Rupert Case Management, Bernstein, and ALDA. He is an investor in and informal advisor to Offline Ventures and a client of EPIQ. The Marson laboratory has received research support from the Parker Institute for Cancer Immunotherapy, the Emerson Collective, Arc Institute, Juno Therapeutics, Epinomics, Sanofi, GlaxoSmithKline, Gilead, and Anthem and reagents from Genscript, 10X, Ultima, Illumina, and Cellanome. A.H. is the founder and chief scientific officer of Cassidy Bio. Q.T. is a co-founder, shareholder, and scientific advisor of Sonoma Biotherapeutics and is a consultant of eGenesis and Qihan Bio. All declared interests had no input in the design, execution, interpretation, or publication of the work in this paper.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.ymthe.2025.12.004>.

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Supplemental Information

Gene-corrected regulatory T cell therapy for IL2RA deficiency

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Cytogenetic analysis of gcTregs manufactured from healthy donor cells

The introduction of double-stranded breaks by Cas9 nucleases can result in chromosomal damage. To evaluate potential gross chromosomal abnormalities resulting from CRISPR-mediated gene correction, we performed cytogenetic analysis on gcTregs manufactured from healthy donor cells. Briefly, 5e6 cryopreserved gcTregs were thawed and rested for 24 hours. Cells were then transferred to the UCSF Cytogenetics Laboratory where they were cultured for 4-5 days then analyzed by Giemsa (GTG) banding for chromosomal abnormalities (Supplemental Figure S2). A normal male karyotype was detected in a total of 27 analyzable metaphase cells (Supplemental Figure S3), indicating that no significant chromosomal damage was detected resulting from gene correction and throughout the manufacturing process.

Table S1: Off-target sites nominated by GUIDE-Seq and COSMID

Table of genomic coordinates for all on- and off-target editing sites detected *in vitro* by GUIDE-Seq (38), *in silico* by COSMID (13), or both (2). A subset of sites identified in GUIDE-seq were also detected in samples from HEK293 cells edited with RNP. A total of 54 sites (1 on-target, 53 off-target) were detected and further validated by rhAmpSeq. Plots shown in Fig 1d were sites that had ≤ 7 mismatches, but all identified sites were included in the final rhAmpSeq validation panel.

Site	Nomination method	Target coordinates (without PAM)(hg38)	rhAmpSeq assay coordinates (hg38)	Strand	Comments
On-target	-	chr10:6021535-6021555	chr10:6021493-6021677	-	
IL2RA_12_C	COSMID	chr1:7630060-7630080	chr1:7629953-7630147	-	
IL2RA_13_C	COSMID	chr10:93749002-93749022	chr10:93748921-93749103	-	
IL2RA_14_C	COSMID	chr13:32858761-32858781	chr13:32858671-32858854	-	
IL2RA_15_C	COSMID	chr14:106102325-106102345	chr14:106102216-106102388	-	
IL2RA_16_C	COSMID	chr16:12557953-12557973	chr16:12557843-12558038	-	
IL2RA_17_C	COSMID	chr18:37489529-37489549	chr18:37489450-37489605	-	
IL2RA_18_C	COSMID	chr3:10170929-10170949	chr3:10170870-10171085	-	
IL2RA_19_C	COSMID	chr5:21998731-21998751	chr5:21998614-21998798	-	
IL2RA_20_C	COSMID	chr10:16361645-16361665	chr10:16361554-16361804	+	
IL2RA_21_C	COSMID	chr12:56397129-56397149	chr12:56397045-56397207	+	
IL2RA_22_C	COSMID	chr7:36448532-36448552	chr7:36448442-36448616	+	
IL2RA_24_C	COSMID	chr8:4374568-4374588	chr8:4374443-4374683	+	
IL2RA_25_C	COSMID	chrX:5820647-5820667	chrX:5820541-5820766	+	
IL2RA_159HT	GUIDE-seq (T-cells)	chr10:80732507-80732530	chr10:80732419-80732654	-	
IL2RA_160HT	GUIDE-seq (T-cells)	chr12:68175801-68175825	chr12:68175754-68175946	-	
IL2RA_161HT	GUIDE-seq (T-cells)	chr14:22547488-22547508	chr14:22547358-22547560	+	
IL2RA_162HT	GUIDE-seq (T-cells)	chr14:69480122-69480145	chr14:69480077-69480259	+	
IL2RA_1_HC	GUIDE-seq (T-cells)	chr1:1037658-1037678	chr1:1037514-1037720	+	
IL2RA_2_HC	GUIDE-seq (T-cells)	chr2:44854693-44854713	chr2:44854640-44854876	+	
IL2RA_3_HC	GUIDE-seq (T-cells)	chr2:188508612-188508631	chr2:188508515-188508687	-	
IL2RA_4_HC	GUIDE-seq (T-cells)	chr22:35118061-35118081	chr22:35117988-35118183	+	
IL2RA_5_HC	GUIDE-seq (T-cells)	chr5:70008524-70008543	chr5:70008408-70008591	-	
IL2RA_6_HC	GUIDE-seq (T-cells)	chr5:70883602-70883621	chr5:70883486-70883669	-	
IL2RA_7_HC	GUIDE-seq (T-cells)	chr6:11886919-11886939	chr6:11886776-11887008	+	
IL2RA_8_HC	GUIDE-seq (T-cells)	chr6:35048311-35048331	chr6:35048206-35048389	+	
IL2RA_163T	GUIDE-seq (T-cells)	chr1:200990180-200990200	chr1:200990138-200990325	-	
IL2RA_164T	GUIDE-seq (T-cells)	chr1:205256302-205256322	chr1:205256244-205256425	-	

IL2RA_165T	GUIDE-seq (T-cells)	chr14:21846935-21846955	chr14:21846849-21847027	+	
IL2RA_166T	GUIDE-seq (T-cells)	chr15:80825862-80825882	chr15:80825789-80825971	-	
IL2RA_167T	GUIDE-seq (T-cells)	chr16:22323642-22323662	chr16:22323584-22323825	-	No amplification in rhAmpSeq
IL2RA_168T	GUIDE-seq (T-cells)	chr17:1725165-1725185	chr17:1725061-1725226	-	
IL2RA_169T	GUIDE-seq (T-cells)	chr17:75475775-75475795	chr17:75475674-75475858	-	
IL2RA_170T	GUIDE-seq (T-cells)	chr17:75489338-75489358	chr17:75489226-75489462	-	
IL2RA_171T	GUIDE-seq (T-cells)	chr19:5145831-5145851	chr19:5145744-5145913	-	
IL2RA_172T	GUIDE-seq (T-cells)	chr19:49960240-49960260	chr19:49960177-49960419	-	
IL2RA_173T	GUIDE-seq (T-cells)	chr19:49970381-49970401	chr19:49970282-49970445	-	
IL2RA_174T	GUIDE-seq (T-cells)	chr19:52270567-52270587	chr19:52270431-52270675	+	
IL2RA_177T	GUIDE-seq (T-cells)	chr2:75340997-75341017	chr2:75340892-75341088	+	
IL2RA_178T	GUIDE-seq (T-cells)	chr2:106486777-106486797	chr2:106486686-106486843	-	
IL2RA_179T	GUIDE-seq (T-cells)	chr2:130610811-130610831	chr2:130610759-130610983	-	
IL2RA_180T	GUIDE-seq (T-cells)	chr2:135825481-135825501	chr2:135825388-135825568	+	
IL2RA_181T	GUIDE-seq (T-cells)	chr2:207266602-207266622	chr2:207266527-207266686	+	
IL2RA_182T	GUIDE-seq (T-cells)	chr2:219011175-219011195	chr2:219011009-219011255	-	
IL2RA_183T	GUIDE-seq (T-cells)	chr20:24318790-24318810	chr20:24318634-24318856	-	
IL2RA_184T	GUIDE-seq (T-cells)	chr20:24956203-24956223	chr20:24956164-24956354	+	
IL2RA_185T	GUIDE-seq (T-cells)	chr3:38365545-38365565	chr3:38365440-38365624	-	
IL2RA_186T	GUIDE-seq (T-cells)	chr3:141573995-141574015	chr3:141573875-141574074	+	
IL2RA_187T	GUIDE-seq (T-cells)	chr3:170062924-170062944	chr3:170062838-170063004	+	
IL2RA_188T	GUIDE-seq (T-cells)	chr7:10959421-10959441	chr7:10959310-10959481	-	
IL2RA_189T	GUIDE-seq (T-cells)	chr7:74446305-74446325	chr7:74446219-74446398	-	
IL2RA_190T	GUIDE-seq (T-cells)	chr8:22396730-22396750	chr8:22396678-22396865	-	
IL2RA_10_HTC	GUIDE-seq (T-cells), COSMID	chr3:126793309-126793328	chr3:126793232-126793409	-	
IL2RA_11_HTC	GUIDE-seq (T-cells), COSMID	chr5:81869231-81869250	chr5:81869120-81869294	-	

Table S2: CyTOF Reagents

Metal tag	Product	Vendor	Catalog no.
(102, 104, 105, 106, 108, 110) Pd	Cell-ID 20-Plex Pd Barcoding Kit	Fluidigm	201060
191Ir	Cell-ID Intercalator-Ir	Fluidigm	201192A
195Pt	Cisplatin	Sigma-Aldrich	P4394

Table S3: CyTOF Panel

Metal tags used to identify targets in CyTOF experiments. *denotes intracellular targets

Metal tag	Marker	Clone	Vendor	Catalog no.
89Y	CD45	HI30	Fluidigm	3089003B
115In	CD7	CD7-6B7	Biolegend	343102
139La	CD33 (Siglec-3)	WM53	Biolegend	303410
140Ce	CD45RO	UCHL1	Biolegend	304202
141Pr	CD3	UCHT1	Fluidigm	3141019B
142Nd	CD19	HIB19	Fluidigm	3142001B
143Nd	CD117	104D2	Fluidigm	3143001B
144Nd	*Helios	22F6	Biolegend	137202
145Nd	CD4	RPA-T4	Fluidigm	3145001B
146Nd	CD8a	RPA-T8	Fluidigm	3146001B
147Sm	*pStat5 [Y694]	47	Fluidigm	3147012C
148Nd	CD226 (DNAM-1)	11A8	Biolegend	338302
149Sm	CD183 (CXCR3)	G025H7	Biolegend	353702
150Nd	FcεR1a	AER-37 (CRA-1)	Fluidigm	3150027B
151Eu	CD123 (IL-3Ra)	6H6	Fluidigm	3151001B
152Sm	*Granzyme B	CLB-GB11	Novus	NBP1-50071
153Sm	CD45RA	HI100	Fluidigm	3153001B
154Eu	TIM3	F38-2E2	Fluidigm	3154010B

155Gd	TIGIT	A15153G	Biolegend	372702
156Gd	CD274 (PD-L1)	29E.2A3	Fluidigm	3156026B
157Gd	CD278 (ICOS)	C398.4A	Biolegend	313502
158Gd	CD27	L128	Fluidigm	3158010B
159Tb	CD197 (CCR7)	G043H7	Fluidigm	3159003C
160Gd	*T-bet	4B10	Fluidigm	3160010B
161Dy	*CD152 (CTLA-4)	14D3	Fluidigm	3161004B
162Dy	*FoxP3	PCH101	Fluidigm	3162011A
163Dy	CD28	CD28.2	BioLegend	302902
164Dy	CD161	HP-3G10	Fluidigm	3164009B
165Ho	CD127	A019D5	Fluidigm	3165008B
166Er	CD154 (CD40L)	24-31	Biolegend	310802
167Er	*GATA-3	TWAJ	Fluidigm	3167007A
168Er	*RORgt	600214	Fluidigm	3168018B
169Tm	CD25	2A3	Fluidigm	3169003B
170Er	TCR Va24-Ja18 (iNKT)	6B11	Fluidigm	3170015B
171Yb	CD196 (CCR6)	G034E3	BioLegend	353402
172Yb	CD38	HIT2	Fluidigm	3172007B
173Yb	CD194 (CCR4)	205410	R&D Systems	MAB1567-100
174Yb	HLA-DR	L243	Fluidigm	3174001B
175Lu	CD279 (PD-1)	EH12.2H7	Fluidigm	3175008B
176Yb	CD56	NCAM16.2	Fluidigm	3176008B
209Bi	CD16	3G8	Fluidigm	3209002B

Table S4: Antibodies used for flow cytometry experiments. Antibodies used for clinical-scale FACS are listed separately in the methods section.

Target	Fluorophore	Clone	Vendor	Catalog #:
CD5	PacBlue	BL1a	Beckman Coulter	A82790
HA-Tag	AF647	6E2	Cell Signaling	3444
IL2RA	BV421	BC96	BioLegend	302630
CD127	FITC	MB15-18C9	Miltenyi	130-113-409
CD4	APC	SK3	BD	340443
CD8	FITC	SK1	BD	347313
FOXP3	AF647	206D	BioLegend	320114
FOXP3	AF488	206D	Biolegend	320112
Helios	PE	22F6	BioLegend	137216
Live/Dead	APC efluor780	N/A	Tonbo	13-0865-T100
Fixable Live/Dead	APC efluor780	N/A	Invitrogen	65-0865-18
7-AAD	N/A	N/A	BD	559925

Table S5: Homology-Directed Repair Template (HDRT) Sequences

Template	Sequence
HDRT-714: c.530 127-F	CCTGCTGAGAGCGTCTGCAAAATGACCCACGGGAAGACAAGATG GACCCAGCCCCAGCTCATATGCACAGGTGAAATGGAGACCAGTCA GTTTCCAGGTAGGGTGGCTCCCTTCTGGGGTCCACAAT
HDRT-715: c.530 172-F	GTTTATTATCAGTGCGTCCAGGGATACAGGGCTCTACACAGAGGT CCTGCTGAGAGCGTCTGCAAAATGACCCACGGGAAGACAAGATG GACCCAGCCCCAGCTCATATGCACAGGTGAAATGGAGACCAGTCA GTTTCCAGGTAGGGTGGCTCCCTTCTGGGGTCCACAAT
HDRT-514: 40-HA-CD5-40	ACCCTCCTCTCTTCTTTCTGCAGTCGCTTCCTGCCTCGGATACCCA TACGATGTGCCTGATTACGCAGGATCACGGCTCAGCTGGTATGAC CCAGGTAAGGAAGAGCCACATG

Table S6: sgRNA Sequences

sgRNA	Sequence (5'-3')
G472: CD5 N2	CAGUCGCUUCCUGCCUCGGA
G494: IL2RA c530	AAAUGACCCACGGGAAGACA

Table S7: Antibodies used for spectral flow experiments in Figure 5. Antibodies used for spectral flow are listed below. Antibodies marked (I) were stained intracellularly.

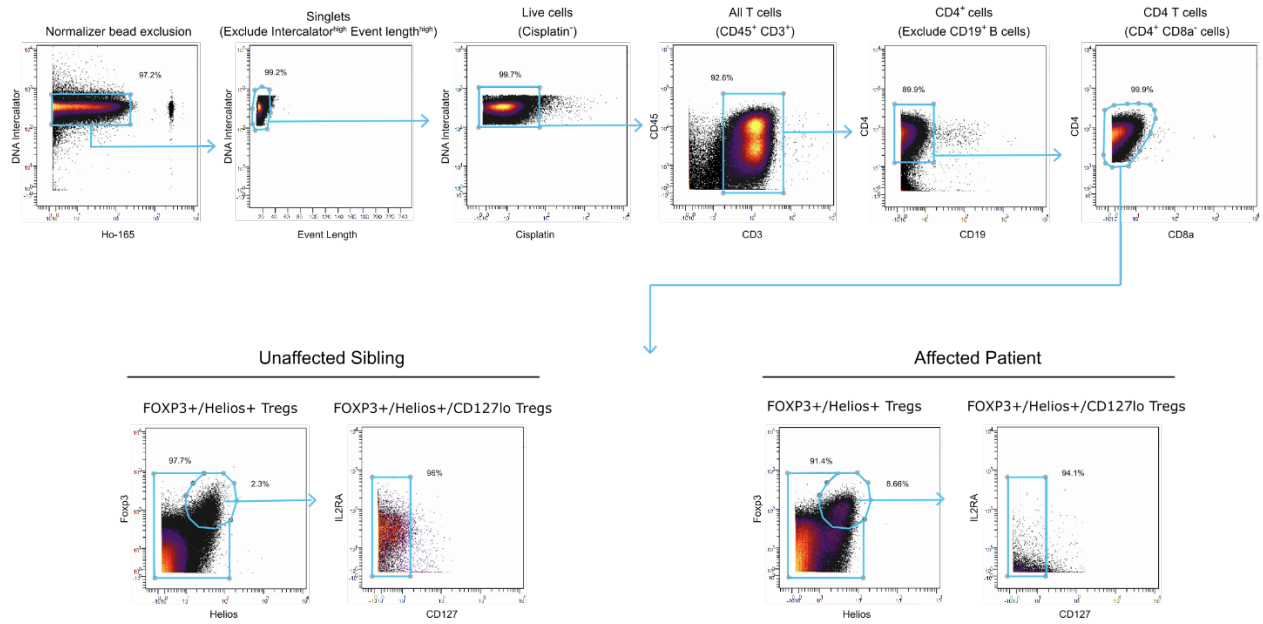
Target	Fluorophore	Clone	Vendor	Catalog #:
CD4	BUV395	SK3	BD Biosciences	563550
CD25	PE	2A3	BD Biosciences	341010
CD127	BV480	HIL-7R-M21	BD Biosciences	566101
CD45RA	BV650	HI100	BD Biosciences	563963
CD45RO	BUV805	UCHL1	BD Biosciences	748367
CCR7	PE-Cy5	G043H7	BioLegend	353272

FOXP3 (I)	AF647	206D	BioLegend	320114
Helios (I)	PE-Cy7	22F6	BioLegend	137236
TCF1/TCF7 (I)	AF488	C63D9	Cell Signaling Technology	6444S
BCL-2 (I)	PE-CF594	BCL-2/100	BD Biosciences	563601
KI-67 (I)	BUV563	SolA15	Invitrogen	365-5698-82
CTLA-4 (I)	PerCP-Cy5.5	14D3	BioLegend	369608
ICOS	BUV737	DX29	BD Biosciences	749665
PD-1	RB744	EH12.1	BD Biosciences	570478
LAG-3	APC-eFluor780	3DS223H	Invitrogen	47-2239-42
TIM3	BV785	F38-2E2	BioLegend	345032
TIGIT	BV605	4E1.2	BioLegend	372712
CD226	RB780	118A	BD Biosciences	755470
CD40L (I)	BV421	24-31	BioLegend	310824
KLRG1	BV711	2F1/KLRG1	BioLegend	138427
CCR4	APC-Fire 810	L291H4	BioLegend	359440
CD39	AF700	A1	BioLegend	328256
Viability	Zombie UV		BioLegend	423108

Table S8. Serum IL-2 levels from the individual affected by compound heterozygous IL2RA deficiency.

Date	Performing laboratory	Serum IL-2 (pg/mL)	Reference range (pg/mL)
6/4/2025	ARUP	5.4	<=2.1
7/3/2024	ARUP	6.7	<=2.1
1/3/2024	ARUP	8.3	<=2.1
10/5/2022	ARUP	4.1	<=2.1
11/8/2021	Yale	2.2	0-1.0

A



B

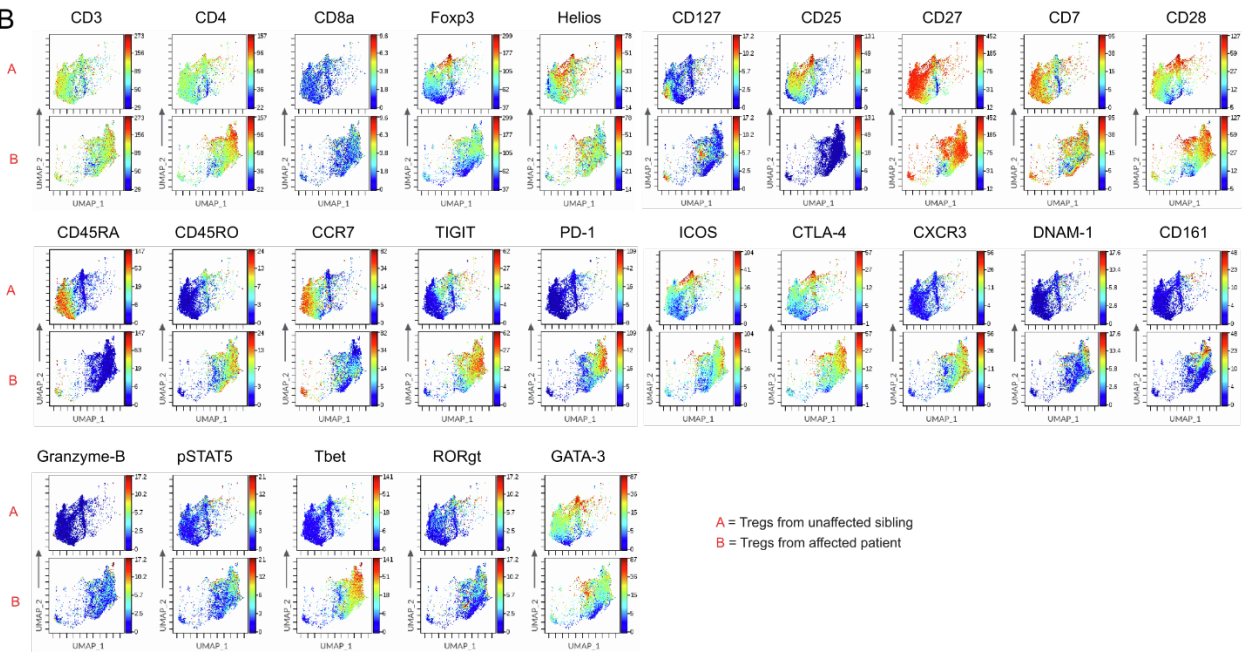


Figure S1: Gating strategy for CyTOF analysis and expression of lineage and functional markers

A) Gating strategy to isolate Treg and Tconv populations for CyTOF analysis. Boxed regions from the patient and an unaffected sibling (A-D) were populations used for functional and lineage marker analysis.

B) UMAP dimensionality reduction for functional and lineage markers in patient (top rows) and unaffected sibling (bottom rows) Tregs.



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Cell line name: HTR-V031 PI or contact person: Brian Shy
Recharging account information : Speedtype MLMJE17579
Type of study: Karyotype Received date: 04/01/2022
Reviewed date: 04/12/2022

Lab No. : HTR-V031
Specimen Type: Enriched T cells
Metaphase Examined: 27
Karyotypes (photos): 7
Banding Technique(s): GTG
Banding Resolution: 500
Result: **46,XY[27]**
Interpretation: **Normal male karyotype**

A normal male karyotype was detected in all 27 analyzable metaphase cells obtained from this specimen.

Cytogeneticist:

Zhongxia(Joe) Qi, Ph.D.

Figure S2: Cytogenetics report outlining analysis method, result, and final interpretation of analysis.

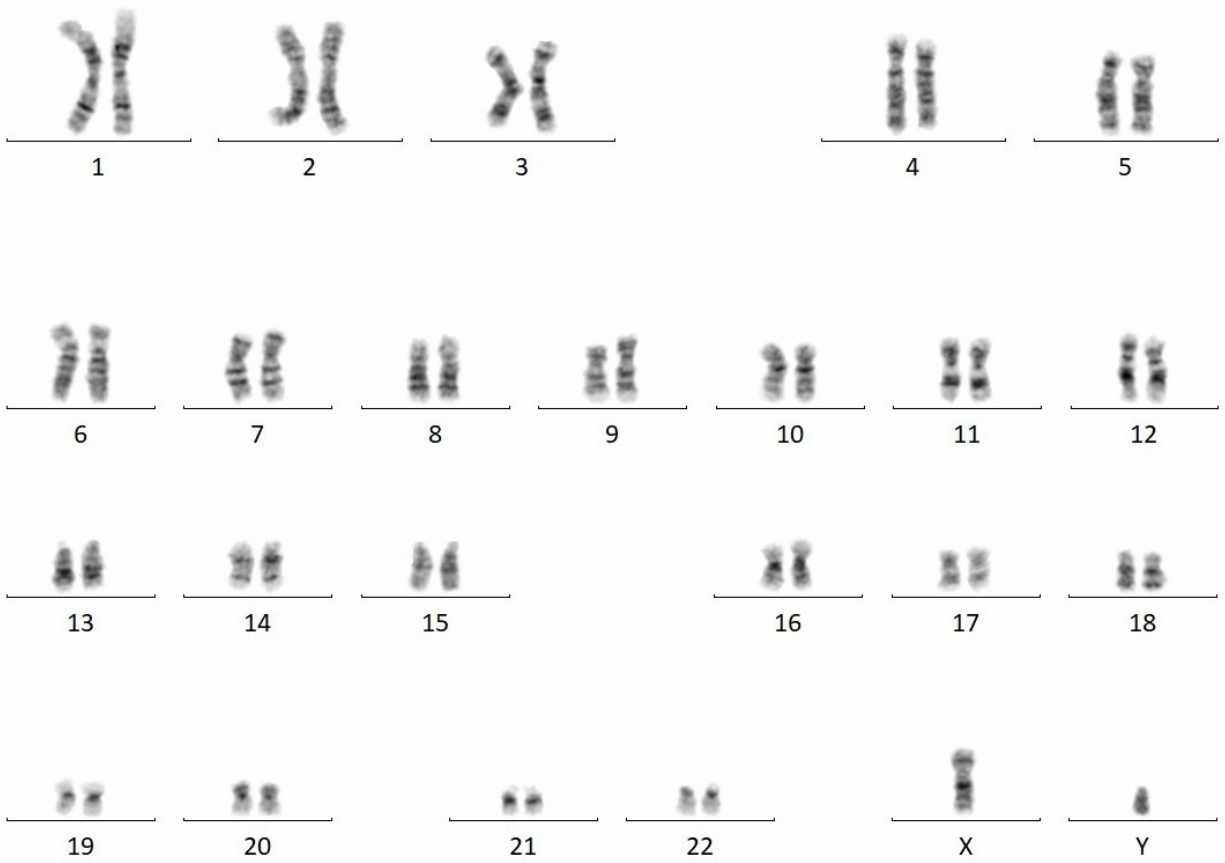


Figure S3: Karyotype of gcTregs manufactured from healthy donor. No major chromosomal abnormalities were detected. Representative karyotype of 27 analyzed metaphase cells.

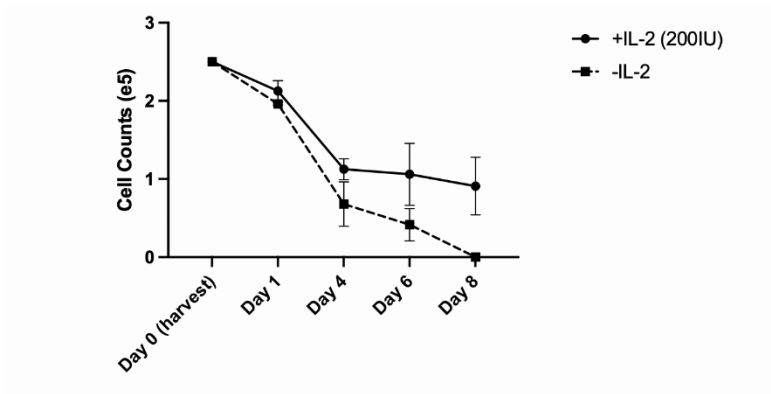


Figure S4: Survival of gcTreg in culture is dependent on IL-2. gcTreg cells from the affected individual were cultured in X-VIVO 15 with human serum and hIL-2 at 200 IU/mL. Total viable cell counts were measured through day 8 in culture. Error bars are standard deviation of technical replicates.

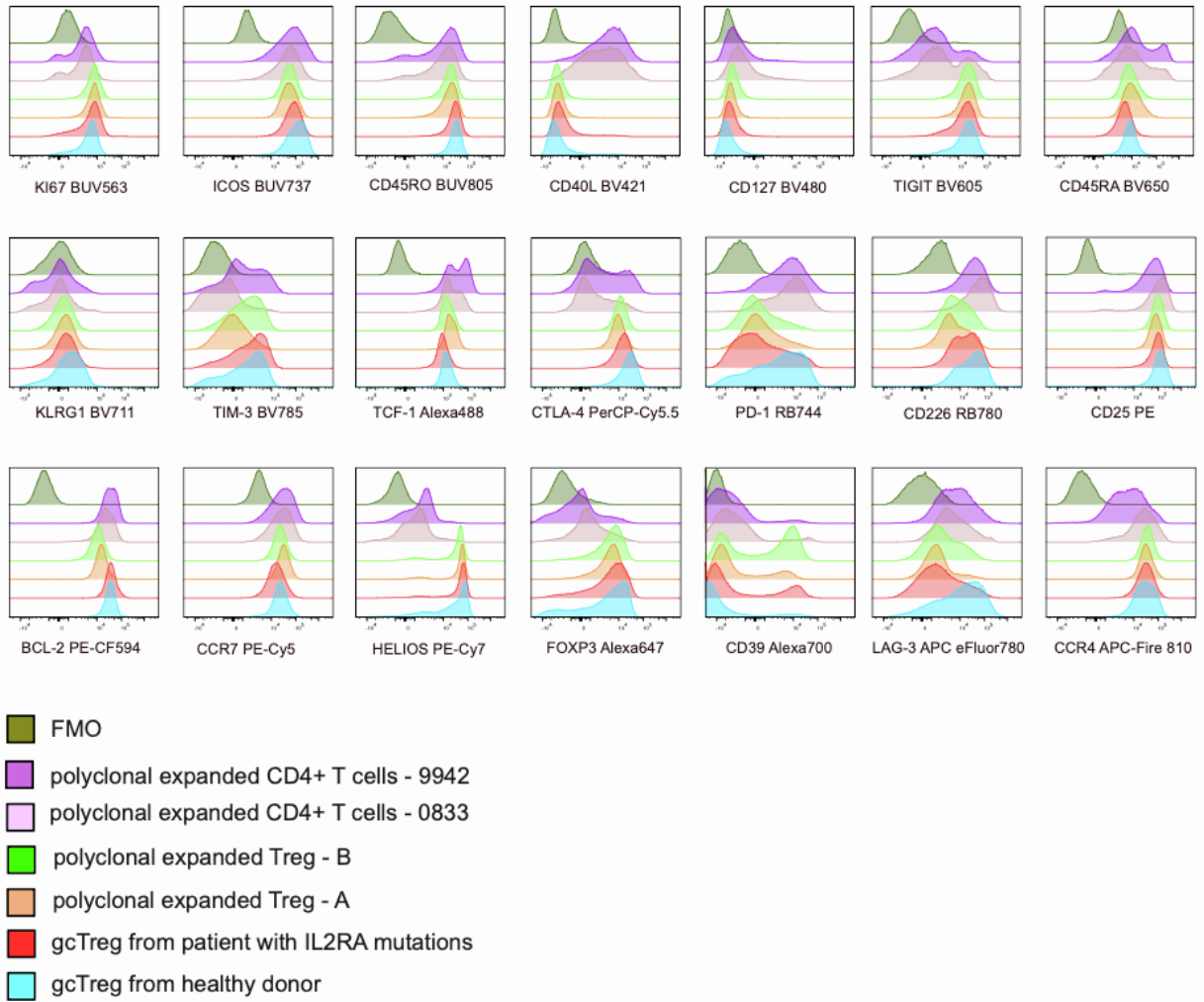


Figure S5: Flow analysis of gcTreg from the affected individual compared to gcTreg from a healthy donor, polyclonal expanded Treg, and polyclonal expanded CD4⁺ T cells . FMO, fluorescence minus one control.

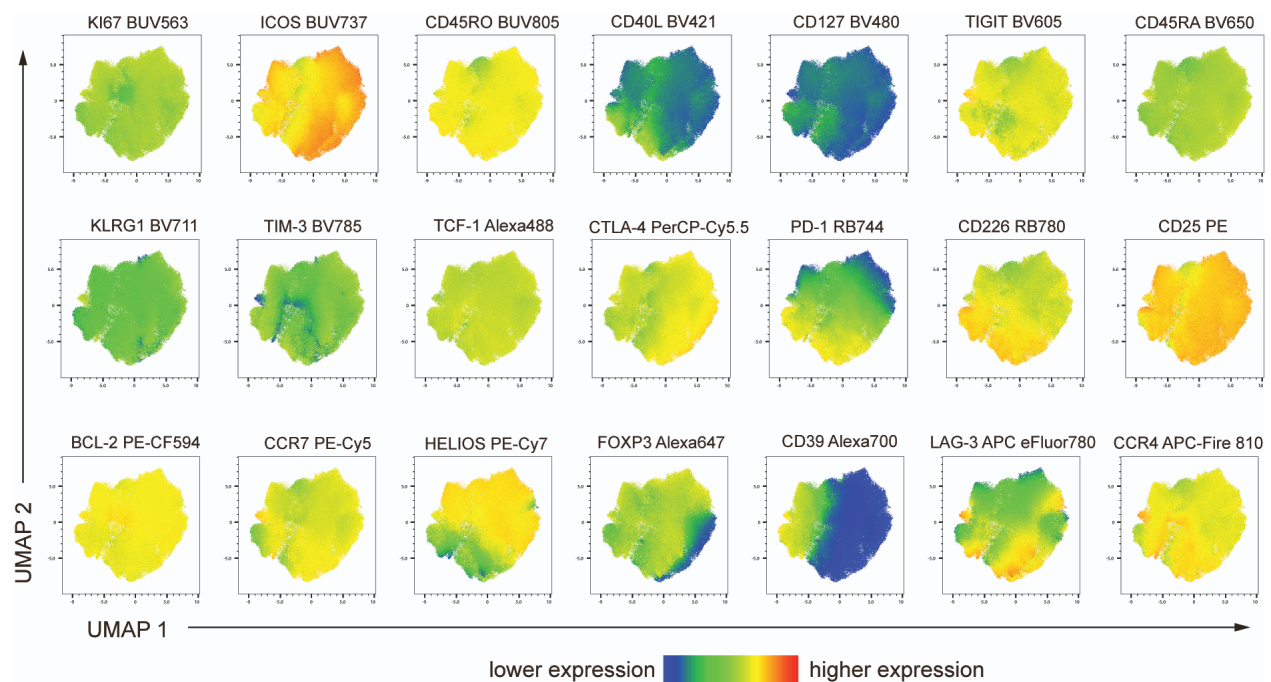


Figure S6: UMAP analysis of gcTreg samples from the individual with IL2RA mutations and a healthy donor shows similar expression profiles for gcTreg from the affected individual and gcTreg from a healthy donor. This figure is based on the gcTreg UMAP analysis in Figure 5B.