Therapeutic gene editing of T cells to correct CTLA-4 (CD152) insufficiency

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One Sentence Summary: A therapeutic approach using edited T cells as gene therapy corrects CTLA-4 insufficiency in vitro and in a mouse model of the disease.

Editor's summary:

Abstract

Heterozygous mutations in *CTLA-4* result in an inborn error of immunity with an autoimmune and frequently severe clinical phenotype. Autologous T cell gene therapy may offer a cure without the immunological complications of allogeneic hematopoietic stem cell transplantation. Here we designed a homology-directed repair (HDR) gene editing strategy that inserts the *CTLA-4* cDNA into the first intron of the *CTLA-4* genomic locus in primary human T cells. This resulted in regulated expression of CTLA-4 in $CD4^+$ T cells, and functional studies demonstrated CD80 and CD86 transendocytosis. Gene editing of T cells isolated from three patients with CTLA-4 insufficiency also restored CTLA-4 protein expression and rescued transendocytosis of CD80 and CD86 in vitro. Lastly, gene-corrected T cells from *CTLA-4*$^{-/-}$ mice engrafted and prevented lymphoproliferation in an in vivo murine model of CTLA-4 insufficiency. These results demonstrate the feasibility of a therapeutic approach using T cell gene therapy for CTLA-4 insufficiency.

INTRODUCTION

CTLA-4 (CD152) insufficiency is an inborn error of immunity (IEI) (primary immunodeficiency) with a severe clinical phenotype that results from heterozygous germline mutations in *CTLA-4 (1,2)*. CTLA-4 insufficiency was first described in 2014 *(1-5)*. CTLA-4 insufficiency has a heterogeneous genetic landscape with no obvious hotspots, although most disease-causing mutations (>80%) are found in exons 2 and 3 ***(****1-3,5-8)*.

CTLA-4 (CD152) is a critical negative immune regulator, expressed constitutively on regulatory T cells (T_{regs}) and on conventional T cells (T_{conv}) after activation *(9,10)*. CTLA-4 competes with CD28 for the shared ligands CD80 and CD86, expressed on antigen presenting cells (APCs). CTLA-4 binds its ligands and then removes them from APCs by the process of transendocytosis (TE), thereby depleting the same ligands required for CD28 co-stimulation, leading to immunosuppression *(3,10,11).* Patients with CTLA-4 insufficiency can exhibit an increased percentage of $CD4^+$ forkhead box P3 $(FOXP3)^+$ T_{reg} cells compared to healthy controls, however within the T_{reg} fraction, lower CTLA-4 expression and a reduction in CD80 and CD86 uptake has been observed, consistent with compromised T_{reg} function *(2, 3, 12)*.

Clinically, CTLA-4 insufficiency is characterized by immune dysregulation due to reduced suppression by T_{regs} and consequent hyperactivation of effector T cells (*1,2).* It typically presents in the first two decades of life with hypogammaglobulinemia, recurrent infections, marked autoimmunity and lymphoproliferation (which can be malignant) resulting in progressive morbidity and premature mortality *(1,3,13)*. Management is challenging, and whilst CTLA-4 fusion protein mimetics abatacept and belatacept can result in clinical improvement, concomitant systemic immunosuppression is usually required to control

autoimmunity *(3,5,13,14)*. Allogeneic hematopoietic stem cell transplantation (alloHSCT) is therefore currently the only curative treatment however, it carries high risk of mortality as well as morbidity from graft failure, graft rejection and graft-versus-host disease (*5,8,15)*.

Autologous gene therapy (GT) is a potential curative approach without the immunological complications of alloHSCT. Gammaretroviral and lentiviral hematopoietic stem cell (HSC) GT has been successfully used to treat other IEIs by introducing a transgene which integrates semi-randomly into the genome with expression driven by an artificial promoter *(16-22)*. However, in disorders where tightly regulated gene expression is required, as is the case in gain-of-function disorders and haploinsufficiency such as in CTLA-4 insufficiency, a specific gene editing approach may be more appropriate to facilitate physiological, dynamic, cell-specific protein expression.

Gene editing technologies such as the clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9) system enable correction of genetic defects whilst preserving the endogenous gene control machinery *(23)*. New genetic material can be inserted by the process of homology directed repair (HDR) at the site of a double-stranded DNA (dsDNA) break using non-integrating templates such as adeno-associated virus 6 (AAV6) vectors *(24)*. Several published pre-clinical studies have demonstrated that IEIs can be corrected using gene editing, thus providing proof-of-principle of autologous GT approaches for these disorders *(25-30)*. Gene edited cellular therapies have entered the clinic for the treatment of monogenic disorders with promising results published for sickle cell disease and beta-thalassemia *(31)*.

Most gene therapy approaches for IEIs modify hematopoietic stem cells (HSCs) to achieve gene expression across all hematopoietic lineages *(19,20,22)*. However, for conditions primarily affecting the lymphoid compartment, such as cluster of differentiation-40 (CD40) ligand deficiency, X-linked lymphoproliferative disease and CTLA-4 insufficiency, restoration of T cell function may offer a cure *(3,26,32,33)*. T cell GT has several advantages over HSC-GT. Firstly, large numbers of T-lymphocytes can be obtained with non-mobilised apheresis. The lymphodepletion required before infusion of a T-cell product is substantially less toxic than the myeloablative regimens required for HSC engraftment. Because T-cells are terminally differentiated, the risk of insertional mutagenesis is reduced (*32)*. Although the pathophysiology of CTLA-4 insufficiency is not strictly confined to the T cell compartment, CTLA-4 functions in a cell-extrinsic manner; thus, we hypothesized that T cell correction could abrogate the clinical phenotype *(11)*.

Here, we report a widely applicable gene editing approach that corrects the immunological defect in CTLA-4-insufficient T cells. We demonstrate functional restoration in patient T cells in vitro and abrogation of the clinical phenotype in an in vivo murine model of the disease. Our data demonstrate the feasibility of a therapeutic approach using T cell gene therapy for CTLA-4 insufficiency.

RESULTS

Targeted genome modification of the human CTLA-4 locus using CRISPR/Cas9 and AAV HDR template

To demonstrate the feasibility of performing gene editing at the *CTLA-4* locus (**Fig. 1A**) and to optimize our editing protocol in human T cells we designed a CRISPR/Cas9/AAV6 approach that inserted a green fluorescent protein (GFP) sequence into the open reading frame (ORF) of *CTLA-4*. We used a 20-nucleotide guide RNA (gRNA) (gRNA 1; GAUGUAGAGUCCCGUGUCCA) that produced a dsDNA break in exon 2 of *CTLA-4* (fig. S1A). This break was targeted for repair using an AAV6 donor template incorporating a promoterless 2A self-cleaving peptide (P2A)-GFP sequence followed by a synthetic woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and polyadenylation signal (pA), flanked by two asymmetrical homology arms [HAs (396bp and 420bp] (**Fig. 1B**). After editing with this gRNA/Cas9/AAV6 approach, successful HDR-mediated integration of the HDR cassette was assessed by flow cytometry examining GFP expression. Rates of HDR (GFP^+) in wild-type (WT) $CD3^+$ cells were 55.8 +/-1.6% (mean +/- SD n=3) (**Fig. 1C** and fig. S1, B and C).

Restoration of CTLA-4 expression following correction of a point mutation

To demonstrate that CRISPR/Cas9/AAV6 gene editing could correct CTLA-4 insufficiency we first set out to repair the disease-causing c.370A>C (p.T124P) point mutation. Because gRNA 1 covers this site, modification by one base was sufficient to retarget the guide to the c.370A>C mutation (gRNA 2: GATGTAGAGTCCCGGGTCCA). A second AAV6 HDR donor was also designed with a codon-divergent CTLA-4 exon 2 sequence (donor 2) to allow identification of HDR-corrected alleles by sequencing (fig. S1D).

T cells isolated from a patient harboring the c.370A>C (p.T124P) point mutation were then edited using gRNA 2/Cas9 RNP and HDR donor 2. Total CTLA-4 median fluorescence intensity (MFI) increased following editing [from 149 +/- 1 (mean+/-SD) in unedited patient cells (*n*=3) to 167.7 +/- 3.8 after editing, compared to 192.8 +/- 22.1 in healthy donor T cells (*n*=5)]. After editing, the difference between healthy control and mutant CTLA-4 MFI was no longer significant (*P*=0.036 before editing, *P*=0.071 after editing) (**Fig. 1D**). Correction of the heterozygous mutation was confirmed after DNA extraction using in-out PCR and Sanger sequencing of the edited locus (fig. S1E). gRNA2 demonstrated specificity for only the mutated allele because no CTLA-4 knockdown was observed in heathy cells treated with this guide (**Fig. 1E**). These data demonstrated the feasibility of targeting patient mutations using mutation-specific CRISPR guides and an AAV6-directed HDR repair approach.

Assessment of universal gene editing strategies for correction of physiological CTLA-4 expression in human T cells

Although promising, the above mutation-specific correction approach for CTLA-4 insufficiency would not be feasible for clinical translation because over fifty distinct mutations have been described, and new variants are being regularly discovered *(5)*. We therefore set out to devise an editing strategy that could correct most disease-causing mutations.

We evaluated several universal editing strategies, first targeting early exon 1 of *CTLA-4*. An AAV6 HDR template was designed with a WT CTLA-4 cDNA in front of the P2A sequence, GFP reporter cassette and WPRE sequence flanked by two asymmetrical HAs (HDR donor 3) **(Fig. 2A)**. The target TGGCTTGCCTTGGATTTCAG (gRNA 3: UGGCUUGCCUUGGAUUUCAG) resulted in knock down of CTLA-4 on assessment by

flow cytometry **(Fig, 2B,** middle plot) and produced on-target "indels" in more than 90% of $CD4^+$ T cells when analyzed by inference of CRISPR edits (ICE) analysis (fig. S1F). This gRNA/AAV6 editing strategy was then tested in healthy human T cells. HDR was assessed by flow cytometry (GFP^+) and showed average editing efficiencies (GFP^+) of 42.5 +/- 8.1% (mean +/- SD, *n*=3), in healthy donor $CD4^+$ T cells **(Fig. 2, B and C).**

In a second approach, we targeted the 3' end of the first intron of *CTLA-4.* This would enable correction of most disease-causing mutations but had the additional advantage of avoiding the introduction of indels in the coding region of the remaining healthy allele, which could worsen disease in heterozygous disease settings. In vitro assessment demonstrated that using intron-targeting gRNAs, CTLA-4 expression remained intact despite the creation of a dsDNA break (fig. S2, A to C). In ICE analysis, one gRNA (gRNA 4: AGCUCCGGAACUAUAAUGAG) efficiently targeted the intron and produced indels in over 90% of cells while CTLA-4 expression remained intact as measured by flow cytometry (fig. S2, A and B).

A new AAV6 HDR donor template was therefore designed incorporating an artificial splice acceptor (SA) sequence, followed by cDNA for exons 2, 3 and 4, P2A sequence, GFP reporter, WPRE and pA sequences flanked by two HAs (donor 4A) (**Fig. 2D**). The SA sequence is required because the gRNA results in a dsDNA break before the endogenous splice acceptor thus allowing the artificial SA sequence in the HDR donor to exploit normal splicing of exon 1 to exon 2 of the repair donor after HDR. A further HDR donor template was also tested in which the WPRE-pA was replaced with the *CTLA-4* 3'UTR, to allow comparison of the WPRE and the 3'UTR on the gene expression profile (donor 4B). Editing efficiencies of 64.6% GFP^+ +/- 3.1% (mean +/- SD) (*n*=3) were achieved using the intronic editing approach **(Fig. 2, C and E),** which were more efficient than the previous approach targeting exon 1 (**Fig. 2C**).

When comparing the two HDR donors, which differed by having a synthetic 3'UTR sequence (WPRE – donor 4A) or the *CTLA-4* 3'UTR (donor 4B), the WPRE donor (donor 4A) reproducibly mediated higher editing efficiencies **(Fig. 2, C and E)**. Confirmation of editing efficiency using gRNA 4 and donor 4A was performed using digital droplet PCR (ddPCR) with probes targeting the edited sequence, demonstrating targeted integration of ≥40% (Table 1, fig. S2D). The intronic gRNA with donor 4A (WPRE) was therefore selected for further validation.

Predicted on-target and off-target activities of gRNAs were initially assessed using *in silico* design tools (see Materials and Methods). However, after demonstration of the superiority of the intronic editing strategy with gRNA 4, genome wide, off-target cleavage activities were formally assessed using capture of a short double-stranded oligonucleotide at double strand breaks (DSBs) through GUIDE-seq (genome wide, unbiased identification of DSBs enabled by sequencing) (see Materials and Methods). GUIDE-seq analysis demonstrated that gRNA 4 had no detectable off-target activity (fig. S2, E and F).

Assessment of CTLA-4-mediated transendocytosis in edited CD4$^+$ T cells and T_{regs}

We assessed the ability of our edited T cells to perform CTLA-4-mediated transendocytosis (TE) using TE assays, whereby CTLA-4 drives the capture of labelled ligands (CD80 and CD86) from donor B cells **(Fig. 3A)** .[11,34] The ability of CD4$^+$ T cells to perform CTLA-4-mediated TE was monitored via transfer of mCherry-labelled CD80 and CD86 ligands from the surface of co-cultured B cells to the T cells, by flow cytometry (**Fig. 3A**). Unedited healthy donor CD4$^+$ cells were compared to cells that were edited with the universal editing approaches (donors 3 and 4A). Knock down of CTLA-4 [91% using gRNA3 (exon 1)] without a repair donor almost entirely abolished TE [92% and 93% reduction in CD80-mCherry and CD86-mCherry uptake respectively (upper right quadrant, second line, **Fig. 3B**)]. TE was restored in

cells now expressing CTLA-4 protein, particularly those edited with the intronic approach (gRNA 4), where donor 4A (WPRE) was the most successful in restoring ligand uptake equivalent to WT unedited cells (gating on edited GFP^+ cells) **(Fig. 3, B and C)**. We also noted that in the absence of WT levels of expression of CTLA-4 (for example gRNA 3 alone) we could detect some CD80 transfer in CTLA-4-negative cells. This occurred because, in the absence of effective CTLA-4 competition, CD28 was free to bind its ligands and some trogocytosis could be detected as previously reported *(35)*. However, this process was typically limited to *CTLA-4* knockout settings and largely prevented by the presence of CTLA-4 expression.

To demonstrate the intracellular transfer of CD80 and CD86 into CTLA-4^+ T_{regs} we amended our editing protocol to enable confocal microscopy to be performed on the cells of interest, using *in vitro* expanded T_{reg} (fig. S3A and Materials and Methods). Using the intronic editing strategy (gRNA 4, donor 4A), HDR rates (determined by %GFP positivity) of up to 35% were achieved (fig. S3B). Confocal microscopy of the cells after 6-hour TE demonstrated correct intracellular localization of CTLA-4 itself (within cytoplasmic vesicles) as well as co-localization of CTLA-4, CD80 and CD86 in GFP^+edited cells, which was indistinguishable from unedited cells (GFP^-) in the same field. These data suggested TE of CD80 and CD86 in the edited T_{regs} was equivalent to that observed in unedited T_{regs}. (fig. S3C). Together these data demonstrated that the intronic editing approach produced functional CTLA-4 that retained a similar ability as unedited healthy T_{regs} to transendocytose CD80 and CD86.

Assessment of functional characteristics in edited $CD4^+$ T cells

To determine the functional characteristics of the edited T cells, we assessed the impact of gene editing on T_{reg} survival. Because T_{regs} require CD28 signalling for their homeostasis, we

incubated edited and unedited T_{regs} with DG75 B cells expressing either CD80, CD86 or no ligand. This also assessed the impact of CTLA-4 expression since CTLA-4 competes with CD28 for ligand binding in this system. Edited T cells were flow cytometrically sorted for GFP and compared with mock edited cells. After 5 days, both unedited and edited $CD4^+$ T cells possessed a robust population of $FoxP3^+$ T_{regs} following stimulation in the presence of CD80 or CD86, indicating that edited cells behaved indistinguishably from unedited cells (fig. S3D). In addition, we observed that in both unedited and edited cells CD86-CD28 costimulation enhanced the expression of CTLA-4 compared to CD80-CD28 costimulation **(Fig. 3D)** in line with previous observations *(36)*. Together, this data indicated that T_{reg} homeostasis, which integrates both CD28 and CTLA-4 functions, was normal in the edited cells T cells.

Next, we assessed the ability of edited T cells to produce cytokines in response to stimulation. Unedited and sorted edited $CD4^+$ T cells were rested for 72 hours and then stimulated for 4 hours with phorbol myristate acetate (PMA)/ionomycin. Cells were then fixed, permeabilized and intracellular cytokine staining performed. This revealed that both Interferon gamma and IL-17 production was similar in edited $CD4^+$ cells compared to unedited controls (fig. S4A, B) again supporting the observation that gene editing does not affect the functional characteristics of T cells in vitro.

Evaluation of universal intronic gene editing strategy in $CD4^+$ patient T cells

Having established the gene editing strategy in healthy control cells we proceeded to test this system in patient-derived material. We obtained cells from three patients with CTLA-4 insufficiency resulting from three different heterozygous mutations (c.370A>C, c.193_203del and c.223C>T). Gene editing of CTLA-4 insufficient $CD4^+$ T cells was performed using the universal intronic approach (HDR donor template 4A – WPRE) and was assessed by flow

cytometry for editing efficiency and restoration of CTLA-4 expression. Similar editing efficiencies to those achieved previously with healthy donor T cells were obtained in patient samples, with HDR rates (determined by %GFP^+) >60% in all patient samples tested **(Fig. 4A)**. Further, editing restored surface CTLA-4 expression in $CD4^+$ T cells to amounts similar to healthy controls **(Fig. 4B)**.

We then tested the ability of edited cells to perform TE, using T_{regs} due to higher constitutive expression of CTLA-4 and higher efficiency of TE in these cells. Following overnight TE assay, cells were fixed and permeabilized and stained for total CTLA-4 and the transcription factor FOXP3 allowing TE to be examined in the T_{reg} fraction. In all three patient samples a reduction in TE compared to the healthy control was noted prior to gene editing (**Fig. 4, C to E,** second rows) however, there was not a complete absence of TE due to the remaining functional allele in these patients. Nonetheless, following intronic editing (gRNA 4, donor 4A), TE was restored to healthy donor frequencies (**Fig. 4, B to F**). As expected, greater detection of CD80 uptake was observed compared to CD86 due to the higher affinity of CTLA-4 for CD80. In addition, we have observed a faster degradation of CD86, which also contributes to its more limited detection *(37).* Together these data demonstrated that the universal gene editing approach could restore CTLA-4 expression and function in patient-derived T cells with three different heterozygous mutations in *CTLA-4.*

Whilst protein expression profiles between WT unedited cells and cells edited with gRNA 4 and HDR donor 4A were similar to each other, we also assessed the effects of gene editing on transcription. Cells were rested for 72 hours following editing (or mock editing) and then restimulated for 48 hours with CD3/CD28 beads. mRNA was extracted and quantified by qPCR. mRNA expression was similar between unedited and edited healthy control $CD4^+$ T

cells (fig. S4C). The same experiment was performed on unedited and edited $CD4^+$ T cells from patients with two different heterozygous mutations in *CTLA-4* that are known to reduce CTLA-4 protein expression (c.370A>C and c.223C>T). Following editing with gRNA 4 and donor 4A, CTLA-4 mRNA expression appeared slightly increased (non-statistically significant *P=0.54*) compared to the unedited heterozygous cells (fig. S4D) supporting the flow cytometric results obtained for the same cells described earlier **(Fig. 3B).**

Assessment of CTLA-4 surface expression kinetics in the resting and activated states

Because CTLA-4 expression is normally tightly controlled, we assessed whether CTLA-4 expression after editing had the same profile as healthy unedited cells. The surface expression of CTLA-4 (staining performed at 37°C to maximize cycling) on unedited and intronic edited (donor 4A) T cells was therefore monitored over a 7-day period. Four days after editing, cells were re-activated with CD3/CD28 beads and surface expression of CTLA-4 was analyzed by flow cytometry at 18-, 24-, 48-, 96- and 168-hours. CTLA-4 expression in healthy control T cells peaked at 18-24 hours and then gradually returned to baseline by 168 hours (**Fig. 5A**). The expression kinetics of CTLA-4 haploinsufficient cells edited with gRNA 4 and donor 4 (WPRE) mimicked those of healthy unedited cells and exhibited consistently higher median fluorescent intensity (MFI) than unedited patient cells **(Fig. 5, B and C**). This demonstrated the preservation of normal expression patterns of CTLA-4, which is one of the key advantages of using the endogenous *CTLA-4* promoter via gene editing over simple lentiviral gene replacement approaches for CTLA-4 insufficiency.

For a point of comparison, we designed a lentiviral vector encoding *CTLA-4* cDNA followed by a P2A-GFP sequence under the influence of a phosphoglycerate kinase (*PGK*) promoter (fig. S5A). Healthy human T cells were transduced (at 80-90% efficiency, vector copy number

3.4) with this lentiviral vector (fig. S5A). Overnight TE assays demonstrated increased TE of ligand compared to WT $CD4^+$ T cells and edited $CD4^+$ T cells (fig. S5B). The kinetics of CTLA-4 surface expression in healthy and patient cells transduced with this vector were assessed and lentivirally transduced cells were observed to have higher expression of CTLA-4 in both the resting and activated state (fig. S5, C and D). Together, the above data demonstrated that the intronic editing approach with gRNA 4 and HDR donor 4 produced CTLA-4 protein that retains the expression kinetics observed in healthy T cells, providing evidence that the endogenous gene control machinery remains intact following gene editing.

Assessment of T cell gene therapy for CTLA-4 insufficiency in vivo using a murine model

The critical regulatory role of CTLA-4 was first identified in *CTLA-4* knockout mice (CTLA-$4^{-/-}$) which exhibit a lethal lymphoproliferative syndrome with multi-organ lymphocytic infiltration (*38,39)*. Because CTLA-4 can regulate T cell responses in a cell-extrinsic manner (consistent with its role on T_{regs}), the presence of CTLA-4-sufficient T cells, in mixed bone-marrow chimeric mice or in adoptive co-transfer models, can correct the disease phenotype *(40)*. We therefore devised a murine approach to test whether gene edited T cells could survive in vivo and control the disease associated with CTLA-4 deficiency. Because the *CTLA-4* gene is disrupted in CTLA-$4^{-/-}$ mice by mutations introduced in exon 2, a similar editing strategy targeting the first intron (of murine *CTLA-4*) was used. Murine CTLA-$4^{-/-}$ T cells were edited (gRNA 5, HDR donor 5) or mock edited and then adoptively transferred into adult $Rag2^{-/-}$ mice without conditioning *(38)*. We have previously demonstrated that co-transfer of CTLA-4-sufficient T_{regs} can control the disease induced by CTLA-4-deficient T cells in this system *(40, 41)*, thus, sufficient restoration of CTLA-4 expression by gene editing would be predicted to protect from lymphoproliferative disease.

A gRNA was selected that causes a dsDNA break in the 3' end of the first intron of murine *CTLA-4*. An AAV6 HDR repair template was designed, replicating the architecture of the human template but containing the murine genomic sequence (donor 5) (fig. S6A). Editing efficiencies were lower than in human T cells, however cycling CTLA-4 molecules could only be detected in the edited, GFP^+ fraction of the CTLA-$4^{-/-}$ cells, confirming successful gene expression (**Fig. 6A**, upper panel). Likewise, intracellular staining revealed restoration of CTLA-4 protein in both T_{reg} ($Foxp3^+$) and T_{conv} ($Foxp3^-$) compartments (**Fig. 6A**, lower panel) as expected. The lower editing efficiencies were mitigated by fluorescence-activated cell sorting (FACS) on GFP positivity to produce a cellular product with a high proportion of edited cells (**Fig. 6B**). The sorted GFP^- cells were used as a control population that were matched for activation/editing conditions, having been derived from the same wells as the GFP^+ cells. Additional controls included mock-edited CTLA-$4^{-/-}$ cells and unmanipulated WT T cells. $6x10^5$ edited or control cells were injected intravenously into $Rag2^{-/-}$ mice (Protocol schematic in fig. S6B). Tail vein bleeds were performed 1, 3 and 4 weeks post adoptive transfer. In the mice that received the GFP^+ edited cells a stable population of GFP^+ cells was detectable at all timepoints demonstrating *in vivo* persistence as well as genetic stability (**Fig. 6C**).

All mice were sacrificed four weeks after cell transfer. To assess lymphoproliferation, the cellularity of peripheral lymph nodes and spleen was analyzed. When peripheral lymph nodes and spleens from all treatment groups were compared, lymphadenopathy and splenomegaly could be observed in mice that had received mock-edited and edited GFP^- T cells (edited, but without repair) while lymph nodes and spleens from mice treated with edited GFP^+ T cells did not differ from those found in the recipients of WT T cells (**Fig. 6D**). Furthermore, lymph nodes and spleens from recipients of edited GFP^+ and WT T cells displayed equal cellularity, whereas lymph nodes and spleens from mice treated with mock-edited and GFP^- T cells

contained substantially greater cell numbers (**Fig. 6E**). To assess lymphocytic organ infiltration, cardiac tissue was analysed: only mice from groups that had received mock-edited or GFP^- T cells showed elevated cardiac tissue infiltration, whereas T_{conv} numbers in mice treated with edited GFP^+ cells did not exceed those seen in WT controls **(Fig. 6F)**. Collectively, these findings indicated that the lymphoproliferative disease that occurred in recipients of CTLA-$4^{-/-}$ cells was being controlled in mice that received edited GFP^+ cells. Subsequent assessment of CTLA-4 expression in cells from the lymph nodes **(Fig. 6G)** and spleens **(Fig. 6H)** of recipient mice revealed that CTLA-4 was expressed in over 70% of lymph node T_{regs} and over 60% of splenic T_{regs} and in 6-15% of T_{conv} in mice that had received edited GFP^+ T cells. Indeed, CTLA-4 expression in edited GFP^+ T_{reg} was only marginally lower than that seen in WT T_{reg}, while expression in edited T_{conv} was equivalent to WT expression. Together these data demonstrated that CTLA-4-edited T cells survived in vivo, expressed CTLA-4 and were able to control the clinical phenotype of CTLA-4 insufficiency, providing a proof-of-concept for this T cell GT approach.

DISCUSSION

Here, we demonstrated that gene editing approaches for CTLA-4 insufficiency resulted in correction of immunological defects and provided a universal editing strategy that is attractive for clinical translation. An autologous T cell GT may improve the clinical phenotype, whilst abrogating many of the immunological complications of alloHSCT as well as removing the need to find a suitably matched donor. Targeting the first intron was superior in terms of editing efficiency with the key advantage of avoiding the introduction of indels which could potentially worsen disease, by targeting the remaining healthy allele. Several studies have demonstrated that gene expression can be enhanced in mammalian cells by the inclusion of an intron, such

as when correcting cytochrome B-245 beta chain (*CYBB*) gene mutations in chronic granulomatous disease and when editing the *CD40 ligand* gene *(42-46)*. However, our intronic editing approach targets the 3' end of the first intron of *CTLA-4*, thus preserving most of the first intronic sequence.

Most gene therapy approaches for IEIs modify hematopoietic stem cells (HSCs). However, for disorders mediated primarily through the lymphoid compartment there are clear advantages of a T cell GT approach, most notably the requirement for less intensive conditioning and higher editing efficiencies *(32)*. T cell GT for IEIs could benefit from the rapidly expanding infrastructure to manufacture genetically engineered T cell products *(47)*. Correction of HSCs enables long term correction due to modification of a self-renewing population of cells *(48)*, however, increasing data suggests that genetically engineered CAR-T cells can also persist long term if sufficient numbers of central and effector memory T cells are modified and transferred *(49)*. Clinical proof-of-principle of T cell gene therapy for IEIs already exists from the early retroviral T cell gene therapy trials for ADA-SCID, which demonstrated persistence of gene marking 10 years after patients received gene-modified T cells *(50)*.

CTLA-4 insufficiency is a complex disorder, and the pathology may not be strictly confined to the CD4 T cell compartment. Abnormalities in other immune cell lineages such as natural killer cells and B cells have been suggested in CTLA-4 insufficiency, although evidence of functional relevance remains limited *(1,3, 51)*. It may therefore be important to extend this work to the correction of autologous HSCs and to compare an HSC GT approach to T cell GT. Although exploration of HSC GT for CTLA-4 insufficiency needs to be explored, we would hope to position a T cell GT strategy ahead of HSC editing due to the reasons outlined in the introduction including the requirement for less intense conditioning, use of non-mobilized

apheresis, reduced risk of mutagenesis and higher editing efficiencies. The cell-extrinsic action of CTLA-4 makes a T cell GT particularly attractive for the disorder.

It is not yet known what degree of correction is required to ameliorate the clinical phenotype in CTLA-4 insufficiency. In humans, alloHSCT can be curative, although in the cases reported the majority have 100% donor chimerism *(5,8)*. However, from murine experiments it is clear that 50:50 chimeras or less can correct disease in CTLA-4$^{-/-}$ mice and that in adoptive transfer models, a single injection of CTLA-4-sufficient T_{regs} can prevent disease caused by CTLA-4-deficient bone marrow *(40, 41, 52)*. The limitation of incomplete correction could be mitigated in a clinical product by selecting cells using a reporter gene as shown in our *in vivo* experiments. In our editing construct we included a GFP tag which could be easily substituted for a clinically appropriate reporter such as truncated nerve growth factor receptor (NGFR) to enable a cell product that contained close to 100% edited cells *(53)*.

There are several limitations to this study and further work is needed before a first-in-human clinical trial. Firstly, our in vivo model used murine biallelic CTLA-4 deficiency in which disease occurs with 100% penetrance; this differs from human CTLA-4 haplo-insufficiency in which the clinical phenotype shows incomplete penetrance. The murine model enabled us to assess the ability of gene-edited T cells to control lymphoproliferative disease and tissue infiltration in vivo, however we recognize that the in vivo function of edited human T cells using our approach has yet to be demonstrated. We chose not to test the adoptive transfer of gene edited human T cells into humanized mice as this was likely to be limited by the development of graft-versus-host disease or impaired persistence of the gene-modified T cells. Although our model adoptively transferring CTLA-4$^{-/-}$ cells into Rag2$^{-/-}$ mice does not fully mimic haploinsufficiency in humans, it was considered the best-established model in which to

test our approach. The deterioration of control mice required animals to be sacrificed at 4 weeks to compare tissues between experimental groups and therefore an important follow up study should observe the long-term effects of gene-edited T cells in vivo. Pre-clinical, good manufacturing practice compliant, scale-up experiments are planned to assess a human T cell product manufactured at scale, which will assess the contributions of different T cell subsets, particularly T_{regs}. The role of CTLA-4 on non-lymphoid immune cells is not currently well described, however it remains possible a T cell gene therapy may not correct all the clinical manifestations of CTLA-4 insufficiency.

Our data provide proof-of-concept that gene editing can restore CTLA-4 function in human T cells demonstrating the potential of this approach to treat CTLA-4 haploinsufficiency. Targeting the first intron of *CTLA-4* was the most effective and widely applicable strategy, and the absence of detectable off-target edits from the gRNA used suggests that the safety of this approach is promising. Further work should assess this editing approach in HSCs. A similar approach could be envisaged to treat other IEIs that are caused by multiple heterozygous mutations.

MATERIALS AND METHODS

Study design

We hypothesized that gene editing using CRISPR/Cas9 and AAV6 could modify the *CTLA-4* locus and by inserting a cDNA template, correct pathogenic heterozygous mutations that result in an autosomal dominantly inherited IEI, CTLA-4 insufficiency. Our goal was to devise a universal editing strategy in T cells that would be attractive for clinical translation**.** Blood samples and biopsies were obtained with ethical approval (National Research ethics numbers

08/H0720/46, 99/095 and 02/208) and informed consent from all patients in accordance with the Declaration of Helsinki. All experiments performed in this project had at least 3 replicates to demonstrate biological reproducibility and enable statistical comparisons. Data describe biological replicates unless stated (the number of replicates with patient cells were limited by sample availability). Given the discovery research nature of the study, no predefined power calculations for sample size were used. There was no randomization or blinding of the operator during the experiments.

gRNA design and validation

CRISPR guide RNAs (gRNAs) were designed using the Benchling online tool (https://www.benchling.com/crispr/). NGG protospacer adjacent motif (PAM) sequences were identified, and gRNAs assessed *in silico* for on-target and off-target activity. The three gRNAs with the highest predicted on-target and lowest off-target activitiy were ordered from Synthego and assessed *in vitro*. Seventy two hours after nucleofection, DNA was extracted using QuickExtract™ (Cambio). Primers were designed and PCR performed on extracted DNA to create amplicons 800bp in length that included the site of the predicted dsDNA break. Amplicons were sent for Sanger sequencing. Sequencing results were then analyzed using the Synthego ICE software (ice.synthego.com). The gRNA that caused the highest percentage of indels (>85%) was selected for the editing approach. The gRNA selected for each approach is detailed in Table 1.

AAV6 donor template manufacture and production

Donor templates were designed using Snapgene software and incorporated asymmetrical homology arms 396bp and 420bp in length which flanked the sequence to be inserted at the site of the dsDNA break. Sequences for the insert were manufactured by Geneart™ (Thermo

Fisher Scientific). This insert was then cloned into an AAV6 vector. AAV vectors were produced with a double transfection method that introduces an inverted terminal repeats (ITR) -containing transfer plasmid and a single helper plasmid, pDGM6 (obtained from the Russell laboratory at the University of Washington with permission) that contains the AAV2 rep and AAV6 cap proteins.[53] Vector production took place in HEK293T cells seeded at $15x10^6$ in Complete Dulbecco's Modified Eagle Medium (DMEM) media (Life Tech) in 15x15cm dishes. 24μg of pDGM6 (per plate) and 12μg of ITR-containing plasmid was used to transfect cells and branched polyethylenimine added at a 4:1 ratio to DNA. 48 hours later, supernatant was harvested, treated with ammonium sulphate (Sigma-Aldrich) (31.3g per 100ml supernatant), pelleted (centrifuge at 8300xg for 30 minutes) and re-suspended in 10ml total volume of 1xTagment DNA (TD) buffer (diluted from 5xTD: 5xPBS, 5mM $MgCl_2$, 12.5mM KCl). This solution was then treated with 50U/ml Benzonase (Sigma-Aldrich) and incubated at 37°C for 30 minutes and stored at 4°C before purification. Simultaneously, cells were harvested using cell scrapers, pelleted (centrifuge 1400xg for 10 minutes), washed and resuspended in 10ml 1xTD buffer. Three freeze-thaw cycles were performed, 0.5% deoxycholic acid sodium salt (VWR Laboratory Supplies) and benzonase 50U/ml added and the solution incubated for 30 minutes at 37°C. The lysate was pelleted (4000xg for 30minutes at 18°C) and supernatant removed and stored at 4°C prior to purification. The two solutions were combined and AAV6 vectors purified by iodixanol density gradient and ultra-centrifugation at 40,000rpm (273,799xg) for 2 hours at 18°C. AAV6 particles were extracted using a needle and syringe between the 40% and 60% gradient interface and dialyzed 3 times in 1 x PBS (Thermo Fisher Scientific) with 5% sorbitol (Sigma-Aldrich) in the third step using 10K MWCO Slide-a-Lyzer dialysis cassettes (Thermo Fisher Scientific). Titration was performed using Quick Titre AAV Quantification Kit (Cell Biolabs, USA) prior to aliquoting and storage at -80°C before use.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from four patients with CTLA-4-haploinsufficiency and six healthy controls and diluted 1:2 with PBS before layering over Ficoll (Sigma-Aldrich) and centrifugation (1060 x g for 22 minutes). The PBMC layer was collected, washed and cells frozen in a solution containing FBS and 20% dimethylsulfoxide (DMSO) and stored in liquid nitrogen until use.

After thawing, T cells were isolated by magnetic-activated cell sorting (MACS) using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec). CD4 selected human cells were cultured in TexMACS Media (Miltenyi Biotec, 130-097-196) supplemented with 1% penicillin/streptomycin (100 U/ml; GIBCO, 15070), human IL-2 (Roche 11147528001) 10U/ml (1000U/ml for T_{regs}), human IL-7 (BD, 554608) 5ng/ml and human IL-15 (BD Biosciences, G243-886) 5ng/ml T cells were activated via CD3/CD28 stimulation by using T cell Transact (Miltenyi Biotec 130-111-160) 1:100 titer.

Electroporation and transduction

HiFi Cas9 (Integrated DNA technologies) and gRNA were mixed at a 1:3 molar ratio and incubated at 25°C for 30 minutes to form ribonucleoprotein (RNP) complexes. A Lonza Nucleofector 4D was used for nucleofection (programme EO-115) with a P3 Primary Cell 4D-Nucleofector Kit (Lonza, V4XP-3032). $1x10^6$ $CD4^+$ or T_{reg} cells per reaction were washed in PBS and resuspended in 15µl/per reaction of P3 nucleofector solution. Cells were mixed 1:1 with RNP solution (30µl total volume) and transferred to the nucleofector strip. Immediately after nucleofection, 80µl of warmed TexMACs media was added and cells transferred to a 24-

well plate containing 920µl of warmed TexMACS Media (Miltenyi Biotec, 130-097-196) supplemented with 1% penicillin/streptomycin (100 U/ml; GIBCO, 15070), human IL-2 (Roche 11147528001) 10U/ml (100U/ml for T_{regs}), human IL-7 (BD, 554608) 5ng/ml and human IL-15 (BD, G243-886) 5ng/ml for $CD4^+$ cells. For isolated T_{regs} IL-2 (100units/ml) and aCD3 (100ng/ml) were used. AAV6 was added at 13,000 MOI (vector genomes/cell). After 24 hours cell density was adjusted to $0.5x10^6$/ml. Cells were phenotyped >48 hours after editing by flow cytometry.

Transendocytosis (TE) assay

Prior to incubation, T cells were labelled with CellTrace Violet labelling kit (ThermoFisher Scientific C34571). Control or edited $CD4^+$ T cells (or T_{regs}) were incubated with ligand donor cells (DG75 B cells) expressing CD80 or CD86 molecules C-terminally tagged with mCherry. Donor and recipient cells were plated at a 5:1 ratio (donor:recipient) in 96-well round bottom plates in TexMACS Media (supplemented with cytokines as detailed previously) and left in an incubator at 37°C overnight. Cells were stained as detailed below and analyzed by flow cytometry. TE uptake was determined by dividing the percentage of mCherry and CTLA-4 double positive cells in the upper right quadrant by the equivalent percentage from the healthy control for that particular experiment

Flow cytometry

Flow cytometric analysis was performed on an LSR Fortessa (BD Biosciences) and data analyzed using FlowJo Version 10.7.0 (Treestar). The following anti-human antibodies were used: anti-CD3-PE-Cy7 (BD Biosciences, clone HIT3a), anti-CD4-V500 (BD Biosciences, clone RPA-T4), anti-CD25-APC (Biolegend, clone BC96), anti-CD152-PE (CTLA-4) (BD Biosciences, clone BNI3), LIVE/DEAD™ Fixable Near-IR dead cell stain kit (Thermo Fisher

Scientific, L10119). Intracellular staining was performed using the eBioscience™ Foxp3/Transcription factor staining buffer set (Thermo Fisher Scientific, 00-5523-00) and anti-FOXP3-APC (Thermo Fisher Scientific, clone 236A/E7) and anti-GFP-FITC (Rockland, 600-402-215).

The following anti-mouse antibodies were used: anti-CD3-BV421 (BD Biosciences, clone 17A2), anti-CD4-BUV737 (BD Biosciences, clone GK1.5), anti-CD4-PerCP-Cy5.5 (BD Biosciences, clone RM4-5), anti-CD152-PE (CTLA-4) (BD Biosciences, clone UC10-4F10-11), anti-FOXP3-APC (Thermo Fisher Scientific, FJK-16s), Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific).

Assessment of nuclease specificity

Small double-stranded oligonucleotides (dsODN) were obtained from Creative Biogene. Healthy $CD4^+$ cells were isolated, activated and cultured as per previously described protocols. GUIDE-seq was performed on cells nucleofected with gRNA 4 (AGCUCCGGAACUAUAAUGAG) only. Editing was performed as per the previously described editing protocol except that 2μl 100nM per reaction of the supplied dsODN was added to the RNP solution at the end of the 30-minute incubation. Seventy two hours after editing, genomic DNA was extracted using a QIAGEN Blood & Cell Culture DNA MiniKit (Qiagen, 13323) as per manufacturer's instructions. DNA was suspended in 1ml/condition of 1xTris-EDTA (TE) buffer, frozen at -20°C and then shipped on dry ice to Creative Biogene who performed amplification and next-generation sequencing and analysis *(55)*.

Assessment of edited efficiency by ddPCR

In/out and ddPCR - Primers for detection of donors 4A at the *CTLA-4* locus were designed using the NIH Primer-BLAST tool (Fwd: ATTGGATCATGGGGGACTCA; Rev: GCACGGTTCTGGATCAATTACA). For ddPCR, the same primers were used with the addition of a probe CTGGCCAGCAGCCGAGGC (5'6-FAM, Internal ZEN and 3' Iowa Black FQ, Integrated DNA Technologies). The genomic reference amplicon primers targeted albumin (Fwd GCTGTCATCTCTTGTGGGCTG, Rev CACAAATTTGGAAACAGAACAGGCATT, amplicon length 1035bp) and probe CCTGTCATGCCCACACAAATCTCTCC (5'HEX, Internal ZEN and 3' Iowa Black FQ, Integrated DNA Technologies). Droplets were generated and analyzed according to the manufacturer's instructions (QX200 system, Bio-Rad). The cycling conditions were [95^{o}C 10mins initiation, 50x (94^{o}C 1min, 60^{o}C 30s, 72^{o}C 6min) 98^{o}C 10mins, store 12 ^{o}C].

Mice

Rag2$^{-/-}$ mice were purchased from Taconic Biosciences. CTLA-4$^{-/-}$ mice were a gift from A. Sharpe (Harvard University). Mice were housed in individually ventilated cages with environmental enrichment in a humidity and temperature-controlled environment with a 14-hour light, 10-hour dark cycle at the University College London Biological Services Unit. Experiments were performed in accordance with Home Office project and personal licenses with approval from University College London Animal Welfare Ethical Review Body. All injections were carried out in the afternoon, in the absence of anesthesia and analgesia, and mice were returned immediately to the home cage following the procedures. The welfare of adoptively transferred animals was monitored at least every 2 to 3 days. There was no blinding. Co-housed littermates were randomized to treatment groups such that treatment groups were spread across cages.

In vivo experiments

Cells were isolated from lymph nodes (LN) of 16-20 day old male or female CTLA-4$^{-/-}$ mice and a negative CD4^{+} selection performed (MACS) (Miltenyi Biotech 130-104-454). Cells were re-suspended in Roswell Park Memorial Institute (RPMI) (Gibco 31870-025) medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine (Gibco) and IL-2 (Roche 11147528001) 30U/ml. Cells were stimulated for 24 hours with CD3/CD28 beads (Dynabeads^{tm} Gibco, 11452D). Beads were removed and cells edited using gRNA GGUCUUGGAAACUAAGCCUG Cas9 RNPs with a Lonza Nucleofector 4D (programme DN100) and P3 Primary Cell 4D-Nucleofector Kit (Lonza, V4XP-3032) before being immediately transduced with AAV HDR donor 5. Cells were rested for 48 hours and then sorted for GFP expression using a FACSAria Fusion (BD Biosciences). 6x10^{5} cells (GFP^{+}, GFP^{-} or mock edited) were then injected intravenously into 6 to 10 week old male or female Rag2$^{-/-}$ mice.

Statistical analysis

All statistical analyses were performed in Prism (v.6, Graphpad software). Paired and unpaired t tests were used to compare two groups of categorical variables. A P value of ≤ 0.05 was considered significant in an analysis. For experiments with multiple replicates under the same conditions the mean and standard deviation were calculated unless otherwise stated. One-way ANOVAs were performed to compare experimental groups in the in vivo experiments.

Supplementary Materials

Materials and methods

Figures S1 to S6.

Data file S1

References

1. H. S. Kuehn, W. Ouyang, B. Lo, E. K. Deenick, J. E. Niemela, D. T. Avery, J. N. Schickel, D. Q. Tran, J. Stoddard, Y. Zhang, D. M. Frucht, B. Dumitriu, P. Scheinberg, L. R. Folio, C. A. Frein, S. Price, C. Koh, T. Heller, C. M. Seroogy, A. Huttenlocher, V. K. Rao, H. C. Su, D. Kleiner, L. D. Notarangelo, Y. Rampertaap, K. N. Olivier, J. McElwee, J. Hughes, S. Pittaluga, J. B. Oliveira, E. Meffre, T. A. Fleisher, S. M. Holland, M. J. Lenardo, S. G. Tangye, G. Uzel, Immune dysregulation in human subjects with heterozygous germline mutations in CTLA-4. *Science* **345**, 1623-1627 (2014); published online EpubSep 26 (10.1126/science.1255904).
2. D. Schubert, C. Bode, R. Kenefeck, T. Z. Hou, J. B. Wing, A. Kennedy, A. Bulashevska, B. S. Petersen, A. A. Schaffer, B. A. Gruning, S. Unger, N. Frede, U. Baumann, T. Witte, R. E. Schmidt, G. Dueckers, T. Niehues, S. Seneviratne, M. Kanariou, C. Speckmann, S. Ehl, A. Rensing-Ehl, K. Warnatz, M. Rakhmanov, R. Thimme, P. Hasselblatt, F. Emmerich, T. Cathomen, R. Backofen, P. Fisch, M. Seidl, A. May, A. Schmitt-Graeff, S. Ikemizu, U. Salzer, A. Franke, S. Sakaguchi, L. S. K. Walker, D. M. Sansom, B. Grimbacher, Autosomal dominant immune dysregulation syndrome in humans with CTLA-4 mutations. *Nat Med* **20**, 1410-1416 (2014); published online EpubDec (10.1038/nm.3746).
3. C. Schwab, A. Gabrysch, P. Olbrich, V. Patino, K. Warnatz, D. Wolff, A. Hoshino, M. Kobayashi, K. Imai, M. Takagi, I. Dybedal, J. A. Haddock, D. M. Sansom, J. M. Lucena, M. Seidl, A. Schmitt-Graeff, V. Reiser, F. Emmerich, N. Frede, A. Bulashevska, U. Salzer, D. Schubert, S. Hayakawa, S. Okada, M. Kanariou, Z. Y. Kucuk, H. Chapdelaine, L. Petruzelkova, Z. Sumnik, A. Sediva, M. Slatter, P. D. Arkwright, A. Cant, H. M. Lorenz, T. Giese, V. Lougaris, A. Plebani, C. Price, K. E.

Sullivan, M. Moutschen, J. Litzman, T. Freiberger, F. L. van de Veerdonk, M. Recher, M. H. Albert, F. Hauck, S. Seneviratne, J. Pachlopnik Schmid, A. Kolios, G. Unglik, C. Klemann, C. Speckmann, S. Ehl, A. Leichtner, R. Blumberg, A. Franke, S. Snapper, S. Zeissig, C. Cunningham-Rundles, L. Giulino-Roth, O. Elemento, G. Duckers, T. Niehues, E. Fronkova, V. Kanderova, C. D. Platt, J. Chou, T. A. Chatila, R. Geha, E. McDermott, S. Bunn, M. Kurzai, A. Schulz, L. Alsina, F. Casals, A. Deya-Martinez, S. Hambleton, H. Kanegane, K. Tasken, O. Neth, B. Grimbacher, Phenotype, penetrance, and treatment of 133 cytotoxic T-lymphocyte antigen 4-insufficient subjects. *J Allergy Clin Immunol* **142**, 1932-1946 (2018); published online EpubDec (10.1016/j.jaci.2018.02.055).

4. N. J. Ramirez, S. Posadas-Cantera, A. Caballero-Oteyza, N. Camacho-Ordonez, B. Grimbacher, There is no gene for CVID - novel monogenetic causes for primary antibody deficiency. *Curr Opin Immunol* **72**, 176-185 (2021); published online EpubJun 18 (10.1016/j.coi.2021.05.010).

5. D. Egg, I. C. Rump, N. Mitsuiki, J. Rojas-Restrepo, M. E. Maccari, C. Schwab, A. Gabrysch, K. Warnatz, S. Goldacker, V. Patino, D. Wolff, S. Okada, S. Hayakawa, Y. Shikama, K. Kanda, K. Imai, M. Sotomatsu, M. Kuwashima, T. Kamiya, T. Morio, K. Matsumoto, T. Mori, Y. Yoshimoto, I. Dybedal, M. Kanariou, Z. Y. Kucuk, H. Chapdelaine, L. Petruzelkova, H. M. Lorenz, K. E. Sullivan, J. Heimall, M. Moutschen, J. Litzman, M. Recher, M. H. Albert, F. Hauck, S. Seneviratne, J. Pachlopnik Schmid, A. Kolios, G. Unglik, C. Klemann, S. Snapper, L. Giulino-Roth, M. Svaton, C. D. Platt, S. Hambleton, O. Neth, G. Gosse, S. Reinsch, D. Holzinger, Y. J. Kim, S. Bakhtiar, F. Atschekzei, R. Schmidt, G. Sogkas, S. Chandrakasan, W. Rae, B. Derfalvi, H. V. Marquart, A. Ozen, A. Kiykim, E. Karakoc-Aydiner, P. Kralickova, G. de Bree, D. Kiritsi, M. G. Seidel, R. Kobbe, J. Dantzer, L. Alsina, T.

Armangue, V. Lougaris, P. Agyeman, S. Nystrom, D. Buchbinder, P. D. Arkwright, B. Grimbacher, Therapeutic options for CTLA-4 insufficiency. *J Allergy Clin Immunol*, (2021); published online EpubJun 7 (10.1016/j.jaci.2021.04.039).

6. S. Hayakawa, S. Okada, M. Tsumura, S. Sakata, Y. Ueno, K. Imai, T. Morio, O. Ohara, K. Chayama, M. Kobayashi, A Patient with CTLA-4 Haploinsufficiency Presenting Gastric Cancer. *J Clin Immunol* **36**, 28-32 (2016); published online EpubJan (10.1007/s10875-015-0221-x).

7. S. Zeissig, B. S. Petersen, M. Tomczak, E. Melum, E. Huc-Claustre, S. K. Dougan, J. K. Laerdahl, B. Stade, M. Forster, S. Schreiber, D. Weir, A. M. Leichtner, A. Franke, R. S. Blumberg, Early-onset Crohn's disease and autoimmunity associated with a variant in CTLA-4. *Gut* **64**, 1889-1897 (2015); published online EpubDec (10.1136/gutjnl-2014-308541).

8. M. A. Slatter, K. R. Engelhardt, L. M. Burroughs, P. D. Arkwright, Z. Nademi, S. Skoda-Smith, D. Hagin, A. Kennedy, D. Barge, T. Flood, M. Abinun, R. F. Wynn, A. R. Gennery, A. J. Cant, D. Sansom, S. Hambleton, T. R. Torgerson, Hematopoietic stem cell transplantation for CTLA-4 deficiency. *J Allergy Clin Immunol* **138**, 615-619 e611 (2016); published online EpubAug (10.1016/j.jaci.2016.01.045).

9. S. Khailaie, B. Rowshanravan, P. A. Robert, E. Waters, N. Halliday, J. D. Badillo Herrera, L. S. K. Walker, D. M. Sansom, M. Meyer-Hermann, Characterization of CTLA-4 Trafficking and Implications for Its Function. *Biophys J* **115**, 1330-1343 (2018); published online EpubOct 2 (10.1016/j.bpj.2018.08.020).

10. B. Rowshanravan, N. Halliday, D. M. Sansom, CTLA-4: a moving target in immunotherapy. *Blood* **131**, 58-67 (2018); published online EpubJan 4 (10.1182/blood-2017-06-741033).

11. O. S. Qureshi, Y. Zheng, K. Nakamura, K. Attridge, C. Manzotti, E. M. Schmidt, J. Baker, L. E. Jeffery, S. Kaur, Z. Briggs, T. Z. Hou, C. E. Futter, G. Anderson, L. S. Walker, D. M. Sansom, Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* **332**, 600-603 (2011); published online EpubApr 29 (10.1126/science.1202947).

12. Hou, T.Z., *et al.* Identifying functional defects in patients with immune dysregulation due to LRBA and CTLA-4 mutations. *Blood* **129**, 1458-1468 (2017).

13. N. Verma, S. O. Burns, L. S. K. Walker, D. M. Sansom, Immune deficiency and autoimmunity in patients with CTLA-4 (CD152) mutations. *Clin Exp Immunol* **190**, 1-7 (2017); published online EpubOct (10.1111/cei.12997).

14. B. Lo, K. Zhang, W. Lu, L. Zheng, Q. Zhang, C. Kanellopoulou, Y. Zhang, Z. Liu, J. M. Fritz, R. Marsh, A. Husami, D. Kissell, S. Nortman, V. Chaturvedi, H. Haines, L. R. Young, J. Mo, A. H. Filipovich, J. J. Bleesing, P. Mustillo, M. Stephens, C. M. Rueda, C. A. Chougnet, K. Hoebe, J. McElwee, J. D. Hughes, E. Karakoc-Aydiner, H. F. Matthews, S. Price, H. C. Su, V. K. Rao, M. J. Lenardo, M. B. Jordan, AUTOIMMUNE DISEASE. Patients with LRBA deficiency show CTLA-4 loss and immune dysregulation responsive to abatacept therapy. *Science* **349**, 436-440 (2015); published online EpubJul 24 (10.1126/science.aaa1663).

15. T. A. Fox, R. Chakraverty, S. Burns, B. Carpenter, K. Thomson, D. Lowe, A. Fielding, K. Peggs, P. Kottaridis, B. Uttenthal, V. Bigley, M. Buckland, V. Grandage, S. Denovan, S. Grace, J. Dahlstrom, S. Workman, A. Symes, S. Mackinnon, R. Hough, E. Morris, Successful outcome following allogeneic hematopoietic stem cell transplantation in adults with primary immunodeficiency. *Blood* **131**, 917-931 (2018); published online EpubFeb 22 (10.1182/blood-2017-09-807487).

16. H. B. Gaspar, S. Cooray, K. C. Gilmour, K. L. Parsley, F. Zhang, S. Adams, E. Bjorkegren, J. Bayford, L. Brown, E. G. Davies, P. Veys, L. Fairbanks, V. Bordon, T. Petropoulou, C. Kinnon, A. J. Thrasher, Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. *Sci Transl Med* **3**, 97ra80 (2011); published online EpubAug 24 (10.1126/scitranslmed.3002716).

17. M. Donald B. Kohn, Kit L. Shaw, PhD, Elizabeth Garabedian, RN, MS, Denise Ann Carbonaro-Sarracino, PhD, Theodore B. Moore, MD, Satiro N. De Oliveira, MD, Gay M. Crooks, MD, John Tse, Sally Shupien, Dayna Terrazas, Alejandra Davila, Amalia Icreverzi, PhD, Allen Yu, Krista M. Chun, Christian E. Casas, Provaboti Barman, PhD, Maritess Coronel, Beatriz Campo Fernandez, PhD, Ruixue Zhang, Roger P. Hollis, PhD, Chilenwa Uzowuru, Hilory Ricketts, Jinhua Xu Bayford, Valentina Trevisan, Serena Arduini, PhD, Frances Lynn, Mahesh Kudari, MBBS, MA, Andrea Spezzi, MD MBBS, Lilith Reeves, MS, MT(ASCP), Kenneth Cornetta, Robert A. Sokolic, MD, Roberta Parrott, BS, Rebecca Buckley, MD, Claire Booth, Fabio Candotti, MD, Harry L. Malech, MD, Adrian J. Thrasher, MBBS, PhD FMedSci, H. Bobby Gaspar, MBBS, PhD, Lentiviral Gene Therapy with Autologous Hematopoietic Stem and Progenitor Cells (HSPCs) for the Treatment of Severe Combined Immune Deficiency Due to Adenosine Deaminase Deficiency (ADA-SCID): Results in an Expanded Cohort. *Blood* **134**, 3345 (2019); published online EpubNovember 13 2019 (https://doi.org/10.1182/blood-2019-123432).

18. E. Mamcarz, S. Zhou, T. Lockey, H. Abdelsamed, S. J. Cross, G. Kang, Z. Ma, J. Condori, J. Dowdy, B. Triplett, C. Li, G. Maron, J. C. Aldave Becerra, J. A. Church, E. Dokmeci, J. T. Love, A. C. da Matta Ain, H. van der Watt, X. Tang, W. Janssen, B. Y. Ryu, S. S. De Ravin, M. J. Weiss, B. Youngblood, J. R. Long-Boyle, S.

Gottschalk, M. M. Meagher, H. L. Malech, J. M. Puck, M. J. Cowan, B. P. Sorrentino, Lentiviral Gene Therapy Combined with Low-Dose Busulfan in Infants with SCID-X1. *N Engl J Med* **380**, 1525-1534 (2019); published online EpubApr 18 (10.1056/NEJMoa1815408).

19. D. B. Kohn, C. Booth, E. M. Kang, S. Y. Pai, K. L. Shaw, G. Santilli, M. Armant, K. F. Buckland, U. Choi, S. S. De Ravin, M. J. Dorsey, C. Y. Kuo, D. Leon-Rico, C. Rivat, N. Izotova, K. Gilmour, K. Snell, J. X. Dip, J. Darwish, E. C. Morris, D. Terrazas, L. D. Wang, C. A. Bauser, T. Paprotka, D. B. Kuhns, J. Gregg, H. E. Raymond, J. K. Everett, G. Honnet, L. Biasco, P. E. Newburger, F. D. Bushman, M. Grez, H. B. Gaspar, D. A. Williams, H. L. Malech, A. Galy, A. J. Thrasher, C. G. D. c. Net, Lentiviral gene therapy for X-linked chronic granulomatous disease. *Nat Med* **26**, 200-206 (2020); published online EpubFeb (10.1038/s41591-019-0735-5).

20. F. Ferrua, M. P. Cicalese, S. Galimberti, S. Giannelli, F. Dionisio, F. Barzaghi, M. Migliavacca, M. E. Bernardo, V. Calbi, A. A. Assanelli, M. Facchini, C. Fossati, E. Albertazzi, S. Scaramuzza, I. Brigida, S. Scala, L. Basso-Ricci, R. Pajno, M. Casiraghi, D. Canarutto, F. A. Salerio, M. H. Albert, A. Bartoli, H. M. Wolf, R. Fiori, P. Silvani, S. Gattillo, A. Villa, L. Biasco, C. Dott, E. J. Culme-Seymour, K. van Rossem, G. Atkinson, M. G. Valsecchi, M. G. Roncarolo, F. Ciceri, L. Naldini, A. Aiuti, Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study. *Lancet Haematol* **6**, e239-e253 (2019); published online EpubMay (10.1016/S2352-3026(19)30021-3).

21. S. Hacein-Bey Abina, H. B. Gaspar, J. Blondeau, L. Caccavelli, S. Charrier, K. Buckland, C. Picard, E. Six, N. Himoudi, K. Gilmour, A. M. McNicol, H. Hara, J. Xu-Bayford, C. Rivat, F. Touzot, F. Mavilio, A. Lim, J. M. Treluyer, S. Heritier, F.

Lefrere, J. Magalon, I. Pengue-Koyi, G. Honnet, S. Blanche, E. A. Sherman, F. Male, C. Berry, N. Malani, F. D. Bushman, A. Fischer, A. J. Thrasher, A. Galy, M. Cavazzana, Outcomes following gene therapy in patients with severe Wiskott-Aldrich syndrome. *JAMA* **313**, 1550-1563 (2015); published online EpubApr 21 (10.1001/jama.2015.3253).

22. D. B. Kohn, C. Booth, K. L. Shaw, J. Xu-Bayford, E. Garabedian, V. Trevisan, D. A. Carbonaro-Sarracino, K. Soni, D. Terrazas, K. Snell, A. Ikeda, D. Leon-Rico, T. B. Moore, K. F. Buckland, A. J. Shah, K. C. Gilmour, S. De Oliveira, C. Rivat, G. M. Crooks, N. Izotova, J. Tse, S. Adams, S. Shupien, H. Ricketts, A. Davila, C. Uzowuru, A. Icreverzi, P. Barman, B. Campo Fernandez, R. P. Hollis, M. Coronel, A. Yu, K. M. Chun, C. E. Casas, R. Zhang, S. Arduini, F. Lynn, M. Kudari, A. Spezzi, M. Zahn, R. Heimke, I. Labik, R. Parrott, R. H. Buckley, L. Reeves, K. Cornetta, R. Sokolic, M. Hershfield, M. Schmidt, F. Candotti, H. L. Malech, A. J. Thrasher, H. B. Gaspar, Autologous Ex Vivo Lentiviral Gene Therapy for Adenosine Deaminase Deficiency. *N Engl J Med* **384**, 2002-2013 (2021); published online EpubMay 27 (10.1056/NEJMoa2027675).

23. M. H. Porteus, A New Class of Medicines through DNA Editing. *N Engl J Med* **380**, 947-959 (2019); published online EpubMar 7 (10.1056/NEJMra1800729).

24. R. O. Bak, D. P. Dever, M. H. Porteus, CRISPR/Cas9 genome editing in human hematopoietic stem cells. *Nat Protoc* **13**, 358-376 (2018); published online EpubFeb (10.1038/nprot.2017.143).

25. C. Y. Kuo, J. D. Long, B. Campo-Fernandez, S. de Oliveira, A. R. Cooper, Z. Romero, M. D. Hoban, A. V. Joglekar, G. R. Lill, M. L. Kaufman, S. Fitz-Gibbon, X. Wang, R. P. Hollis, D. B. Kohn, Site-Specific Gene Editing of Human Hematopoietic

Stem Cells for X-Linked Hyper-IgM Syndrome. *Cell Rep* **23**, 2606-2616 (2018); published online EpubMay 29 (10.1016/j.celrep.2018.04.103).

26. N. Hubbard, D. Hagin, K. Sommer, Y. Song, I. Khan, C. Clough, H. D. Ochs, D. J. Rawlings, A. M. Scharenberg, T. R. Torgerson, Targeted gene editing restores regulated CD40L function in X-linked hyper-IgM syndrome. *Blood* **127**, 2513-2522 (2016); published online EpubMay 26 (10.1182/blood-2015-11-683235).

27. R. Rai, M. Romito, E. Rivers, G. Turchiano, G. Blattner, W. Vetharoy, D. Ladon, G. Andrieux, F. Zhang, M. Zinicola, D. Leon-Rico, G. Santilli, A. J. Thrasher, A. Cavazza, Targeted gene correction of human hematopoietic stem cells for the treatment of Wiskott - Aldrich Syndrome. *Nat Commun* **11**, 4034 (2020); published online EpubAug 12 (10.1038/s41467-020-17626-2).

28. S.S., De Ravin, L. Li, X. Wu, U. Choi, C. Allen, S. Koontz, J. Lee, N. Theobald-Whiting, J. Chu, M. Garofalo, C. Sweeney, L. Kardava, S. Moir, A. Viley, P. Natarajan, L. Su, D. Kuhns, K.A. Zarember, M. V. Peshwa, H.L. Malech, CRISPR-Cas9 gene repair of hematopoietic stem cells from patients with X-linked chronic granulomatous disease. *Sci Transl Med* **9 (372)** (2017); published online EpubJan (10.1126/scitranslmed.aah3480).

29. M. Goodwin, E. Lee, U. Lakshmanan, S. Shipp, L. Froessl, F. Barzaghi, L. Passerini, M. Narula, A. Sheikali, C. M. Lee, G. Bao, C. S. Bauer, H. K. Miller, M. Garcia-Lloret, M. J. Butte, A. Bertaina, A. Shah, M. Pavel-Dinu, A. Hendel, M. Porteus, M. G. Roncarolo, R. Bacchetta, CRISPR-based gene editing enables FOXP3 gene repair in IPEX patient cells. *Sci Adv* **6**, eaaz0571 (2020); published online EpubMay (10.1126/sciadv.aaz0571).

30. C. Clough, Y.Wang, I.F. Khan, S. Singh, K. Hung, D.J. Rawlings, 132. Targeting the *BTK* locus in primary human hematopoietic cells with TALENs and AAV donor

template. *Mol Ther* **24 Suppl 1**, S1-S304 (2016); published online EpubApr (https://doi.org/10.1016/S1525-0016(16)32941-0).

31. H. Frangoul, D. Altshuler, M. D. Cappellini, Y. S. Chen, J. Domm, B. K. Eustace, J. Foell, J. de la Fuente, S. Grupp, R. Handgretinger, T. W. Ho, A. Kattamis, A. Kernytsky, J. Lekstrom-Himes, A. M. Li, F. Locatelli, M. Y. Mapara, M. de Montalembert, D. Rondelli, A. Sharma, S. Sheth, S. Soni, M. H. Steinberg, D. Wall, A. Yen, S. Corbacioglu, CRISPR-Cas9 Gene Editing for Sickle Cell Disease and beta-Thalassemia. *N Engl J Med* **384**, 252-260 (2021); published online EpubJan 21 (10.1056/NEJMoa2031054).

32. N. Panchal, S. Ghosh, C. Booth, T cell gene therapy to treat immunodeficiency. *Br J Haematol* **192**, 433-443 (2021); published online EpubFeb (10.1111/bjh.17070).

33. N. Panchal, B. Houghton, B. Diez, S. Ghosh, I. Ricciardelli, A. J. Thrasher, H. B. Gaspar, C. Booth, Transfer of gene-corrected T cells corrects humoral and cytotoxic defects in patients with X-linked lymphoproliferative disease. *J Allergy Clin Immunol* **142**, 235-245 e236 (2018); published online EpubJul (10.1016/j.jaci.2018.02.053).

34. T. Z. Hou, O. S. Qureshi, C. J. Wang, J. Baker, S. P. Young, L. S. Walker, D. M. Sansom, A transendocytosis model of CTLA-4 function predicts its suppressive behavior on regulatory T cells. *J Immunol* **194**, 2148-2159 (2015); published online EpubMar 1 (10.4049/jimmunol.1401876).

35. I. Hwang, J. F. Huang, H. Kishimoto, A. Brunmark, P. A. Peterson, M. R. Jackson, C. D. Surh, Z. Cai, J. Sprent. T cells can use either T cell receptor or CD28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells. *J Exp Med* **191** (7), 1137-1148 (2000); (10.1084/jem.191.7.1137).

36. N. Halliday, C. Williams, A. Kennedy, E. Waters, A. M. Pesenacker, B. Soskic, C. Hinze, T. Z. Hou, B. Rowshanravan, D. Janman, L. S. K. Walker, D. M. Sansom,

CD86 Is a Selective CD28 Ligand Supporting FoxP3^{+} Regulatory T Cell Homeostasis in the Presence of High Levels of CTLA-4. *Front Immunol* **11**, 600000 (2020)10.3389/fimmu.2020.600000).

37. A. Kennedy, E. Waters, B. Rowshanravan, C. Hinze, C. Williams, D. Janman, T.A. Fox, C. Booth, A. Pesenacker, N. Halliday, B. Soskic, S. Kaur, O.S. Quereshi, E.C. Morris, S. Ikemizu, J. Huo, S.J. Davis, E. Boucrot, L.S.K. Walker, D.M. Sansom. (2022) Differences in CD80 and CD86 transendocytosis reveal CD86 as a key target for CTLA-4 immune regulation. *Nature Immunology* 23 (9): 1365-1378.

38. E. A. Tivol, F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, A. H. Sharpe, Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* **3**, 541-547 (1995); published online EpubNov (10.1016/1074-7613(95)90125-6).

39. P. Waterhouse, J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, T. W. Mak, Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* **270**, 985-988 (1995); published online EpubNov 10 (10.1126/science.270.5238.985).

40. L. S. Walker, D. M. Sansom, The emerging role of CTLA-4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol* **11**, 852-863 (2011); published online EpubNov 25 (10.1038/nri3108).

41. C. J. Wang, R. Kenefeck, L. Wardzinski, K. Attridge, C. Manzotti, E. M. Schmidt, O. S. Qureshi, D. M. Sansom, L. S. Walker, Cutting edge: cell-extrinsic immune regulation by CTLA-4 expressed on conventional T cells. *J Immunol* **189**, 1118-1122 (2012); published online EpubAug 1 (10.4049/jimmunol.1200972).

42. R. L. Brinster, J. M. Allen, R. R. Behringer, R. E. Gelinas, R. D. Palmiter, Introns increase transcriptional efficiency in transgenic mice. *Proc Natl Acad Sci U S A* **85**, 836-840 (1988); published online EpubFeb (10.1073/pnas.85.3.836).

43. R. D. Palmiter, E. P. Sandgren, M. R. Avarbock, D. D. Allen, R. L. Brinster, Heterologous introns can enhance expression of transgenes in mice. *Proc Natl Acad Sci U S A* **88**, 478-482 (1991); published online EpubJan 15 (10.1073/pnas.88.2.478).

44. A. R. Buchman, P. Berg, Comparison of intron-dependent and intron-independent gene expression. *Mol Cell Biol* **8**, 4395-4405 (1988); published online EpubOct (10.1128/mcb.8.10.4395).

45. C. L. Sweeney, J. Zou, U. Choi, R. K. Merling, A. Liu, A. Bodansky, S. Burkett, J. W. Kim, S. S. De Ravin, H. L. Malech, Targeted Repair of CYBB in X-CGD iPSCs Requires Retention of Intronic Sequences for Expression and Functional Correction. *Mol Ther* **25**, 321-330 (2017); published online EpubFeb 1 (10.1016/j.ymthe.2016.11.012).

46. V. Vavassori, E. Mercuri, G. E. Marcovecchio, M. C. Castiello, G. Schiroli, L. Albano, C. Margulies, F. Buquicchio, E. Fontana, S. Beretta, I. Merelli, A. Cappelleri, P. M. Rancoita, V. Lougaris, A. Plebani, M. Kanariou, A. Lankester, F. Ferrua, E. Scanziani, C. Cotta-Ramusino, A. Villa, L. Naldini, P. Genovese, Modeling, optimization, and comparable efficacy of T cell and hematopoietic stem cell gene editing for treating hyper-IgM syndrome. *EMBO Mol Med* **13**, e13545 (2021); published online EpubMar 5 (10.15252/emmm.202013545).

47. B. L. Levine, J. Miskin, K. Wonnacott, C. Keir, Global Manufacturing of CAR T Cell Therapy. *Mol Ther Methods Clin Dev* **4**, 92-101 (2017); published online EpubMar 17 (10.1016/j.omtm.2016.12.006).

936 48. B. C. Reinhardt, O. Habib, K. L. Shaw, E. K. Garabedian, D. A. Carbonaro-Sarracino,
937 D. R. Terrazas, B. Campo-Fernandez, S. De Oliveira, T. B. Moore, A. Ikeda, B. C.
938 Engel, G. M. Podsakoff, R. P. Hollis, A. Fernandes, C. R. Jackson, S. A. Shupien, S.
939 Mishra, A. Davila, J. Mottahedeh, A. Vitomirov, W. Meng, A. M. Rosenfeld, A. M.
940 Roche, P. Hokama, S. Reddy, J. K. Everett, X. Wang, E. T. Luning Prak, K. Cornetta,
941 M. Hershfield, R. Sokolic, S. S. De Ravin, H. L. Malech, F. D. Bushman, F. Candotti,
942 D. B. Kohn, Long-term Outcomes after Gene Therapy for Adenosine Deaminase
943 Severe Combined Immune Deficiency (ADA SCID). *Blood*, (2021); published online
944 EpubMay 11 (10.1182/blood.2020010260).
945 49. L. Biasco, N. Izotova, C. Rivat, S. Ghorashian, R. Richardson, A. Guvenel, R. Hough,
946 R. Wynn, B. Popova, A. Lopes, M. Pule, A. J. Thrasher, P. J. Amrolia, Clonal
947 expansion of T memory stem cells determines early anti-leukemic responses and
948 long-term CAR T cell persistence in patients. *Nat Cancer* **2**, 629-642 (2021);
949 published online EpubJun (10.1038/s43018-021-00207-7).
950 50. A. Aiuti, S. Vai, A. Mortellaro, G. Casorati, F. Ficara, G. Andolfi, G. Ferrari, A.
951 Tabucchi, F. Carlucci, H. D. Ochs, L. D. Notarangelo, M. G. Roncarolo, C.
952 Bordignon, Immune reconstitution in ADA-SCID after PBL gene therapy and
953 discontinuation of enzyme replacement. *Nat Med* **8**, 423-425 (2002); published online
954 EpubMay (10.1038/nm0502-423).
955 51. V. Lougaris, G. Tabellini, M. Baronio, O. Patrizi, L. Gazzurelli, N. Mitsuiki, M. R.
956 Pozzi, B. Grimbacher, S. Parolini, A. Plebani, CTLA-4 regulates human Natural
957 Killer cell effector functions. *Clin Immunol* **194**, 43-45 (2018); published online
958 EpubSep (10.1016/j.clim.2018.06.010).

52. M. F. Bachmann, G. Kohler, B. Ecabert, T. W. Mak, M. Kopf, Cutting edge: lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous. *J Immunol* **163**, 1128-1131 (1999); published online EpubAug 1

53. M. Valtieri, R. Schiro, C. Chelucci, B. Masella, U. Testa, I. Casella, E. Montesoro, G. Mariani, H. J. Hassan, C. Peschle, Efficient Transfer of Selectable and Membrane Reporter Genes in Hematopoietic Progenitor and Stem-Cells Purified from Human Peripheral-Blood. *Cancer Research* **54**, 4398-4404 (1994); published online EpubAug 15

54. P. Gregorevic, M. J. Blankinship, J. M. Allen, R. W. Crawford, L. Meuse, D. G. Miller, D. W. Russell, J. S. Chamberlain, Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nature Medicine* **10**, 828-834 (2004); published online EpubAug (10.1038/nm1085).

55. S.Q. Tsai, Z. Zheng, N.T. Nguyen, M. Liebers, V.V. Topkar, V. Thapar, N. Wyvekens, C. Khayter, A.J. Lafrate, L.P. Le, M.J. Aryee, J.K. Joung, GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology* **33**, 187-197 (2015); published online (https://doi.org/10.1038/nbt.3117).

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Author contributions:

Conceptualization of this work was performed by TAF, ECM, CB, SOB, DMS and LSKW. The methodology for this work was devised by TAF, ECM, CB, SOB, DMS, LSKW, PG, BH, OP, EW, AK, LP, CH, CW, AMP, AM, and PG. Experimental work was performed by TAF, BCH, ASA, AM, LP, NME, OP, EW, CH, CW and AK. The project was supervised by : ECM, CB, SOB, DMS, LSKW. The in vivo murine experiments were designed and supervised by LSKW. The original draft of this manuscript was written by TAF, ECM, CB, DMS and LSKW. Reviewing and editing of this manuscript was performed by PG, BH, OP, EW, LP, CH, CW, AMP, AM, SOB and PG.

Competing interests:

ECM is a Founder shareholder of Quell Therapeutics Ltd and has received honoraria from Orchard Therapeutics, GlaxoSmithKline and AstaZeneca.

A patent on the intronic gene editing approach has been filed in the United Kingdom (assigned patent number: 2112922.6 “CRISPR Cas9 Gene Therapy”).

Data and materials availability: All data associated with this study are present in the paper or supplementary materials.

Table 1: Summary of gRNAs used for each approach and the corresponding rAAV6 HDR donor.

gRNA name	gRNA sequence	gRNA target in *CTLA-4*	Donor name	Donor summary	Donor use
gRNA 1	GAUGUAGAGUCCCGUGUCCA	Mid exon 2 p.T124P c.370A>C locus	Donor 1	HA-P2A-GFP-WPRE-HA	Demonstrate editing at exon 2 p.T124P c.370A>C locus
gRNA 2	GATGTAGAGTCCCGGGTCCA	Mid exon 2 p.T124P c.370A>C locus	Donor 2	HA-codon divergent exon 2- HA	Repair of p.T124P c.370A>C mutation
gRNA 3	UGGCUUGCCUUGGAUUUCAG	Early exon 1	Donor 3	HA-CTLA-4-P2A-GFP-WPRE-HA	Universal editing strategy with insertion of replacement sequence in early exon 1.
gRNA 4	AGCUCCGGAACUAUAAUGAG	3' end of intron 1	Donor 4A	HA- SA-Exons 2, 3, 4 -P2A – GFP-WPRE-HA	Universal editing strategy with insertion of replacement sequence in intron 1 to avoid indels in coding DNA.
			Donor 4B	HA- SA-Exons 2, 3, 4 -P2A – GFP-3'UTR-HA	Universal editing strategy with insertion of replacement sequence in intron 1 to avoid indels in coding DNA.
gRNA 5	GGUCUUGGAAACUAAGCCUG	3' end of intron 1 in murine *CTLA-4*	Donor 5	HA- SA-Exons 2, 3, 4 (murine) - P2A – GFP-WPRE-HA	Intronic editing to restore CTLA-4 in murine *CTLA-4*$^{-/-}$ cells.

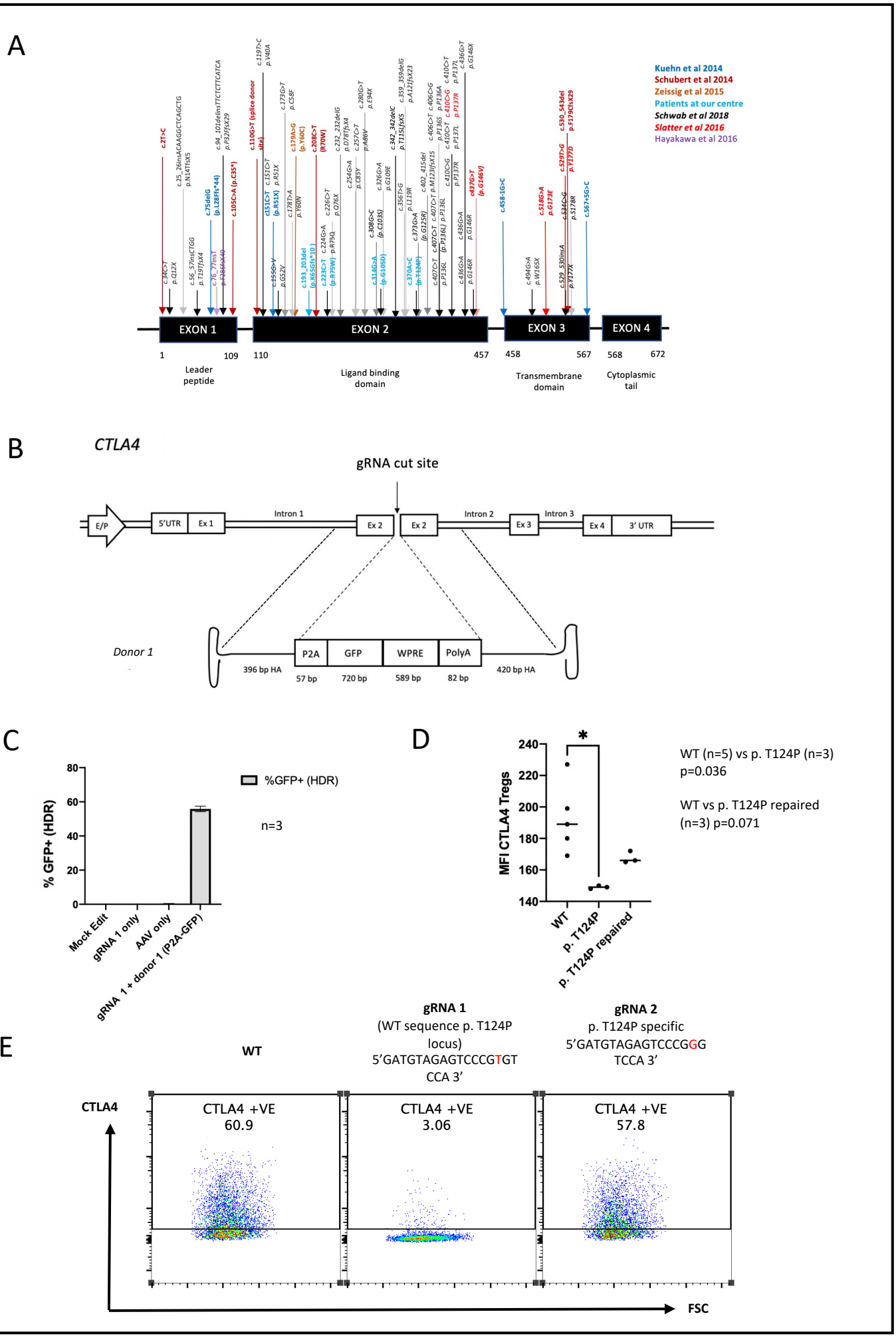


Figure 1: Targeting the *CTLA-4* locus with CRISPR/Cas9 and repair of a point mutation: (A) Schematic representation of the mutational landscape of CTLA-4 insufficiency. Mutations are colour coded by citation (key, bottom right). (B) Schematic representation of HDR donor 1 (P2A-GFP-WPRE-PolyA). (C) Average HDR rate (*n*=3, percentage GFP^+ in cells from six separate healthy donors) (Mean 55.83 SD 1.626). (D) Median fluorescent intensity (MFI) of CTLA-4 from 5 separate healthy controls and 3 separate samples from a single patient with p.T124P c.370A>C, unedited or edited. A significant difference was seen in CTLA-4 MFO between WT and p. T124P heterozygous mutant cells (*P*=0.036, Mann-Whitney test). After editing the difference in MFI was no longer significant (*P*=0.071). (E) Flow cytometry plot demonstrating surface CTLA-4 expression in cells from a healthy individual in an unedited control (left), edited with a gRNA specific for the WT CTLA-4 sequence (gRNA 1) with resulting knock down of CTLA-4 protein (centre) and a population edited with a gRNA specific for the p.T124P c.371A>C (gRNA 2) (right) demonstrating minimal activity on the WT sequence.

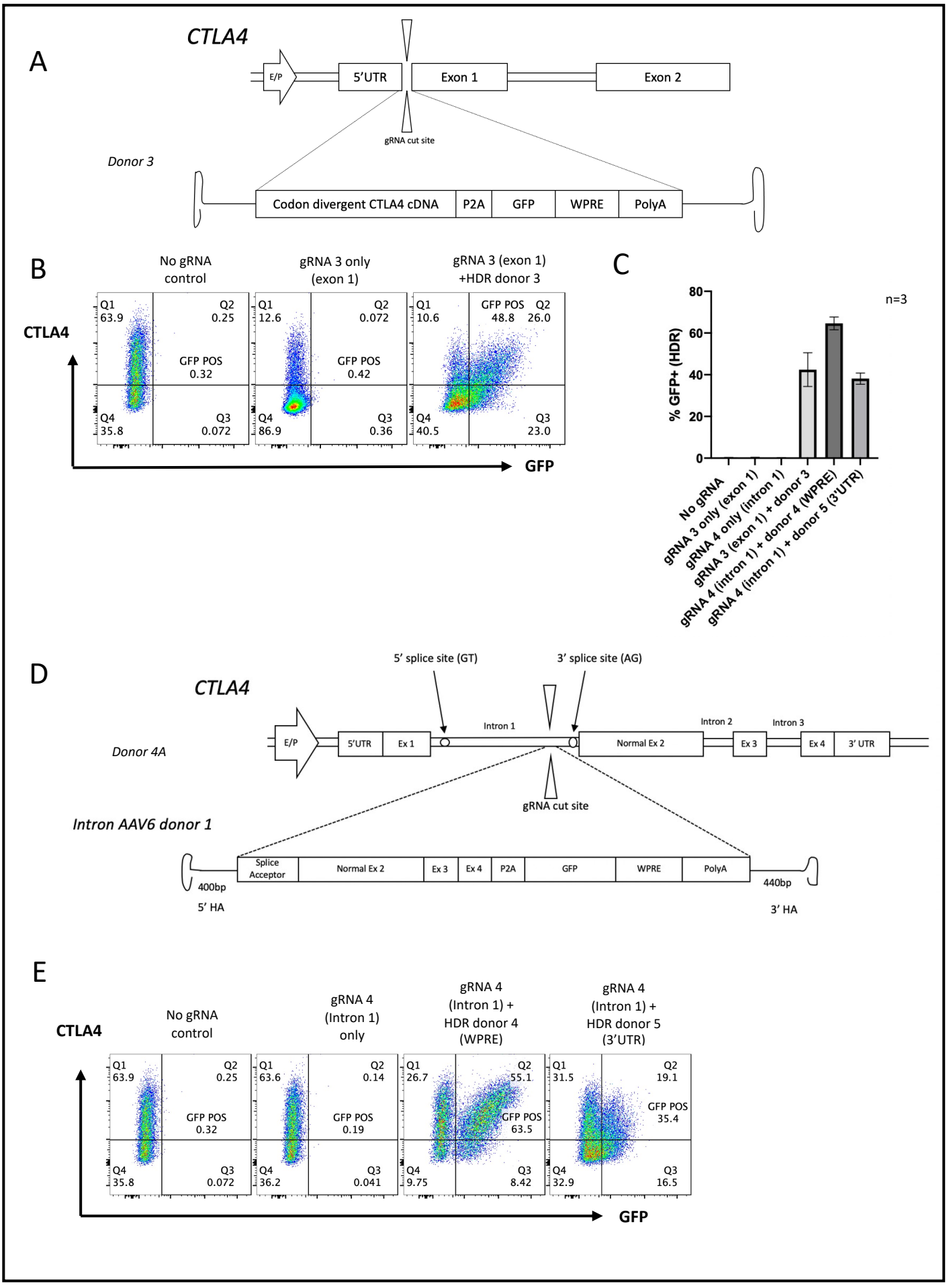


Figure 2: Universal editing strategies: (A) Schematic representation of the editing strategy using gRNA 3 (exon 1) and donor 3 (HA-CTLA-4-P2A-GFP-WPRE-HA). (B) Representative flow cytometry plots of the editing strategy shown in (A) demonstrating a non-edited control (left), gRNA only control with resulting knock down of CTLA-4 (centre) and HDR mediated by the CTLA-4 cDNA-P2A-GFP-WPRE AAV6 donor (48.8% GFP positive cells). (C) Schematic representation of the intronic editing strategy (donor 4 HA-splice acceptor-CTLA-4 exons 2, 3, 4-P2A-GFP-WPRE-HA) (D) Representative flow cytometry plots showing CTLA-4 expression and GFP expression (HDR) in cells edited with the gRNA 3/Cas9 RNP alone (intron 1) (centre left), and then with transduction of donor 4 (WPRE) and donor 5 (3'UTR) (far right). (E) Mean HDR rate (*n*=3, percentage GFP^+ in cells from separate healthy donors). Exon 1 approach (gRNA 3 + donor 3) mean=42.47% GFP^+, SD 8.13, intronic WPRE donor (gRNA 4 + donor 4A) mean=64.63% GFP^+, SD 3.06, Intron 3'UTR donor (gRNA 4, donor 4B) mean=38.13% GFP^+, SD 2.70.

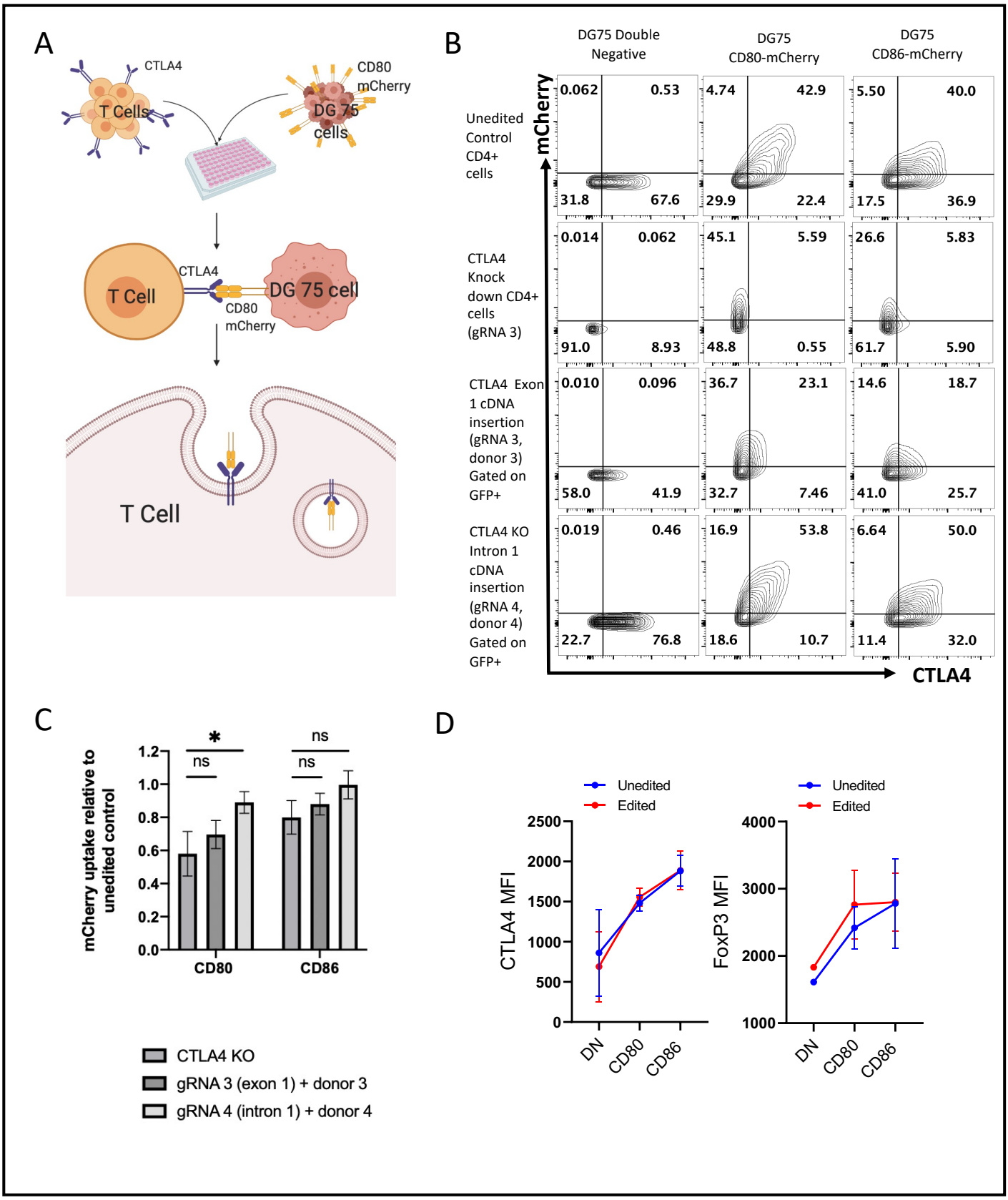


Figure 3: Functional characteristics of edited T cells: (A) Schematic representation of transendocytosis assay. Cells (edited or unedited controls) are incubated in a 5:1 ratio with DG75 cells expressing either fluorescent labelled (mCherry) CD80 or CD86. Uptake of ligand can then be assessed by flow cytometry (mCherry uptake into T cells). Created with biorender.com (B) Representative FACS plots demonstrating TE of mCherry-bound CD80 and CD86 (top right quadrant each plot) in healthy control $CD4^+$ T cells (top row), $CD4^+$ cells that have undergone knock out of CTLA-4 (upper middle row) and $CD4^+$ cells that have undergone repair using the different editing strategies (gated on edited GFP^+ cells). DG75 cells that did not express either ligand were used as a negative control (left column). (C) mCherry uptake relative to the unedited control with the different universal editing strategies in healthy $CD4^+$ cells (CTLA-4 KO mean = 0.69, SD = 0.155, *N*=3, gRNA 3 + donor 3 mean = 0.79, SD = 0.13, *n*=3, gRNA 4 +donor 4 mean = 0.94, SD = 0.08, *n*=3). One-way ANOVA; *, *P*< 0.05; ns, not significant. (D) Graphs showing increase in CTLA-4 and FOXP3 MFI in unedited cells (blue) and edited cells (red) when co-stimulated with CD80 and CD86.

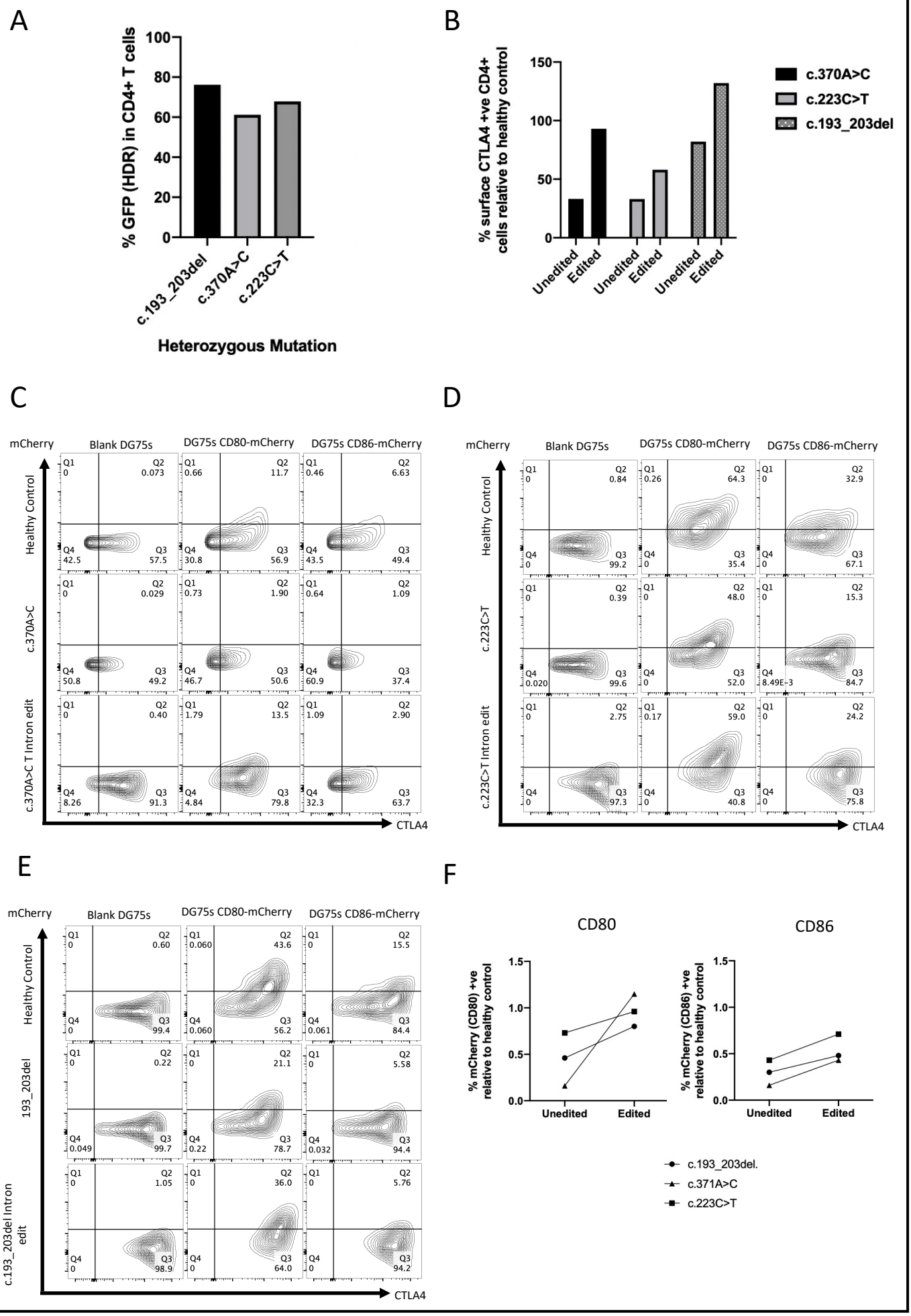


Figure 4: Restoration of CTLA-4 expression and function in CD4⁺ cells from patients with CTLA-4 insufficiency: (A) HDR rates (% cells GFP⁺) in edited CD4⁺ cells from patients with CTLA-4 haploinsufficiency resulting from three different mutations. (B) Graph showing restoration of surface CTLA-4 in heterozygote mutant CD4⁺ cells following editing with gRNA 4 and HDR donor 4. %CTLA-4 positive relative to a healthy control assessed at the same time are shown. GFP⁺ edited cells are compared to mock edited cells. (C to E) Overnight TE assays gated on CD3⁺ CD4⁺ FOXP3⁺ cells in healthy control (top rows), patient cells with three different mutations (middle rows) and patient cells corrected with the intronic editing strategy (bottom rows). (F) Graph showing the increase in ligand acquisition (% cells mCherry and CTLA-4 positive) in patient cells after editing relative to healthy control.

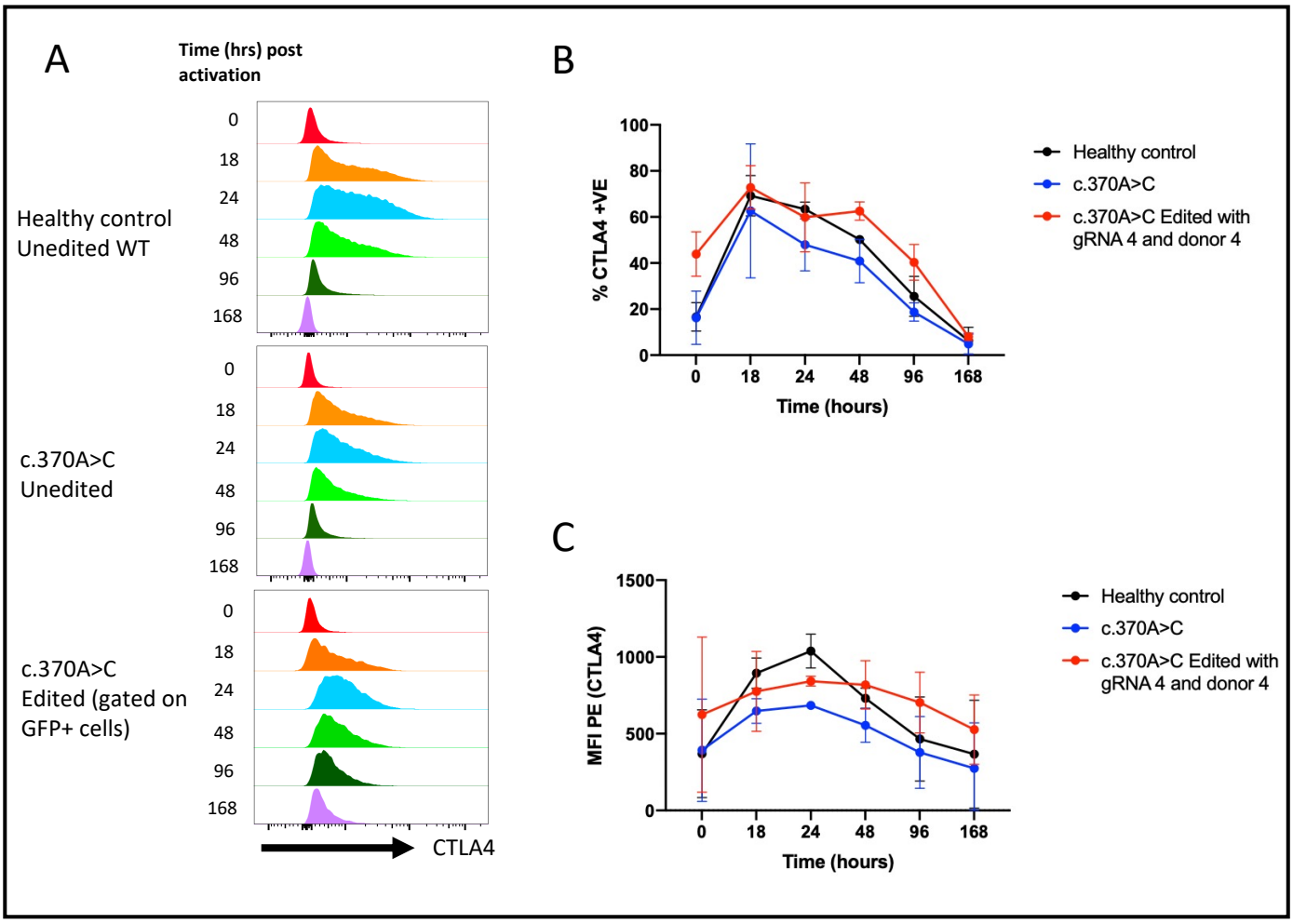


Figure 5: Kinetics of CTLA-4 surface expression in resting and activated states: (A) Representative time course of CTLA-4 expression (MFI histogram) on healthy control $CD4^+$ cells (top), c.371A>C heterozygous mutant cells (middle) and c.371A>C heterozygous mutant cells edited with gRNA 4/Cas9 RNP and donor 4. (B) Percentage and MFI (C) of CTLA-4 surface expression over time (*n*=3 for all conditions).

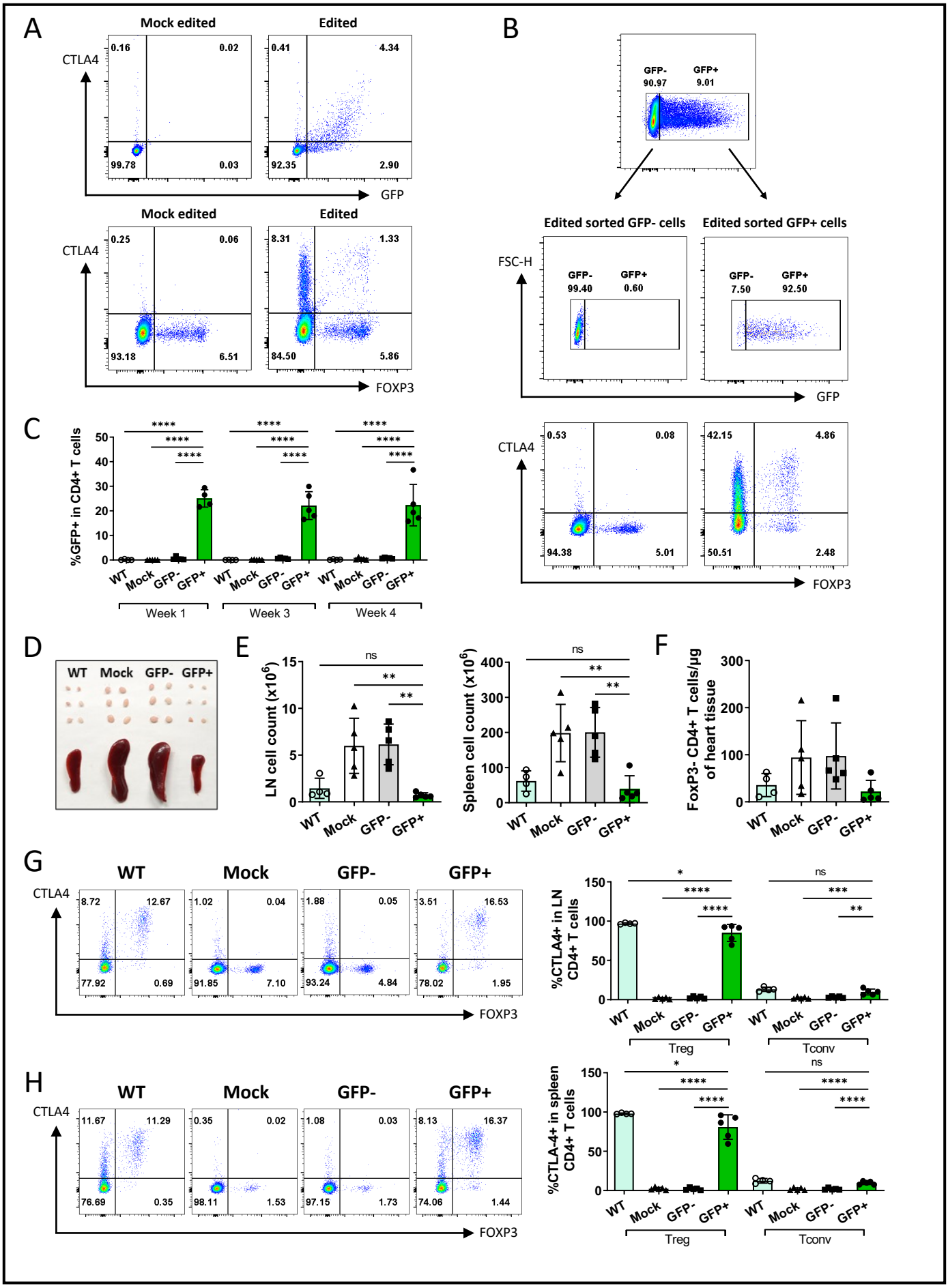
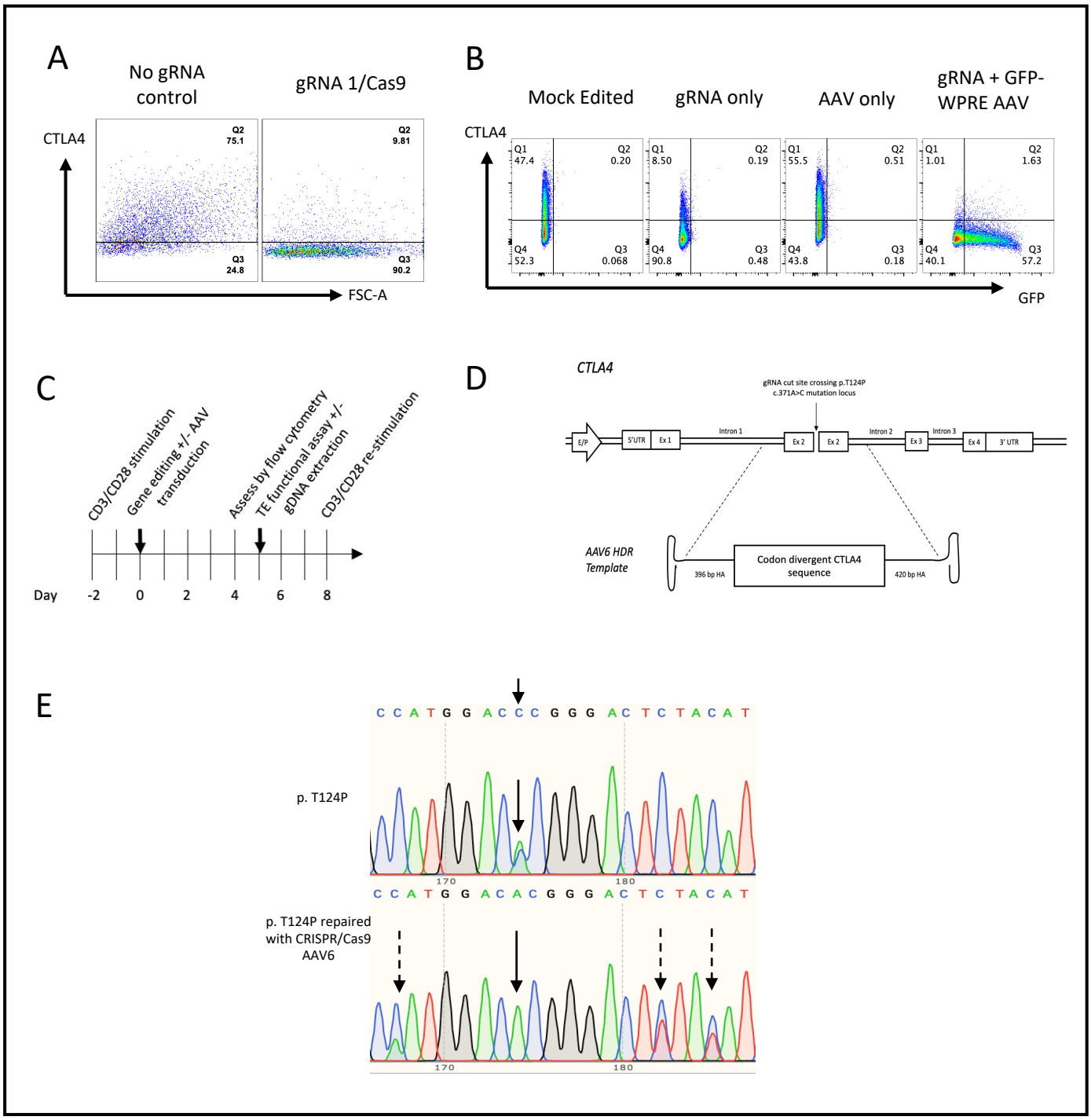
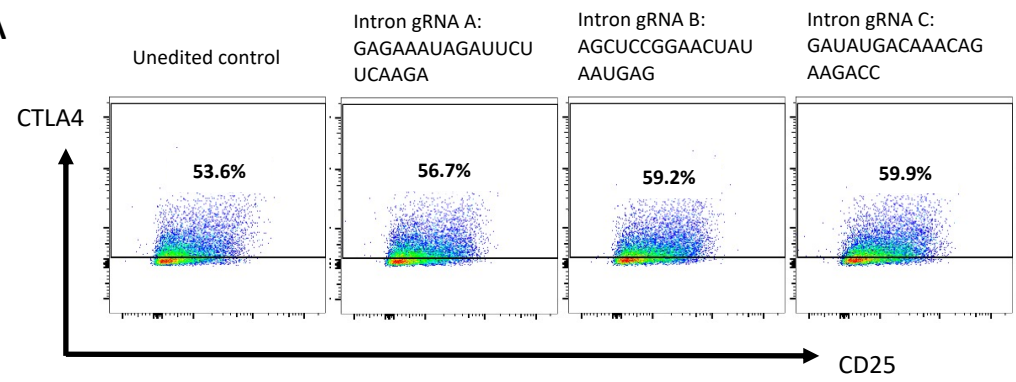


Figure 6: Assessment of T cell GT for CTLA-4 insufficiency using an in vivo murine model: (A) FACS plots demonstrating typical editing efficiencies achieved in murine CTLA-4$^{-/-}$ T cells (GFP$^+$ cells, upper plots) with restoration of CTLA-4 expression in both the FOXP3$^+$ and FOXP3$^-$ compartments (lower plots). (B) FACS plots after sort demonstrating % GFP$^+$ in the sorted edited cells (upper plots) and CTLA-4 expression in FOXP3$^-$ and FOXP3$^+$ cells in the two sorted populations (GFP$^-$ left lower plot, GFP$^+$ left upper plot). (C) Serial tail vein bleeds demonstrating persistence and stability of the GFP$^+$ population after adoptive transfer. (D) Lymph node and spleen size in mice that received WT T cells (left) mock edited and GFP$^-$ cells (middle) and GFP$^+$ enriched edited cells (right). (E) Lymph node (left) and spleen (right) cell counts in mice that received WT T cells, mock edited, GFP$^-$ and GFP$^+$ T cells. (F) Number of T_{conv} per µg of heart tissue in mice that received WT, mock edited, GFP$^-$ and GFP$^+$ T cells. (G) Representative FACS plots (left) and collated data (right) showing CTLA-4 expression in lymph node T_{reg} and T_{conv} cells. (H) Representative FACS plots (left) and collated data (right) showing CTLA-4 expression in spleen T_{reg} and T_{conv} cells. Data collated from two independent experiments; *n*=4-5. One-way ANOVA; mean ± SD are shown; ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant ($P \geq 0.05$).



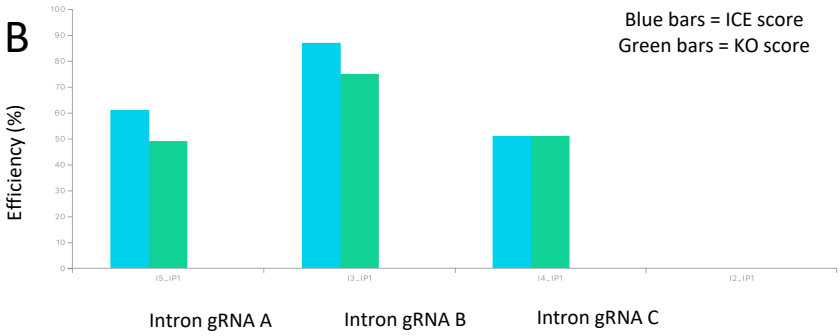
Supplementary Figure 1: Editing the CTLA4 locus and correction of point mutations: *(A) FACS plots demonstrating reduction in CTLA4 expression following nucleofection of gRNA 1/Cas9 RNP to wild type CD4+ T cells. (B) Example FACS plots demonstrating mock nucleofection (Cas9 only)(far left), gRNA +Cas9 (no AAV6) (centre left), AA6 HDR donor only (centre right) and GFP transgene expression in the far-right plot following nucleofection with RNP followed by AAV6 transduction. (C) Experimental timeline for editing experiments. Nucleofection of gRNA/Cas9 ribonucleoprotein complexes was followed immediately (<15 mins) by AAV6 transduction. Phenotypic characterisation of edited populations +/- functional assays and DNA extraction took place >72 hours post nucleofection. (D) Schematic representation of the codon divergent CTLA4 HDR repair template for correction of the p.T124P c.371A>C mutation. (E) Sanger sequence traces from unedited p.T124P c.371A>C cells (top) with the black arrow highlighting the heterozygous mutation. Following editing (bottom trace) the wild type sequence has been restored and the codon divergent repair template has introduced new mutations in the population (black arrows with dashed line).*

A

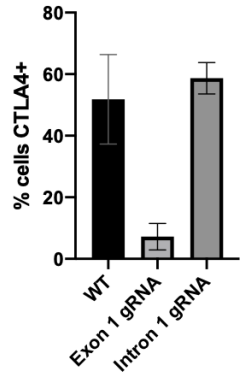


Three intronic gRNAs were assessed in WT T cells. Intron gRNA B created the highest number of indels and thus is referred to as gRNA4 throughout the manuscript.

B



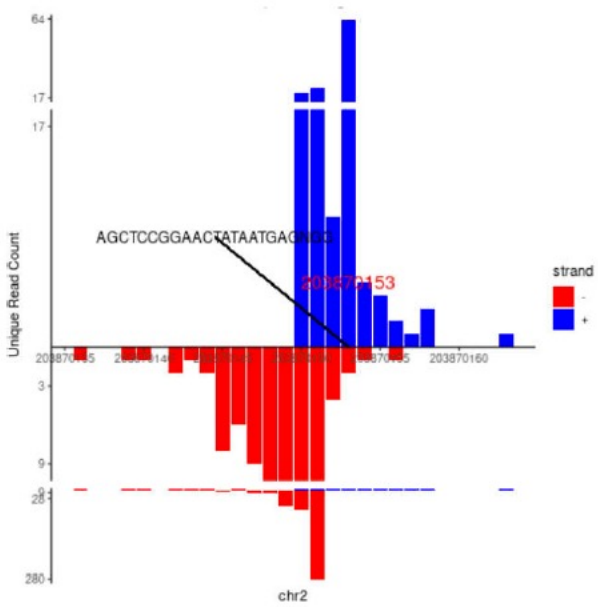
C



D

Sample	Unedited (% integration)	Edited (% integration)
1	0%	40%
2	0%	41%
3	0%	41%

E



F

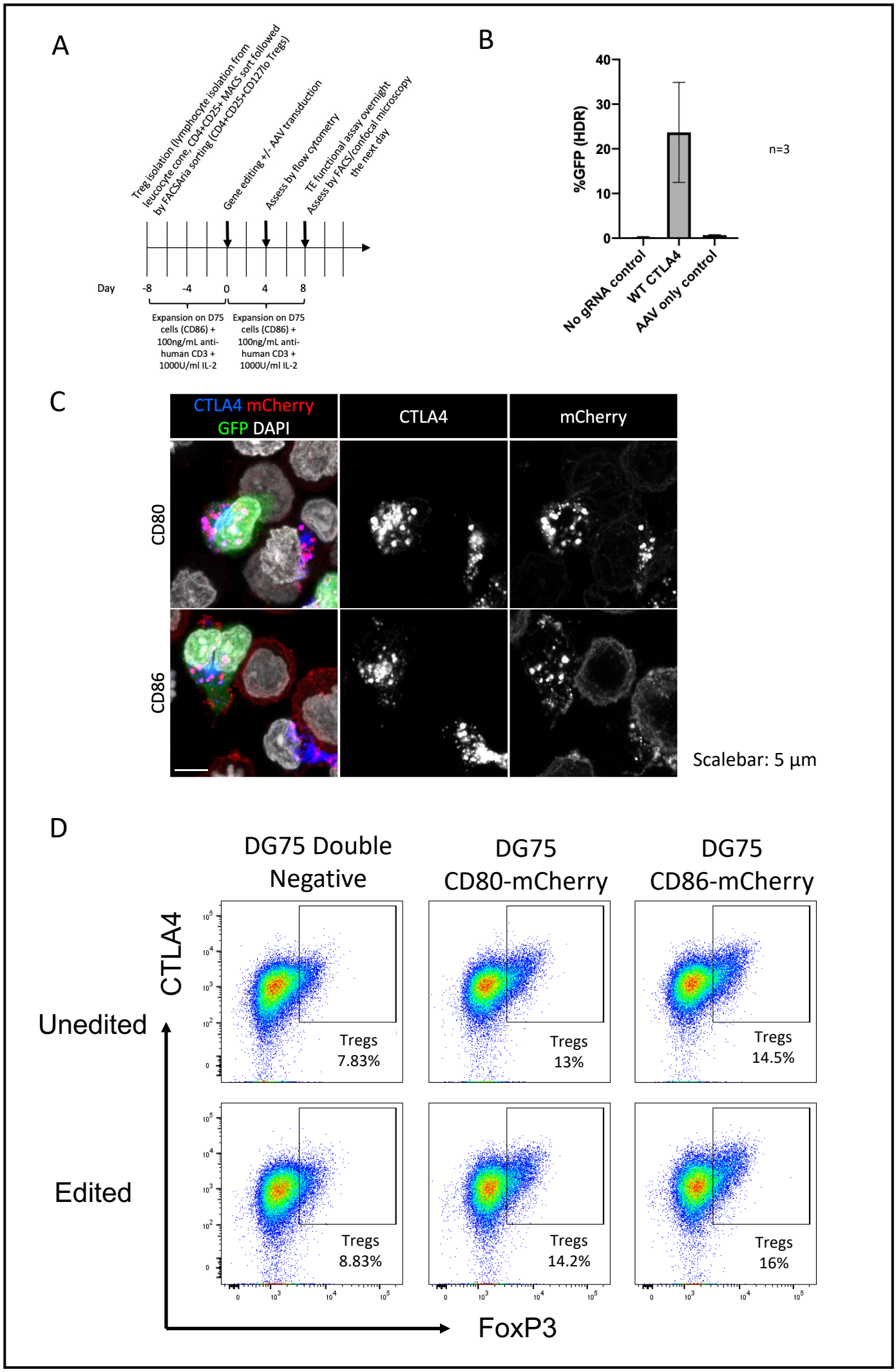
Off-target statistics

Sample	off-target numbers
CTLA4	0

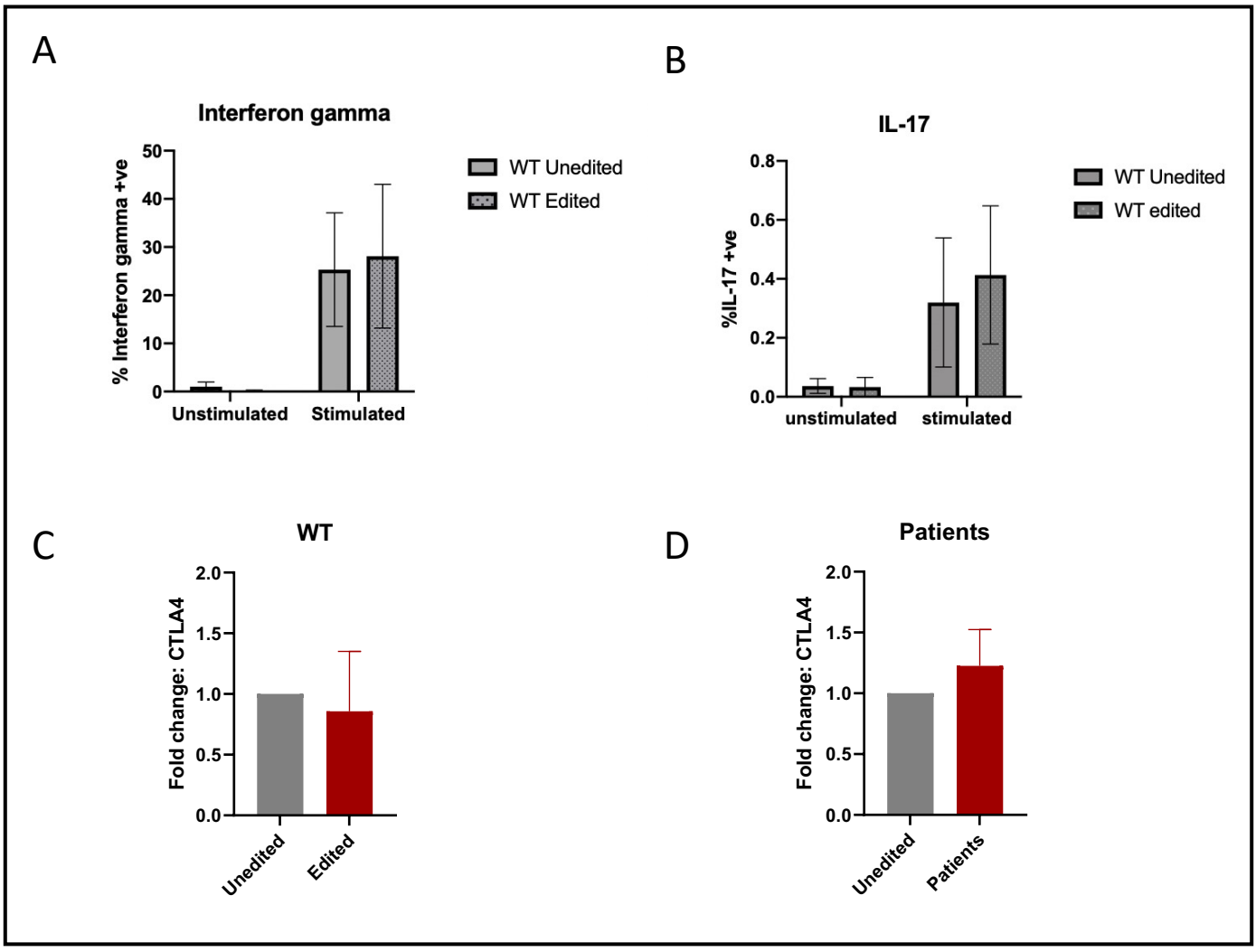
On-target Detail

Sample	BED Chromosome	On-target position	Strand	On-target Sequence	Mismatch
CTLA4	chr2	203870144 - 203870167	-	AGCTCCGGAACTATAATGAGTGG	0

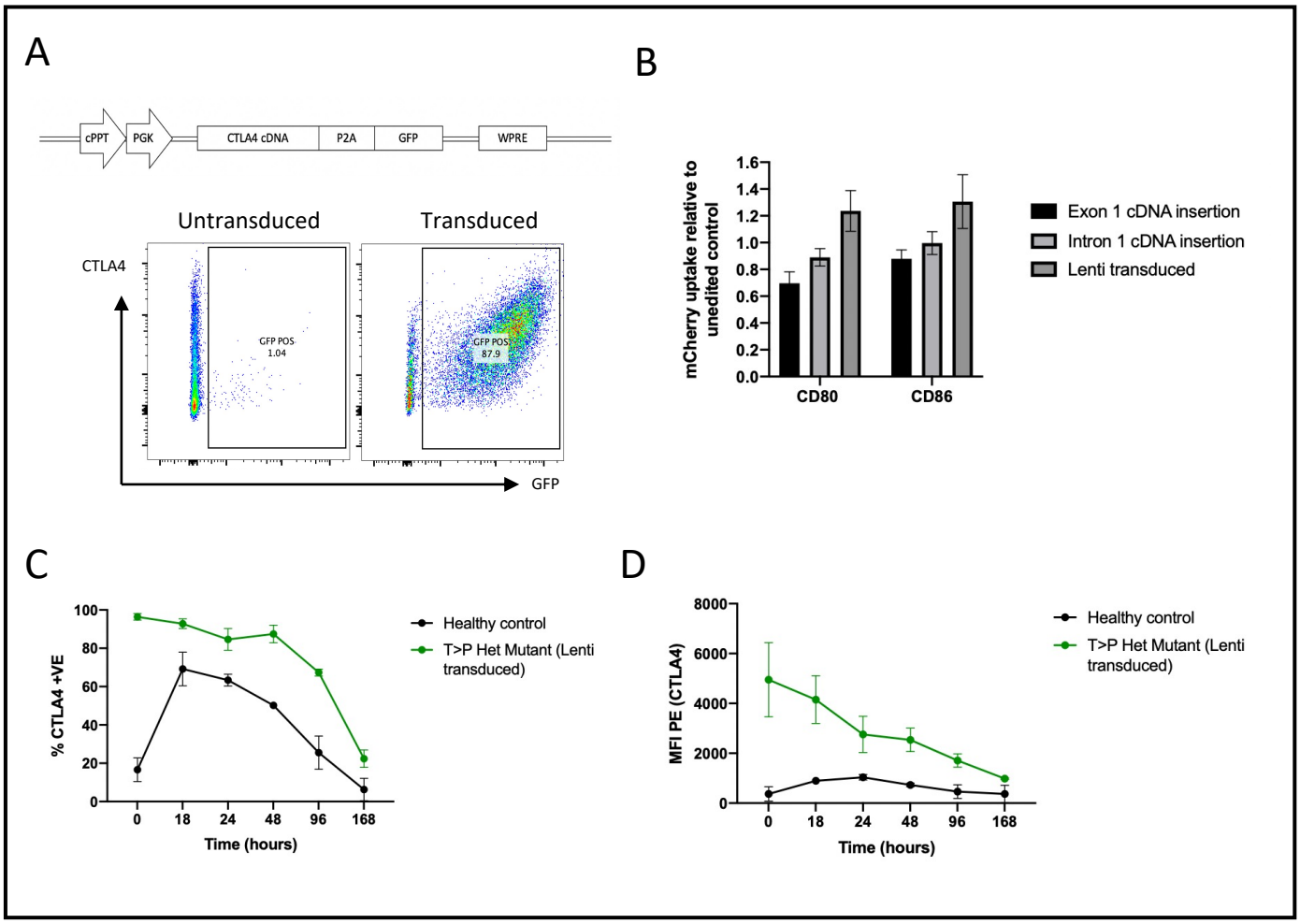
Supplementary Figure 2: Universal editing approach supplementary data: *(A) Representative FACs plots demonstrating normal CTLA4 surface expression following nucleofection of 3 different gRNAs targeting intron 1. (B) Results of the ICE analysis demonstrating the presence of indels in these same population of cells. The blue bars represent the ICE Score - The editing efficiency (percentage of the pool with non-wild type sequence). In the ICE algorithm, potential editing outcomes are proposed and fitted to the observed data using linear regression. The green bars represent the knockout (KO) score - the proportion of cells that have either a frameshift or 21+ bp indel. (C) Summary of experiments (n=3) showing the difference in surface CTLA4 expression between wild type unedited cells, cells edited with gRNA 3 (exon 1) and gRNA 4 (intron 1). (D) % integration calculated from ddPCR results following editing with gRNA 4 and donor 4. (E) GUIDE-seq analysis data showing on-target nuclease specificity of gRNA 4 and (F) off target specificity.*



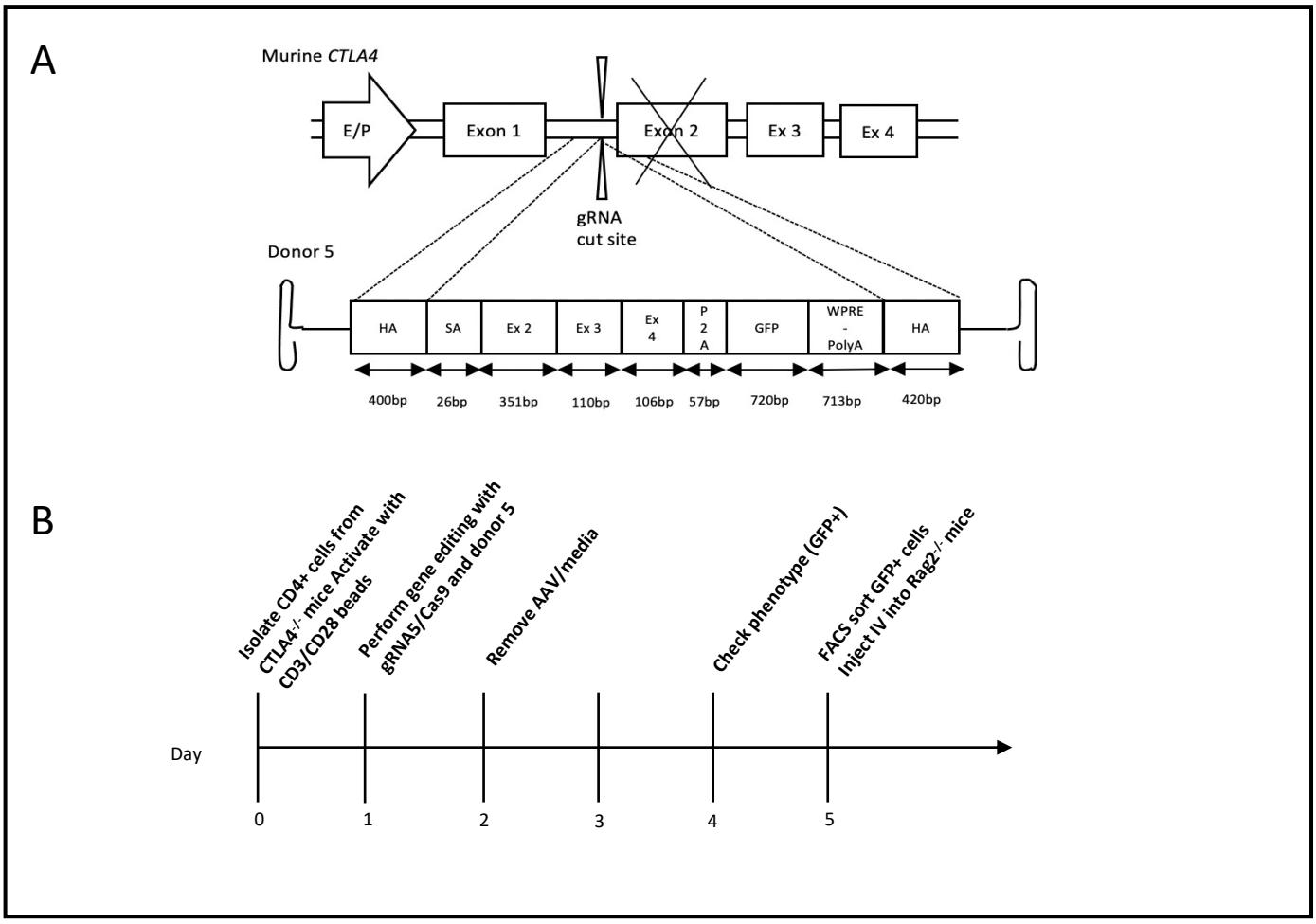
Supplementary Figure 3: Editing the Treg fraction alone: *(A) Schematic of experimental protocol for expansion and editing of Tregs. (B) HDR rates in healthy donor Tregs across three separate experiments from three healthy donors. (C) Confocal microscopy images of edited Treg (green and centre) following 6 hour TE. Co-localisation of CTLA4 with ligands CD80-mCherry and CD86-mCherry is observed. (D) Frequency of FOXP3+ amongst dividing unedited and edited T cells following incubation with either CD80 or CD86. CD86-CD28 interactions drive accumulation or Treg.*



Supplementary Figure 4: Stimulation and cytokine staining of edited cells: *(A) Interferon gamma production (% cells positive) in unedited and edited CD4+ T cells (n=3). (B) IL-17 production (% cells positive) in unedited and edited CD4+ T cells. (C) CTLA4 mRNA was assessed by RT-PCR in WT and (D) patient (left) unedited and edited T cells. Fold changes were calculated using the ΔΔCt method normalized to the levels of GAPDH and relative to unedited control.*



Supplementary Figure 5: Lentivirus gene addition approach: *(A) schematic representation of the PGK-CTLA4-P2A-GFP lentivirus vector (top) and representative FACS plot showing GFP expression following transduction of CD4+ cells with this vector. (B) Results of overnight TE assay with DG 75 cells expressing CD80-mCherry and CD86-mCherry demonstrated increased TE of ligand in the lentivirus transduced cells compared to wild type CD4+ T cells and edited CD4+ T cells. mCherry uptake relative to the unedited control in healthy CD4+ cells (gRNA 3 + donor 3 mean = 0.79, SD = 0.13, n=3, gRNA 4 +donor 4 mean = 0.94, SD = 0.08, n=3, lenti transduced mean=1.27, SD=0.05, n=3). (C) Percentage CTLA4 and (D) MFI of CTLA4 over time following stimulation demonstrating the difference in CTLA4 expression kinetics between lentivirus transduced cells and untransduced healthy control CD4+ cells.*



Supplementary Figure 6: Assessment of T cell GT for CTLA4 insufficiency using an in vivo murine model: *(A) Schematic representation of the murine T cell intronic editing strategy (donor 5 HA-splice acceptor-murine CTLA4 exons 2, 3, 4-P2A-GFP-WPRE-HA). (B) Schematic representation of the murine T cell editing protocol.*

Supplementary Methods

T_{reg} isolation and expansion

For T_{reg} isolation, $CD4^+$ T cells were enriched by addition of RosetteSep™ Human $CD4^+$ T Cell Enrichment Cocktail (Stemcell Technologies) to leukocyte cones diluted 1:5 with phosphate buffered saline (PBS), prior to as per manufacturer's instructions. Blood was layered over Ficoll-Paque PLUS (GE Healthcare) and centrifuged at 1200g for 25 minutes with slow acceleration and no brake. The CD4 enriched layer was collected, washed twice in PBS, before isolation by immunomagnetic positive selection using human CD25 MicroBeads II (Miltenyi Biotec) according to the manufacturer's instructions. Enriched $CD4^+CD25^+$ cells were stained using an antibody cocktail [Anti-CD4 (RPA-T4), anti-CD25 (3G10) and anti-CD127 (A019D5)] and FACSAria sorting was used to sort $CD4^+CD25^+CD127$lo T_{regs}. Sorted T_{regs} were expanded by plating at a 1:1 ratio with irradiated DG75 cell lines stably expressing CD86 co-stimulatory ligand in the presence of 1000 IU/ml IL2 (PeproTech) and 1µg/ml of anti-human-CD3 (OKT3, Biolegend), with IL2 replenished every 2-3 days.

T_{reg} stimulation assay

DG75 cell lines underwent CRISPR-Cas9 HDR editing to knock-out endogenous CD80 and CD86 co-stimulatory ligands (DG75-DN). Edited DG75 were transduced to stably express CD80-GFP or CD86-GFP. Stably transduced DG75-DN, -CD80-GFP or CD86-GFP were irradiated at 7500rads, and incubated with edited or unedited T cells at a 1:1 ratio in the presence of 1000 IU/ml IL2 (PeproTech) and 1µg/ml of anti-human-CD3 (OKT3, Biolegend) for 5 days, with IL2 replenished every 2-3 days. At Day 5 post-stimulation, T cells were fixed and permeabilized using the FoxP3 staining kit (eBioscience) and stained with anti-CTLA4-PE (clone BNI3; BD Biosciences) and anti-FoxP3 Pe-Cy7 (236A/E7; Thermo Fisher Scientific) before flow-cytometric analysis using an LSR Fortessa (BD Biosciences).

Imaging

DG75 expressing mCherry-tagged CD80 or CD86 and T_{reg} were seeded in a 2:1 ratio into 96 well U-bottom plates for 6 hours, washed with ice-cold PBS, resuspended in ice-cold 4 % paraformaldehyde (PFA) (Thermo Fisher Scientific, 28908) in PBS, transferred into 0.01% Poly-L-Lysine coated wells of an imaging plate (Greiner 655866, Sigma Aldrich A-005-C) and spun at 500 g for 20 min. PFA was quenched with 50 mM NH4Cl in PBS. Cells were then washed three times with PBS, permeabilized with 0.1% Saponin in PBS and stained with anti-CTLA4 clone C19 (Santa Cruz Biotechnology, sc-1628) in 0.1% Saponin supplemented with 5% bovine serum albumin (BSA) (staining buffer) at 4°C overnight. After three Saponin washes, cells were incubated with donkey-anti-goat secondary AlexaFluor647 antibody (4µg/ml Thermo Fisher Scientific, A-21447) and DAPI (2µg/ml, D9542) in staining buffer for 40 min at room temperature, washed three times in Saponin, two times in PBS and two times in miliQ water and mounted in Mowiol mounting medium with 2.5% 1,4-diazabicyclo-[2,2,2]-octane (DABCO). Imaging was performed on a Nikon Eclipse Ti confocal inverted laser scanning microscope equipped with a 60X oil-immersion objective (NA 1.4).

Determination of mRNAexpression by RT-PCR

Total RNA was extracted from cells using RNeasy miniprep kit (Qiagen) as per manufacturer's instructions. RNA was converted to complementary DNA using LunaScript RT SuperMix Kit (New England Biolabs) as per manufacturer's instructions. Relative expression of *CTLA4* were measured by qPCR using CFX96 Touch Real-Time PCR Detection System (BioRad), TaqMan Fast Universal PCR Master Mix (2X) No AmpErase UNG (Applied Biosystems) and TaqMan FAM-labelled probes (Thermo Fisher Scientific: CTLA4 Hs00175480_m1, GAPDH:

Hs02786624_g1). Fold changes were calculated using the ΔΔCt method and results were normalized to the amounts of GAPDH.