Molecular Therapy
Methods & Clinical Development
Original Article

A novel FOXP3 knockout-humanized mouse model for pre-clinical safety and efficacy evaluation of Treg-like cell products

Yohei Sato,[1] Abinaya Nathan,[2] Suzette Shipp,[1] John Fraser Wright,[2] Keri Marie Tate,[3] Prachi Wani,[3] Maria-Grazia Roncarolo,[1,2,4] and Rosa Bacchetta[1,2]

[1]Department of Pediatrics, Division of Hematology, Oncology, Stem Cell Transplantation and Regenerative Medicine, Stanford University School of Medicine, Lorry I. Lokey Stem Cell Research Building, 265 Campus Drive West, Room 3039, Stanford, CA 94305, USA; [2]Center for Definitive Curative Medicine (CDCM) Stanford University School of Medicine, Lorry I. Lokey Stem Cell Research Building, 265 Campus Drive West, Room 3039, Stanford, CA 94305, USA; [3]Laboratory for Cell and Gene Medicine (LCGM) Stanford University School of Medicine, Lorry I. Lokey Stem Cell Research Building, 265 Campus Drive West, Room 3039, Stanford, CA 94305, USA; [4]Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Lorry I. Lokey Stem Cell Research Building, 265 Campus Drive West, Room 3039, Stanford, CA 94305, USA

Forkhead box P3 (FOXP3) is an essential transcription factor for regulatory T cell (Treg) function. Defects in Tregs mediate many immune diseases including the monogenic autoimmune disease immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which is caused by *FOXP3* mutations. Treg cell products are a promising modality to induce allograft tolerance or reduce the use of immunosuppressive drugs to prevent rejection, as well as in the treatment of acquired autoimmune diseases. We have recently opened a phase I clinical trial for IPEX patients using autologous engineered Treg-like cells, $CD4^{LVFOXP3}$. To facilitate the pre-clinical studies, a novel humanized-mouse (hu-mouse) model was developed whereby immune-deficient mice were transplanted with human hematopoietic stem progenitor cells (HSPCs) in which the *FOXP3* gene was knocked out (FOXP3KO) using CRISPR-Cas9. Mice transplanted with *FOXP3KO* HSPCs had impaired survival, developed lymphoproliferation 10–12 weeks post-transplant and T cell infiltration of the gut, resembling human IPEX. Strikingly, injection of $CD4^{LVFOXP3}$ into the FOXP3KO hu-mice restored *in vivo* regulatory functions, including control of lymphoproliferation and inhibition of T cell infiltration in the colon. This hu-mouse disease model can be reproducibly established and constitutes an ideal model to assess pre-clinical efficacy of human Treg cell investigational products.

INTRODUCTION

Regulatory T cells (Tregs) play a central role in controlling immune responses[1,2] and their clinical application as "living drugs" has gained increasing interest in the past decade.[3] Currently, there are about 30 clinical trials registered on clinicaltrial.gov using Treg cells products, mostly for the prevention of graft-versus-host disease (GVHD) or organ rejection, but also for type 1 diabetes, rheumatoid arthritis, and skin autoimmunity. Most of the Treg products used to date derive from peripherally isolated Treg and are infused fresh or after expansion *in vitro*. Both allogeneic and autologous Treg cell products, polyclonal rather than antigen specific, have been developed and demonstrated safety in phase I clinical trials.[4] In addition, engineered Treg products are now being tested in the clinic. Indeed, polyclonal autologous $CD4^{LVFOXP3}$ Treg-like cells to treat immunedyregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) as the first indication (NCT05241444),[5] and chimeric antigen receptor (CAR)-Treg human leukocyte antigen (HLA)-A2 specific for the prevention of organ rejection (NCT04817774)[6] have just entered the clinical phase. Although generally found to be safe, many questions remain such as optimal cell dose, long-term survival, expansion, elimination and tracking *in vivo*, and the impact on the recipient's immune response to pathogens or immune surveillance.

During investigational new drug (IND)-enabling studies for the $CD4^{LVFOXP3}$ in IPEX, monogenic autoimmune disease due to Forkhead box P3 (FOXP3) mutations and dysfunctional Treg cells, we developed humanized-mouse models by transplanting human peripheral blood mononuclear cells (PBMCs) or CRISPR-Cas9 *FOXP3* gene edited hematopoietic stem progenitor cells (HSPCs) to enable *in vivo* functional characterization of the product. We have previously generated IPEX-like mouse models via *FOXP3* knock-out (KO) or knockdown (KD) in human HSPCs using various molecular approaches, including (shRNA), TALENs and CRISPR-Cas9.[7,8] Although informative, the FOXP3KO or KD short hairpin RNA efficiencies in these prior experiments did not result in a full KO of FOXP3, limiting the applicability of the model. Specifically, using a single-guide RNA (sgRNA) targeting the *FOXP3* gene and the CRISPR-Cas9 strategy,

Received 31 August 2023; accepted 3 November 2023;
https://doi.org/10.1016/j.omtm.2023.101150.

Correspondence: Rosa Bacchetta, MD, Lorry I. Lokey Stem Cell Research Building, 265 Campus Drive West, Room 3039, Stanford, CA 94305, USA.
E-mail: rosab@stanford.edu

we obtained ~40% FOXP3 gene disruption in edited HSPCs, which was insufficient to confer severe lymphoproliferation when transplanted into the immunodeficient NSG mice.[8] Here, we describe a strategy and protocol that dramatically increases FOXP3KO efficiency and leads to significant disease manifestations in transplanted hu-mice, rendering this a more robust model of Treg-deficient autoimmunity. Importantly, we also show that the investigational product, $CD4^{LVFOXP3}$, can rescue in this model the FOXP3KO-mediated disease features. The KO strategy targets the FOXP3 locus in HSPCs with multiple sgRNAs and achieves >75% FOXP3KO efficiency by creating a 122-bp deletion in the target locus. Immunodeficient NSG mice transplanted with these *FOXP3*KO HSPCs developed severe lymphoproliferation 10–12 weeks after transplantation and T cell infiltration in the gut, resulting in increased morbidity and mortality compared with mice transplanted with *FOXP3* wild-type (WT) HSPCs. We demonstrate that the administration of $CD4^{LVFOXP3}$ cells increased survival when compared with the uninjected *FOXP3KO* mice. Moreover, $CD4^{LVFOXP3}$ injection did not prevent immune reconstitution but did prevent $CD4^+$ T cell lymphoproliferation and organ infiltration. These data confirm that (i) these *FOXP3KO* hu-mice generated by high and efficient CRISPR-Cas9 mediated gene disruption are a valuable pre-clinical model to test the safety and efficacy of Treg-like cell products; and (ii) the administration of $CD4^{LVFOXP3}$ is safe and efficacious in preventing $CD4^+$ lymphoproliferation in a hu-mouse model. The proposed experimental protocol is well adapted in pre-clinical experiments to test different types of human Treg cell products and compare their efficacy and safety. This model could also be suitable to investigate *in vivo* challenge with pathogens and tumor antigens.

RESULTS

High efficiency FOXP3 KO by multiple sgRNA CRISPR-Cas9-mediated gene disruption in HSPCs to generate *FOXP3* KO hu-mice

With the objective of achieving high-efficiency FOXP3KO in HSPCs, we screened nine sgRNAs targeting exons 1 and 3 of the *FOXP3* locus separately or in combination *in vitro* (Figure 1A and Table S1). The highest insertion or deletion (INDEL) efficiency (75.0% ± 5.0%, mean ± SEM; n = 3) was obtained using the combination of sgRNA 4, 5, and 6 targeting exon1 in human HSPCs (1e5 cells/condition, n = 3 healthy donors) (Figure 1B). These sgRNAs can create a ~112-bp deletion adjacent to the start codon, disrupting the open reading frame and limiting the potential for residual FOXP3 protein expression (Figure 1C). We next tested whether, in contrast with our prior attempts, the increased efficiency of FOXP3KO in HSPCs confers a more robust IPEX-like phenotype when transplanted into immunodeficient mice.

FOXP3KO HSPCs were generated by electroporation of the ribonucleoprotein (RNP) complex (*S. Pyogenes* (sp) Cas9 protein and sgRNAs 4, 5, and 6) into HSPCs (2e6 cells/condition). The average INDEL frequencies in HSPCs measured by PCR-based INDEL frequency analysis at days 3–5 post editing was 75.3% ± 2.8% (mean ± SEM, n = 6), which was similar to the INDEL frequencies obtained in the small scale (1e6) sgRNA screening (Figure 2B). Neonatal NSG mice (2–3 days old) were injected intrahepatically with *FOXP3KO* HSPCs (1.5e5 cells/animal) after 1 Gy irradiation (Figure 2A).

The *FOXP3KO* hu-mice showed significantly reduced weight gain and increased mortality compared with the *FOXP3* WT hu-mice (body weight at 16 weeks, *FOXP3* WT hu-mice = 21.1 ± 0.4 g, *FOXP3* KO hu-mice = 18.9 ± 0.4 g, n = 16 per condition, mean ± SEM, $p < 0.05$; Survival rates at 16 weeks: *FOXP3* WT hu-mice = 100%, *FOXP3* KO hu-mice = 75%, n = 16 per condition, $p < 0.05$) (Figure 2C). These data indicated that *FOXP3* KO hu-mice had higher mortality and morbidity compared with the *FOXP3* WT hu-mice.

The *FOXP3* INDEL frequencies, measured in the infused cells and at week 16 after transplantation, remained constant throughout the *in vivo* experiment (16 weeks after transplantation 74.7% ± 2.6%, mean ± SEM, n = 6), indicating persistency and as well as selective advantage of the KO cells. The NSG mice receiving *FOXP3KO* HSPCs consistently developed $CD4^+$ T cell lymphoproliferation between 8 and 12 weeks after transplantation (Figure 2D). Therefore, we used this model to test the efficiency of the autologous engineered $CD4^{LVFOXP3}$ Treg-like cells to prevent the FOXP3KO aberrant immune phenotype.

$CD4^{LVFOXP3}$ cells generated from peripheral and cord blood by cyclic guanosine monophosphate-compatible protocol have Treg-like phenotype and function *in vitro*

Building on the *in vitro* and *in vivo* IND-enabling studies, a $CD4^{LVFOXP3}$ cell manufacturing protocol was developed and optimized to be appropriate for clinical grade production (Figure S1) using the cyclic guanosine monophosphate-compatible lentiviral vector, lentiviral (LV)-FOXP3.[4] The resulting $CD4^{LVFOXP3}$ cells obtained from PB (n = 25) were comparable with those obtained in the research laboratory, as previously described.[4,7] $CD4^{LVFOXP3}$ cells displayed a Treg-like phenotype, analogous to freshly isolated Tregs (Figures S2 and S3A), which was in contrast to control cells transduced with LV-NGFR ($CD4^{LVNGFR}$) or untransduced ($CD4^{UT}$) that were obtained in parallel (Table S2).

Similar to *in vitro* activated Tregs, $CD4^{LVFOXP3}$ cells had reduced proliferative capacity, unlike $CD4^{LVNGFR}$ and $CD4^{UT}$ cells (Figure S3B). Moreover, $CD4^{LVFOXP3}$ cells showed a dose-dependent suppressive function that was significantly higher than $CD4^{LVNGFR}$ cells and similar to that of *in vitro* activated Tregs (Figure S3C). As previously described,[9,10] the $CD4^{LVFOXP3}$ cells had a Treg-like cytokine production profile including reduced production of proinflammatory cytokines, IL-2, IL-4, IL-17A, and interferon (IFN)-γ that was similar to that of *in vitro* activated Tregs (n = 4), and again contrasted with that observed from $CD4^{LVNGFR}$ and $CD4^{UT}$ cells (n = 8) (Figure S3D). While $CD4^{LVFOXP3}$ cells have reduced production of the above proinflammatory cytokines, the production of IL-10 and IL-22 was preserved, as is the case in *in vitro* activated Tregs (Figure S3D).

To test the $CD4^{LVFOXP3}$ cells in FOXP3KO hu-mice, $CD4^{LVFOXP3}$ cells were generated from umbilical cord blood donor cells autologous

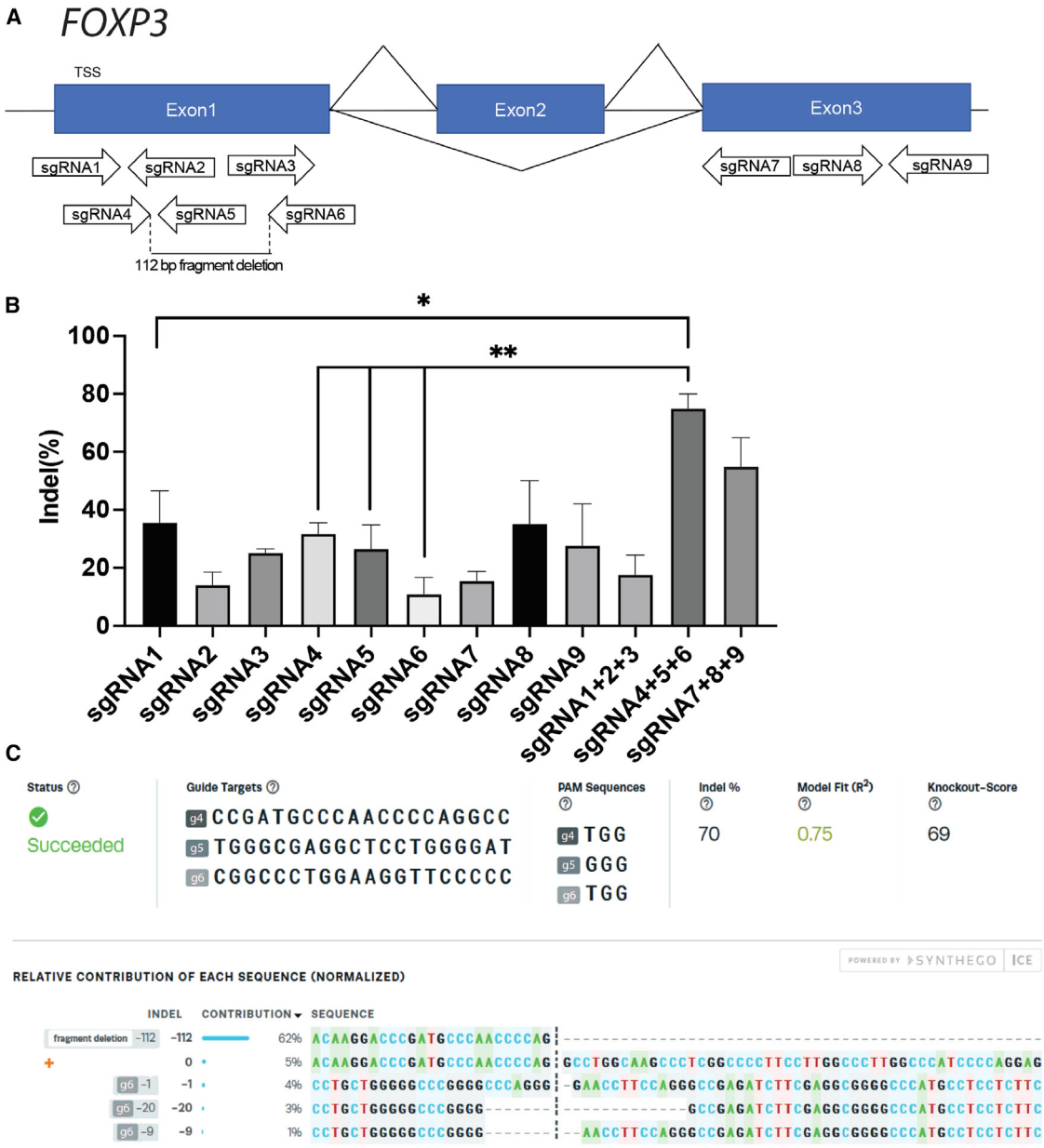


Figure 1. FOXP3 locus can be efficiently targeted by multiple sgRNAs

(A) sgRNAs against FOXP3 exon1 and exon3. TSS, transcription start site. (B) Screening of the sgRNAs against FOXP3 locus. (C) Representative image of multiple sgRNA FOXP3 KO (sgRNA4, 5, and 6) analyzed by ICE software. Significance determined by one-way ANOVA followed by Tukey's multiple comparison tests. *p < 0.05. Data are representative of three independently repeated experiments.

to the CD34^{+} HSPCs used in the various *in vivo* hu-mice experiments (n = 15). The phenotypic and functional characteristics of CD4^{LVFOXP3} cells generated from umbilical cord blood (CB-CD4^{LVFOXP3}) resembled those of CD4^{LVFOXP3} cells obtained from PB (Figures S4A and S4B).

Collectively, CD4^{LVFOXP3} cells generated either from PB or umbilical cord blood according to a good manufacturing practices-compatible production protocol, acquire phenotypical and functional characteristics comparable to freshly isolated and *in vitro* activated Tregs.

Autologous CD4^{LVFOXP3} cells can mitigate lymphoproliferation in FOXP3KO hu-mice

To test whether *FOXP3KO* hu-mice are a suitable model to assess the *in vivo* suppressive function of CD4^{LVFOXP3} cells, autologous CD4^{LVFOXP3} cells (1e6 cells/condition) were injected in the *FOXP3KO*

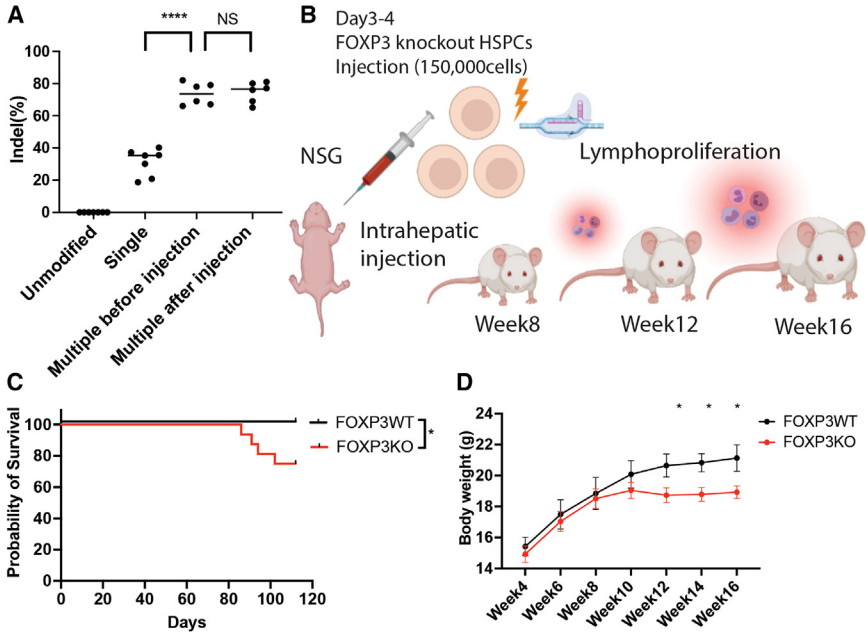


Figure 2. In *FOXP3* KO hu-mice morbidity and mortality

(A) Frequency of INDEL were measured by ICE analysis of HSPCs (n = 6–7/conditions, mean ± SEM). (B) Schematic representation of the experimental setup. NSG, NOD.Cg-Prkdc scid Il2rg tm1Wjl/SzJ. (C) Survival curve and (D) body weight of *FOXP3* KO hu-mice model comparing mice transplanted with *FOXP3* WT HSPC (FOXP3WT), *FOXP3* KO HSPC alone (FOXP3KO) (n = 16/group). Significance determined by one-way ANOVA followed by Tukey's multiple comparison tests. *p < 0.05, ****p < 0.0001. Data are representative of four independently repeated experiments.

hu-mice at week 12 (Figure 3A), when $CD4^+$ T cell lymphoproliferation starts to manifest. Mice injected with $CD4^{LVFOXP3}$ cells were monitored for 4 additional weeks. Administration of $CD4^{LVFOXP3}$ cells significantly improved overall body weight and survival of the *FOXP3KO* hu-mice (Figures 3B and 3C). In contrast, $CD3^+/CD4^+$ T cell peripheral lymphoproliferation in *FOXP3KO* hu-mice who did not receive $CD4^{LVFOXP3}$ cells, progressively developed from week 12 through week 16 (frequency of $CD4^+$ T cells out of $CD3^+$ T cells: week 12 = *FOXP3* WT hu-mice 24.4% ± 4.6%, *FOXP3* KO hu-mice 61.5% ± 4.3%; week 16 = *FOXP3* WT hu-mice 25.2% ± 5.4%, *FOXP3* KO hu-mice 70.5% ± 3.9%, n = 16 per condition, mean ± SEM) (Figures 3D and 3E).

Injection of autologous $CD4^{LVFOXP3}$ cells in *FOXP3KO* hu-mice suppressed the $CD3^+/CD4^+$ T cell lymphoproliferation up to 37% at sacrifice (week 16), reaching a frequency of $CD3^+/CD4^+$ T cells similar to control hu-mice transplanted with WT *FOXP3* HSPCs (n = 16 per condition) (Figures 3D, 3E, and S5A). In contrast with the $CD4^+$ T cell compartment, the $CD8^+$ T cells did not expand *in vivo* in the *FOXP3KO* hu-mice and their frequency remained similar among the three different conditions.

At sacrifice, we observed an increase in the $CD4^+$ T cell compartment in the spleen of *FOXP3KO* hu-mice, which was significantly ameliorated in the hu-mice that received $CD4^{LVFOXP3}$ cell infusion (frequency of $CD4^+$ T cells out of CD3+ T cells in week 16: *FOXP3* WT hu-mice 46.5% ± 2.8%, *FOXP3KO* hu-mice 66.7% ± 2.7%, *FOXP3KO* hu-mice injected with $CD4^{LVFOXP3}$ cells 49.4% ± 3.9%, n = 12–16 per condition, mean ± SEM) (Figures 4A, 4B, and S5B). Moreover, in the *FOXP3KO* hu-mice, we detected reduced naive $CD4^+$ T cells and increased effector and central memory $CD4^+$ T cells as compared with the *FOXP3* WT hu-mice (Figure 4C), whereas in the $CD4^{LVFOXP3}$-treated *FOXP3KO* hu-mice, the proportion of the memory cell compartments normalized (Figure 4C).

Analysis of the spleen showed that $CD45^+$ human cell engraftment and multilineage reconstitution ($CD13^+$, $CD19^+$, $CD56^+$) were not significantly affected in the *FOXP3KO* hu-mice, with or without $CD4^{LVFOXP3}$ treatment (Figures S5A and S5B). Therefore, the *FOXP3KO* hu-mice primarily resulted in the expansion of mainly memory $CD3^+/CD4^+$ T cell compartment, which was selectively suppressed and normalized by the $CD4^{LVFOXP3}$ treatment. Overall, the effect of $CD4^{LVFOXP3}$ was more robust in this FOXP3KO humanized-mouse (hu-mouse) model than previously observed.[8]

To further validate the effects of CRISPR-Cas9 mediated *FOXP3*KO, we analyzed the Treg phenotype in the spleen of *FOXP3*KO hu-mice at sacrifice. We detected Tregs ($CD4^+CD25^+CD127^-$) that expressed FOXP3 in the spleen of *FOXP3* WT hu-mice, while FOXP3 expression was absent in Tregs in the *FOXP3*KO hu-mice (Figures 5A and 5B). These data demonstrate that FOXP3 expression is fully abrogated and result in severe immune dysregulation in the *FOXP3*KO hu-mice, thus providing a model that is suitable for pre-clinical functional studies of Treg-like cell products.

$CD4^{LVFOXP3}$ cells mitigated gut infiltration of FOXP3-deficient T cells

We previously showed that *FOXP3* partial KO resulted in the mild $CD4^+$ T cells infiltration in the colon.[8] We hypothesized that (i) the lack of the human MHC molecules in the NSG mouse recipient of the human cells made it difficult for FOXP3 deficient T cells to migrate into the colon and (ii) incomplete FOXP3KO resulted in the insufficient dysregulation of engrafting T cells in the humanized mice. As it is difficult to obtain human HSPCs with the specific HLA subtypes (i.e., HLA-DR1 and HLA-DR4) that are identical to those expressed by immunodeficient human-HLA transgenic mice (i.e., NSG-DR1 and NSG-DR4), it was not feasible to conduct pre-clinical safety studies using these models. Therefore, we focused on

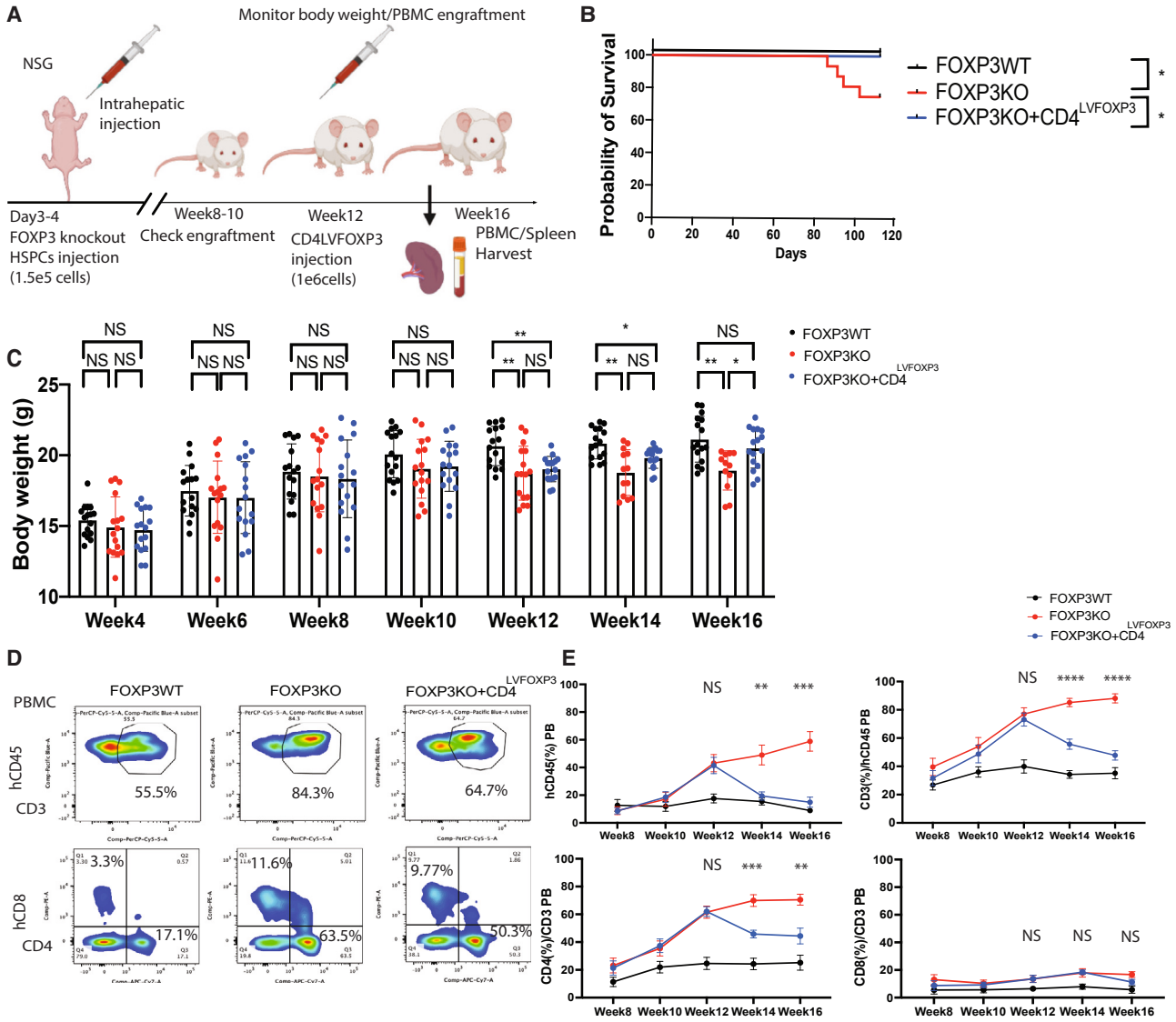


Figure 3. CD4^{LVFOXP3} cells can ameliorate CD4 dominant lymphoproliferation in *FOXP3* KO hu-mice

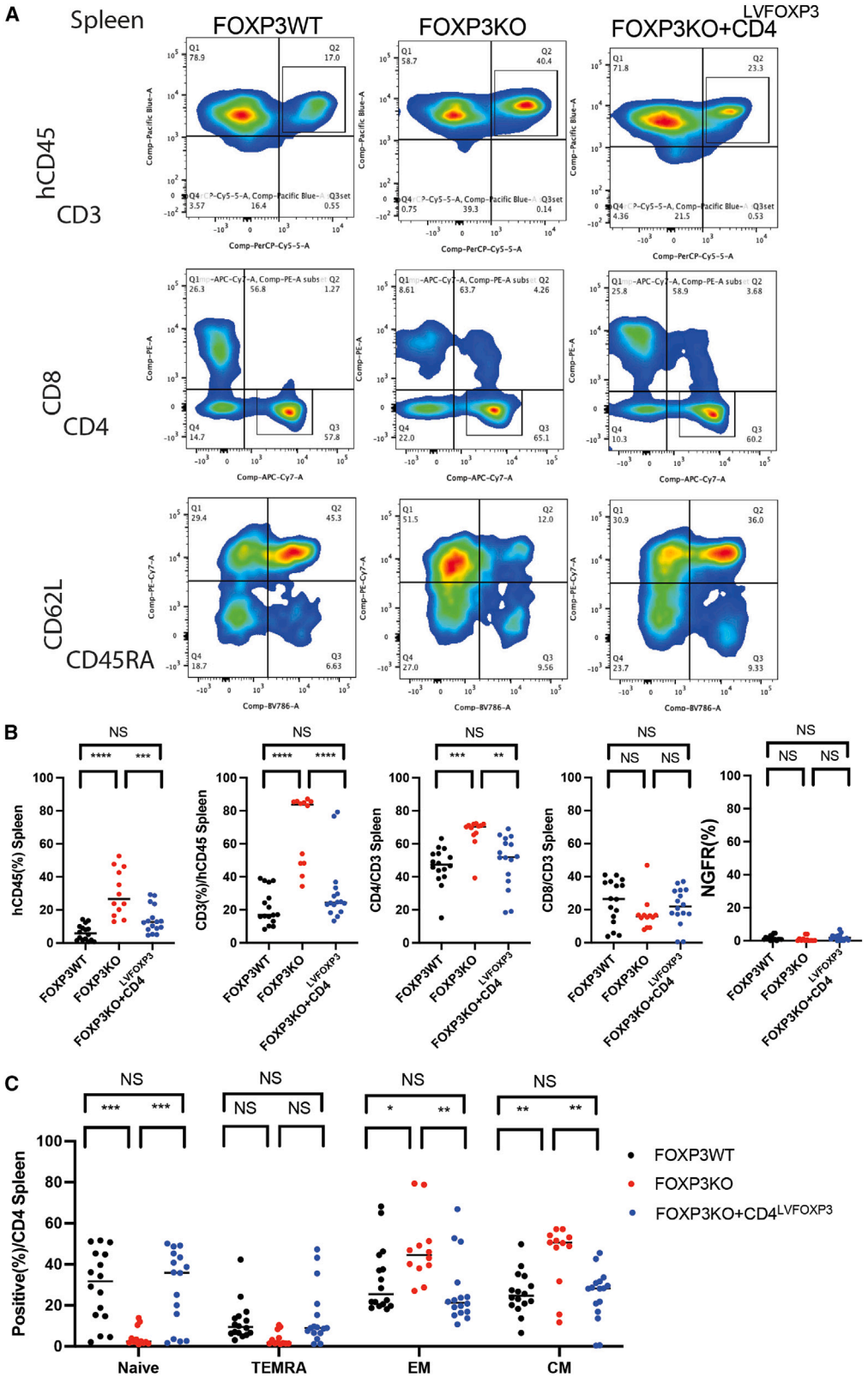
(A) Schematic representation of the experimental setup. (B) Survival curve and (C) body weight of *FOXP3* KO hu-mice model comparing mice transplanted with *FOXP3* KO HSPC alone (FOXP3KO) and *FOXP3* KO HSPC treated by CD4^{LVFOXP3} (FOXP3KO + CD4^{LVFOXP3}) (n = 16/group). (D) Representative flow cytometry dot plots. (E) Percentage of hCD45$^+$/CD3$^+$/CD4$^+$/CD8$^+$ cells in PB between weeks 8 and 16. (n = 12–16, mean ± SEM). Data are representative of four independently repeated experiments. All phenotypes were performed by FACS. Significance determined by one-way ANOVA followed by Tukey's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

addressing the second hypothesis by using the effective FOXP3KO in HSCPs from a single cord blood sample. The multiple sgRNA strategy was sufficient to produce 8–12 FOXP3KO hu-mice, as well as 3–5 FOXP3 WT hu-mice across different HSPCs donors (n = 6) and CD4^{LVFOXP3} cells all being generated from the same donor.

Therefore, we histologically analyzed the colon in the *FOXP3*KO hu-mice obtained from cord blood cells. We did not detect CD3$^+$/CD4$^+$ T cells in the colon of FOXP3 WT hu-mice, regardless of the level of human cell engraftment (Figure 6A). However, CD3$^+$/CD4$^+$ T cell infiltration was observed in the colon of the *FOXP3*KO hu-mice, but not when these mice were treated with CD4^{LVFOXP3} cells (Figure 6B). These data support that infusion of the engineered CD4^{LVFOXP3} cells could be clinically beneficial.

CD4^{LVFOXP3} cells are responsive to anti-CD25 antibody (basiliximab)-mediated reduction *in vivo*

Basiliximab is a clinically approved antibody specific for IL-2R alpha chain (CD25) and used as an immunomodulant in GVHD or graft rejection to prevent or control undesired excessive immune responses



(legend on next page)

driven by activated CD25$^+$ T cells.[11,12] CD4^{LVFOXP3} cells, similar to freshly isolated Tregs, express high levels of membrane-bound CD25, which is directly upregulated by FOXP3.[8,13] Thus, we tested whether basiliximab administration could be used to decrease the number of CD4^{LVFOXP3} cells, as a rescue approach to mitigate excessive immunosuppression *in vivo* of the CD4^{LVFOXP3} cell infusion.

To determine whether CD4^{LVFOXP3} cells can be efficiently reduced *in vivo*, hu-mice were established by intrahepatic injection of human CD34$^+$ HSPCs from 3 different donors into 3- to 4-day-old NSG-SGM3 pups after 1 Gy irradiation as previously described (Figure 7A). At weeks 8–10 after injection, average human cell engraftment (%hCD45) in PB from hu-mice was 59.0% ± 4.3% (mean ± SEM, n = 18). Thereafter, autologous CD4^{LVFOXP3} cells derived from three healthy donors were injected into the hu-mice 10 weeks post-transplant (n = 12). After 72 h from the CD4^{LVFOXP3} cells injection, basiliximab (25 μg per mouse) was administered to one-half of the mice that received CD4^{LVFOXP3} cells (6 mice out of the total 12 mice receiving CD4^{LVFOXP3} cells) and the effect on the CD4^{LVFOXP3} cell persistence were assessed 7 days after the basiliximab administration (at sacrifice).

At sacrifice, there were no significant changes in the peripheral engraftment among different groups. NGFR$^+$ cells were clearly detected in the PB and spleen after CD4^{LVFOXP3} cell infusion (NGFR$^+$ cells out of hCD4$^+$ cells in PB, control 1.4% ± 0.3%, CD4^{LVFOXP3} cells 5.7% ± 1.7%, mean ± SEM, n = 6) (Figure 7B). Upon basiliximab administration, the percentage of NGFR$^+$ cells was reduced by 85% in the PB (NGFR$^+$ cells out of hCD4$^+$ cells in PB, CD4^{LVFOXP3} cells treated with basiliximab, 0.8% ± 0.3%, mean ± SEM, n = 6) (Figures 7B and S7). Similarly, in the spleen the percentage and absolute counts of NGFR$^+$ cells after CD4^{LVFOXP3} cell infusion (NGFR$^+$ cells out of hCD4$^+$ cells in spleen, control 1.3 ± 0.5%, CD4^{LVFOXP3} cells 5.0% ± 1.2%, mean ± SEM, n = 6) were significantly reduced by basiliximab administration (NGFR$^+$ cells out of hCD4$^+$ cells in spleen, CD4^{LVFOXP3} cells treated with basiliximab, 0.3% ± 0.1%, mean ± SEM, n = 6) (Figures 7C and S8). In the spleen, we also observed a significant reduction in CD3$^+$, CD4$^+$ and CD8$^+$ T cells after CD4^{LVFOXP3} cells infusion, which was normalized by basiliximab administration (Figures 7C and S8). In contrast, injection of CD4^{LVFOXP3} cells with or without basiliximab did not alter the percentage of CD19, CD13, and CD56$^+$ cells, indicating that multilineage differentiation capacity of HSPCs at week 11 was not impaired (Figure 7D).

These data suggest that basiliximab administration could be used *in vivo* as a safety procedure to decrease the number of CD4^{LVFOXP3} cells.

DISCUSSION

In vivo animal models are a critical component of pre-clinical studies for gene and cell therapy products.[14,15] Among various animal models, humanized mice models are often used to test the efficacy and safety of translational biomedical products, including cell and gene therapy approaches.[16] The xeno-GvHD mice model, frequently used in pre-clinical studies, can assess the efficacy of polyclonal expanded Tregs to control a xenogenic immune reaction, resembling GVHD.[17–19] However, this model does not have the intrinsic autologous immune dysregulation, such as the lymphoproliferative phenotype, observed in the IPEX syndrome. In addition, the xeno-GvHD model cannot allow for a broad evaluation of the effects of CD4^{LVFOXP3} cells on lymphoid reconstitution, due to the limited time of mice survival and the infusion of only mature T cells in this model.[10] Therefore, to overcome these limitations, we previously developed a hu-mice model with *FOXP3* KD and partial FOXP3 KO in HSPC.[8,20] But while these unique hu-mice models allowed us to validate *in vivo* safety and efficacy profile of autologous CD4^{LVFOXP3} cells that are currently in phase I clinical trial (NCT 05241444),[5] they did not fully recapitulate the IPEX disease phenotypes. Here, we developed an improved *FOXP3* KO strategy in HSPC by combining multiple sgRNA, which completely abrogates FOXP3 expression in the resulting Treg population of the hu-mice. Moreover, in this newly refined model, we observe infiltration of HSPC-derived immune reconstituting CD4$^+$ T cells in the gut tissue, which is a typical disease hallmark of patients with IPEX syndrome and could not be observed in the previous partial *FOXP3KO* hu-mice. At the same time, we showed that transfer of the CD4^{LVFOXP3} cells in the improved FOXP3KO hu-mice, controlled the lymphoproliferation, especially of the memory CD4$^+$ T cell compartment, and organ infiltration, thus significantly prolonging animal survival. Notably, transfer of CD4^{LVFOXP3} cells did not impact reconstitution of the other main lymphocyte subsets. These findings, in addition to further supporting the clinical translation of CD4^{LVFOXP3} cells for IPEX, demonstrate the suitability of this model for pre-clinical IND-enabling studies to test different types of Treg cells under development for clinical application independently of the disease indication.

In this study, it was not possible to perform TCR analysis of T cell compartment of our FOXP3 KO humanized mice due to the limited cell numbers. However, in our previous work,[7] in which we downregulated FOXP3 expression in human HSC by shRNA, we observed peripheral effector T cell expansion with polyclonal TCR repertoire and increased diversity (reduced clonality) suggesting altered negative selection. To characterize more in detail the FOXP3KO T cell compartment was beyond the scope of the current work, however, it could be speculated that T cells in our FOXP3 KO mice might contain more autoreactive T cells. In addition, our previous studies in T cell clones obtained from IPEX patients with a FOXP3-null mutation showed

Figure 4. CD4^{LVFOXP3} cells can ameliorate imbalance between naive/memory CD4$^+$ T cell populations in *FOXP3* KO hu-mice

(A) Representative flow cytometry dot plots. (B) Percentage of hCD45$^+$/CD3$^+$/CD4$^+$/CD8$^+$ cells in spleen in week 16 (n = 12–16, Median). (C) Naive/memory phenotype of CD4$^+$ T cells in spleen in week 16 (n = 12–16, median). Data are representative of four independently repeated experiments. All phenotypes were performed by FACS. Significance determined by one-way ANOVA followed by Tukey's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

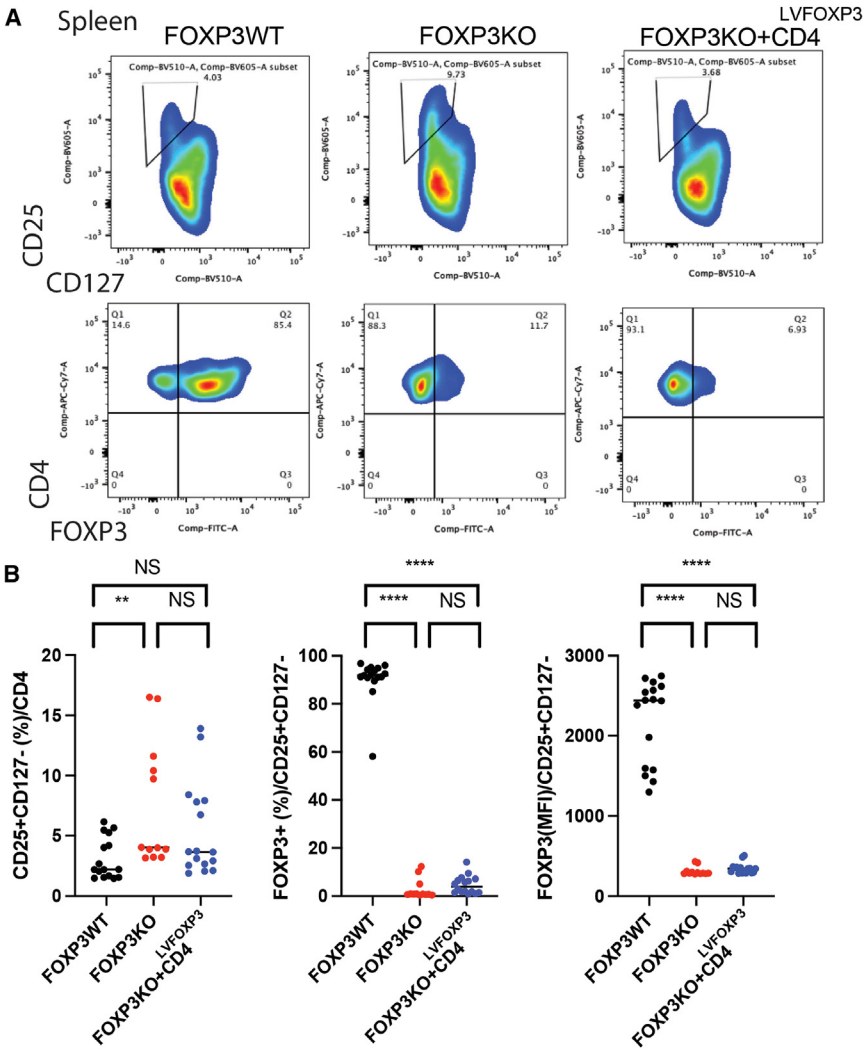


Figure 5. FOXP3 expression was abrogated in the Treg population in *FOXP3* KO hu-mice

(A) Representative flow cytometry dot plots. Frequency of $CD25^{+}CD127^{-}$ cells out of total $CD4^{+}$ T cells (top columns) and $FOXP3^{+}$ cells out of $CD25^{+}CD127^{-}$ cells (bottom columns). (B) Percentage of Tregs ($CD4^{+}CD25^{+}CD127^{-}$), Percentage and MFI of $FOXP3^{+}$ Tregs ($FOXP3^{+}/CD4^{+}CD25^{+}CD127^{-}$) in spleen in week 16 (n = 12–16, median). Data are representative of four independently repeated experiments. All phenotypes were performed by FACS. Significance determined by one-way ANOVA followed by Tukey's multiple comparison tests. $^{**}p < 0.01$, $^{****}p < 0.0001$.

intrinsic increased proliferation.[21] Indeed, FOXP3 has both an extrinsic regulatory role mediated by Treg cells and an intrinsic one, directly downregulating proliferation.[22] Last, we think that xeno-GVHD reactions in *FOXP3*KO hu-mice could be excluded by the high purity of our initial $CD34^{+}$ cells and by the different kinetics and timing of the lymphoproliferation which usually occurs much faster than the engraftment and development of transplanted HSPCs.

The morbidity of *FOXP3*KO hu-mice is not as severe as classical IPEX syndrome, which originally described early onset and limited survival, if not treated. However, due to the increasing patient numbers, the moderate to mild cases of IPEX patients were reported in the recent articles.[23] In addition, Scurfy mice, which are a conventional animal model for IPEX syndrome, showed severe mortality, but it is almost impossible to conduct a safety study in such a short-lived mice model.[24] Therefore, our model has advantages in the longevity and capacity for testing human T cell responses and Treg cell therapy, which will be used in the clinics.

We previously showed that FOXP3 partial KO resulted in mild $CD4^{+}$ T cell infiltration in the colon.[8] We have now obtained a stronger disease phenotype in terms of lymphoproliferation and gut infiltration, which is more suitable to functionally test Treg cells *in vivo*. However, our model is still not able to generate full multi-organ autoimmune damage as observed in IPEX disease, which would likely require the presence of human HLA molecules in the NSG mouse recipient organs matching the human cells used for the immune reconstitution (i.e., NSG-DR1 and NSG-DR4). As it is difficult to obtain human HSPCs with the specific HLA subtypes (i.e., HLA DR1 and HLA-DR4) that are identical to those expressed by immunodeficient human-HLA transgenic mice, we focused on using the best effective FOXP3KO in HSCPs from a single cord blood sample. The multiple sgRNA strategy was sufficient to produce 8–12 FOXP3KO hu-mice, as well as 3–5 FOXP3 WT hu-mice across different HSPCs donors (n = 6) and $CD4^{LVFOXP3}$ cells all being generated from the same donor.

Indeed, despite the lack of human HLA molecules in the murine tissue, which could result in more prominent and specific autoimmunity, including massive T cell infiltration in the systemic organs and auto-antibody production,[25] we have observed significant gut infiltration and 20%–25% overall mortality at week 16 in the *FOXP3*KO hu-mice. This lymphoproliferative and infiltrative phenotype observed in *FOXP3*KO hu-mice recapitulates the major immunological spectrum of IPEX syndrome. Compared with the traditional colitis models, such as dextran sodium sulfate-induced colitis and T cell transfer colitis in SCID mice, the colon infiltration in our model showed mild phenotype based on the weight reduction and pathological change. In addition, the lack of normal microbiota might contribute to the suitability of this model, while there are numbers of benefits using this mice model especially in the pre-clinical studies. In addition, the preservation of T, natural killer, and B cell reconstitution and differentiation, suggest that

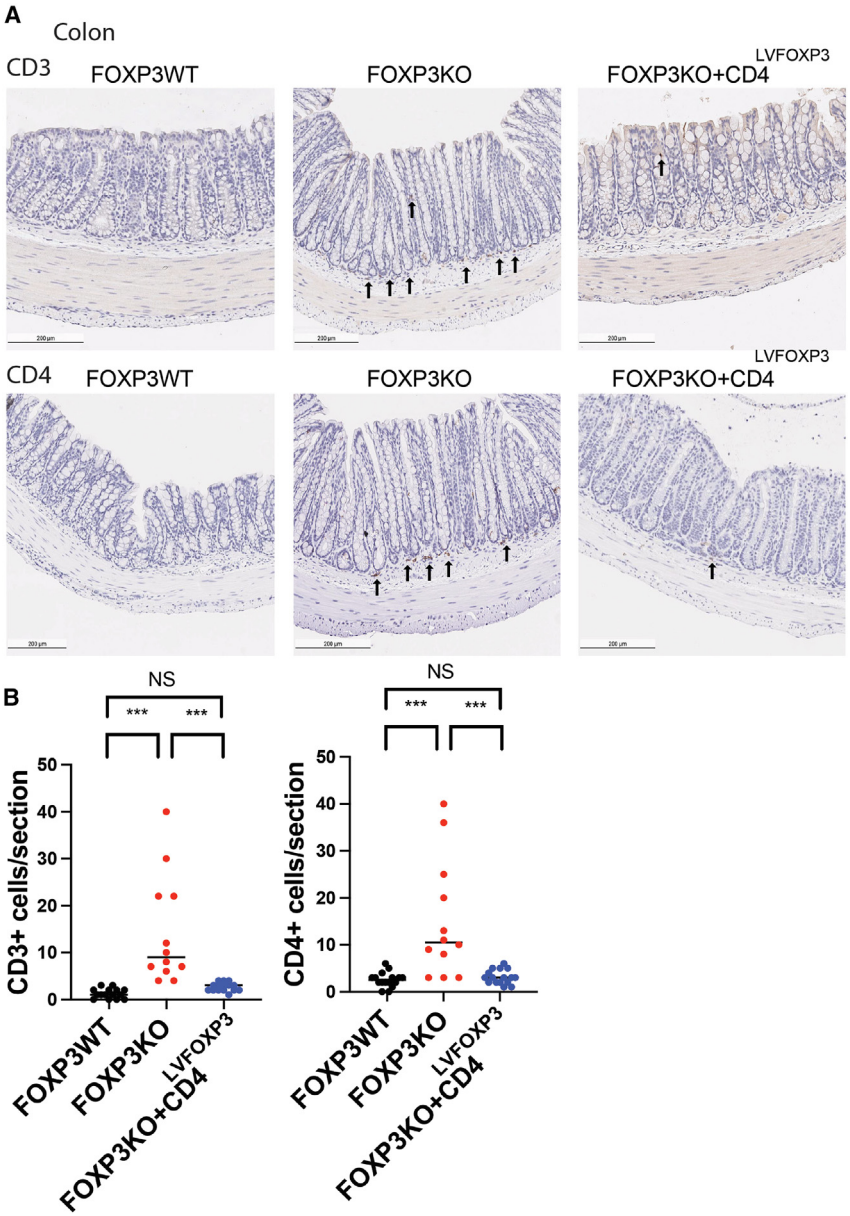


Figure 6. CD4^{LVFOXP3} cells can ameliorate gut infiltration of CD3$^+$/CD4$^+$ T cell populations in *FOXP3* KO hu-mice

(A) Representative immunohistochemistry (IHC) analysis of colon histology (CD3$^+$/CD4$^+$). (B) The quantification of CD3$^+$/CD4$^+$ T cells detected in colon in week 16 (n = 12–16, median). Data are representative of four independently repeated experiments. Significance determined by one-way ANOVA followed by Tukey's multiple comparison tests. ***$p < 0.001$.

this model is suitable for antigenic challenges toward which the impact of Tregs could be evaluated. Therefore, based on their robustness and reproducibility, *FOXP3KO* hu-mice could be used in the pre-clinical safety assessment. This model could address, for example, whether Treg-like cell products will affect the host immune system, especially in fighting against pathogens and tumor antigens. Currently, different products are candidates for Treg based cell therapy and are in the clinic or at different stages toward clinical trial development, including expanded Tregs and other gene-engineered Treg-like cell products such as CD4^{IL10} cells[26] and CAR-Tregs.[27,28] Our invaluable *FOXP3KO* humanized mice with multilineage human cell engraftment and phenotypic features of the prototype of genetic autoimmunity, may be ideal for testing other engineered Treg-cell therapy products and generating relevant pre-clinical data comparing different products. These assessments could include different cell doses, or the possibility to expand or eliminate Treg products vivo, as we show here using basiliximab.

Moreover, HSPCs based hu-mice models are critical tools in the pre-clinical examination of treatments aimed at other genetic diseases and other non-genetic diseases, including cancer and hematologic malignancy.[29,30] For example, several bispecific antibodies and immune checkpoint blockade inhibitors were tested in HSPC-based hu-mice models to confirm the safety and efficacy in human immune system.[31] Moreover, CRISPR-Cas9 disruption of other genes contributing to the immune regulation, such as CTLA-4 and CD25 and the generation of CRISPR-Cas9-edited HSPC-based hu-mice models could serve as ideal pre-clinical mice models for other genetic autoimmune diseases characterized by Tregopathy.[32]

Treg-based therapy is growing in the field of cell and gene therapy. Initially, FOXP3 was identified as a master regulator of Tregs and shown to provide suppressive function by inducing Treg related gene expression network such as CD25 and CTLA-4.[33] LV FOXP3 gene transfer and CRISPR-Cas9-mediated gene editing are still investigated to generate Treg-like cell products.[9,34] Moreover, FOXP3 gene transfer is shown to stabilize Treg-like phenotype and function of TCR-transduced Tregs[35] and CAR-Tregs.[36] Therefore, the necessity of suitable humanized animal model could enhance the clinical applications of Treg-related cell products.

In conclusion, the data presented significantly contributes to support nonclinical studies toward the clinical use of CD4^{LVFOXP3} cells in the first-time in human ongoing trial to treat patients with IPEX syndrome. Further, these data support the possibility of future pre-clinical use in the examination of other more common immune-mediated diseases caused by insufficient or dysfunctional Tregs.

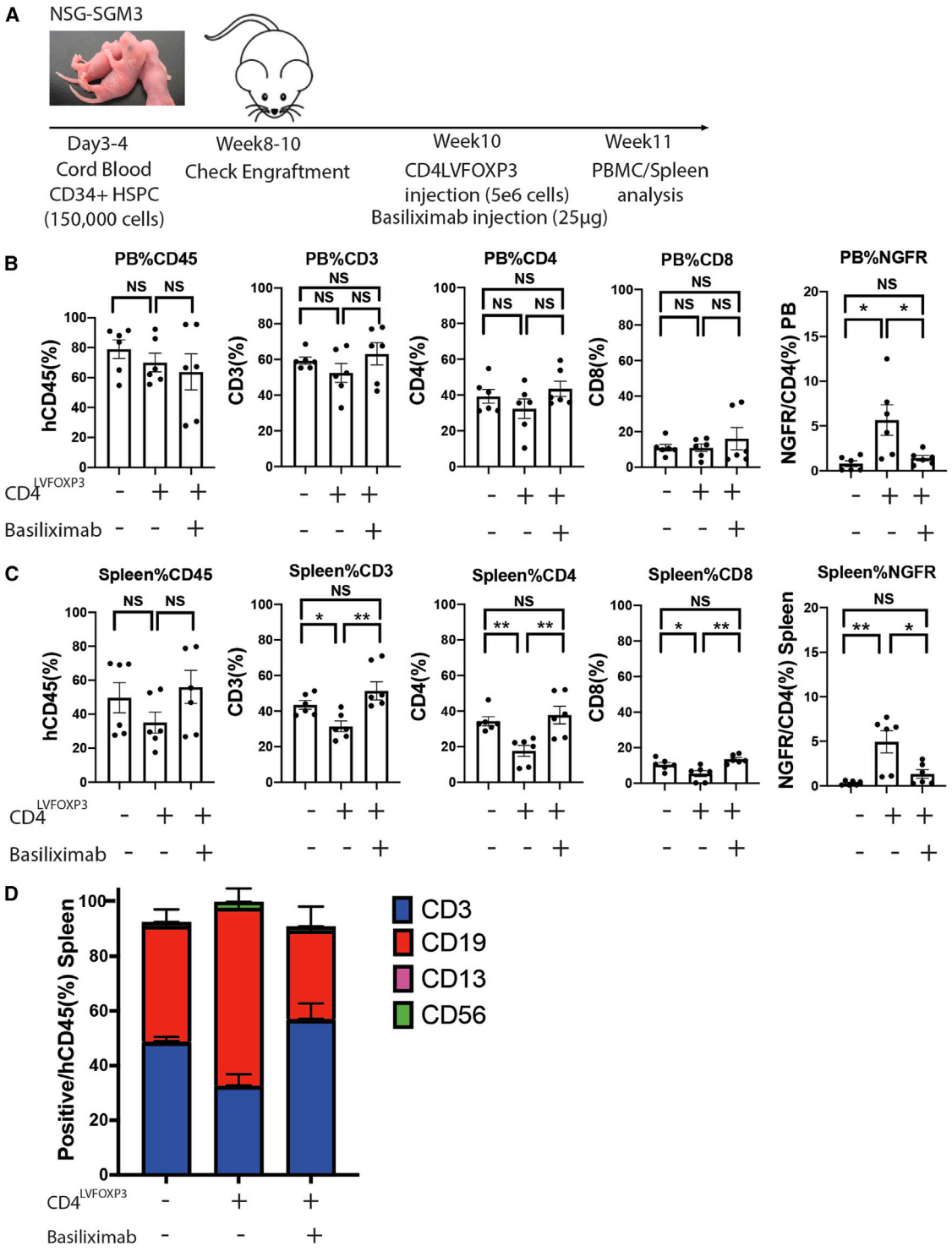


Figure 7. CD4^{LVFOXP3} cells can be reduced by basiliximab *in vivo*

(A) Schematic representation of the experimental setup. Percentage of human (h)CD45$^+$/CD3$^+$/CD4$^+$/CD8$^+$/NGFR$^+$ cells in (B) PB and (C) spleen (n = 6, mean + SEM). (D) Multilineage engraftment of hu-mice injected with CD4^{LVFOXP3} cells with basiliximab (n = 6, mean + SEM). Data are representative of two independently repeated

(legend continued on next page)

MATERIAL AND METHODS

Screening of sgRNA by ICE analysis

The *FOXP3* gene was disrupted by CRISPR-Cas9-mediated non-homologous end-joining. Freshly isolated cord blood-derived $CD34^+$ hematopoietic stem cells from male donors were obtained through the Stanford Binns Program as previously described.[37] After thawing, $CD34^+$ hematopoietic stem cells (2e5 cells/condition) were expanded for 2 days with StemSpan SFEM II medium (Stem Cell Technologies) supplemented with cytokine cocktails (100 ng/mL Flt3L, 100 ng/mL SCF, 100 ng/mL TPO, 100 ng/mL IL-6, 35 nM UM-171, and 0.75 μ M SR-1). The RNP complex was created by incubation of the sgRNA targeting *FOXP3* (TriLink Biotechnology or Synthego) with SpCas9 protein (Integrated DNA Technologies) at room temperature for 15 min. The RNP complex was electroporated into HSPC with program DZ-100 by nucleofector 4D (Lonza); transfected cells were expanded for additional 2 days. As an unmodified control, $CD34^+$ HSPC were cultured in parallel without transfection of RNP complex. Genomic DNA was extracted from the HSPC and INDEL frequencies were analyzed by ICE software (Synthego).

FOXP3KO hu-mice

Two million HSPCs were edited with the RNP complex created by incubation of the sgRNA targeting *FOXP3* (CRISPR Gene Knockout Kit, Synthego) with SpCas9 protein (Integrated DNA Technologies) at room temperature for 15 min. HSPCs were electroporated with RNP complex using program DZ-100 on the LONZA 4D-Nucleofector unit. Transfected cells were expanded for additional 2 days. *FOXP3KO* HSPCs (150,000 cells) were dissolved in 30 μL PBS and intrahepatically transplanted to the neonatal NSG mice (days 2–3) after sublethal irradiation (1 Gy). Twelve weeks after injection, $CD4^{LVFOXP3}$ cells (10^6 cells) generated from the same cord blood donor were injected peritoneally. PB samples were obtained biweekly by saphenous vein bleeding from 8 week after HSPC transplantation. At 16 weeks after injection, mice were sacrificed and spleen were analyzed by fluorescence-activated cell sorting (FACS). The experimental protocol was approved by Stanford University's Administrative Panel on Lab Animal Care.

LV production

Transfer vector plasmid (pLVFOXP3) was modified to enhance the safety including the modification of WPRE element (replacement to mut6 WPRE) from the original bidirectional vector.[8] LV vector was produced by co-transfection of transfer vector plasmids (pLVFOXP3) and helper plasmids including pMDLg/pRRE(gag/pol), pILVV01(REV), pMD2G(VSVG), and pAdVantage as described previously.[8]

Cell transduction

PBMCs were isolated by Ficoll-Paque PLUS from healthy donors' Buffy coat (purchased at the Stanford Blood Bank) or umbilical cord blood (obtained through the Stanford Binns Program). $CD4^+$ T cells were positively isolated by MACS isolation kit. Two million $CD4^+$ T cells were activated by TransAct (Miltenyi Biotechnologies, research grade) at a concentration of 1:100 at day 0. Lentiviral vectors were added at a multiplicity of infection of 20 at day 2. Transduced $CD4^+$ T cells were re-stimulated by TransAct at the concentration of 1:500 at day 11. Transduced $CD4^+$ T cells were expanded for additional 10 days and harvested on day 21 for the further *in vitro* and *in vivo* readouts.

Proliferation and suppression assays

For the proliferation assay, $CD4^+$ T cells labeled by CellTrace CFSE (Life Technologies) were activated by Dynabead Human T cell Activator CD3/CD28 at 1:25 bead:cell ratio. Ninety-six hours after stimulation, proliferation of $CD4^+$ T cells were counted by FACS.

For the suppression assay, responder $CD4^+$ T cells (50,000 cells) are labeled with CFSE and suppressor $CD4^+$ T cells (12,500–50,000 cells) are labeled with CellTrace Violet (LifeTechnologies) were co-cultured at different concentrations (1:0.25–1:1) after activation by Dynabead Human T cell Activator CD3/CD28 at 1:25 bead: cell ratio. Ninety-six hours after stimulation, proliferation of responder $CD4^+$ T cells were counted by FACS.

ELISA

$CD4^+$ T cells were cultured at a density of 1 million cells/mL in a round bottom 96 well plate and activated using the Dynabead Human T cell Activator CD3/CD28 (Gibco) at a 1:25 bead: cell ratio. Culture supernatant was collected at 24 h (IL-2) and 72 h (IL-4, IL-10, IFN-γ, IL-17A, and IL-22) after stimulation. Cytokine concentrations including IL-2, IL-4, IL-10 and IFN-γ were measured by Human OptEIA ELISA kit (BD Bioscience). Cytokine concentrations including IL-17A and IL-22 concentrations were measured by Human DuoSet ELISA kit (R&D Systems).

FACS

$CD4^+$ T cells were re-suspended in FACS buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) and stained using an antibody cocktail for 30 min, see Table S3 for antibody list. After surface staining, intracellular staining (FOXP3) was done by Foxp3/Transcription Factor Staining buffer set (eBioscience). Data were acquired by FACSAria II (BD Biosciences) and analyzed by FlowJo 10.4 software (FlowJo LLC).

Reduction of $CD4^{LVFOXP3}$ cells by basiliximab

HSPCs (150,000 cells) were intrahepatically transplanted into neonatal NSG mice (days 2–3) after sublethal irradiation (1 Gy). Ten weeks after HSPC injection, $CD4^{LVFOXP3}$ cells (5×10^6 cells) generated from the same cord blood donor were injected intraperitoneally. At 72 h after

experiments. All phenotypes were performed by FACS. Significance determined by one-way ANOVA followed by Tukey's multiple comparison tests. *$p < 0.05$, **$p < 0.01$. Bacchetta and colleagues generated FOXP3 KO hu-mice transplanted with human HSPCs in which *FOXP3* gene was knocked out using CRISPR-Cas9. Mice transplanted with FOXP3KO-HSPCs developed lymphoproliferation and T cell infiltration of the gut. Injection of $CD4^{LVFOXP3}$ engineered Tregs into FOXP3KO hu-mice restored *in vivo* regulatory functions.

$CD4^{LVFOXP3}$ cell infusion, 25 μg basiliximab was injected into mice that received $CD4^{LVFOXP3}$ cells. Seven days after basiliximab injection, PB and the spleen were harvested and analyzed by FACS.

Statistics

GraphPad Prism Software ver7.0 (GraphPad software) was used for statistical analyses. All statistical analyses were performed with the two-tailed Student *t*-test or Mann-Whitney *U* test. Mice survival was analyzed with the log rank test. A p values of <0.05 was considered significant.

Study approval

Buffy coats samples of healthy subjects were obtained from Stanford Blood Center in accordance with institutional review board guidelines. Umbilical cord blood samples were provided from Binns Program for Cord Blood Research and approved by Stanford Internal Review Board. The *in vivo* experimental protocols were approved by Stanford University's Administrative Panel on Lab Animal Care.

DATA AND CODE AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2023.101150.

ACKNOWLEDGMENTS

We thank Dr. Neehar Bhatia (Laboratory for Cell and Gene Medicine, Stanford University School of Medicine) for the insightful advice on the clinical translation of the project. We thank the Stanford Institute for Stem Cell Biology and Regenerative Medicine FACS Core for the technical assistance. We thank Stanford Veterinary Service Center (VSC) for supporting the animal experiments. We acknowledge Stanford's Binns Program for Cord Blood Research for providing cell products for our research. We thank Dr. Laura Passerini (San Raffaele Telethon Institute for Gene Therapy) for setting up the initial *in vivo* experimental protocol to test basiliximab. We gratefully acknowledge Dr. Rhonda Perriman for thoughtful feedback and revision of the manuscript. R.B., Y.S., and A.N. were supported by the California Institute of Regenerative Medicine (CIRM) CLIN-1 grant (11591 to RB). R.B. is an Anne T. and Robert M. Bass Faculty Scholar in Pediatric Cancer and Blood Diseases, Stanford University School of Medicine.

AUTHOR CONTRIBUTIONS

Y.S. conducted experiments, analyzed data, and wrote the manuscript. A.N. managed the project and review the manuscript. S.S. supported animal experiments. J.F.W., P.W., and K.M.T. provided experimental support and suggestions during development and reviewed the paper. M.G.R. provided critical insights. R.B. designed experiments, supervised data analysis and results interpretation, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. *155*, 1151–1164.
2. Sakaguchi, S., Miyara, M., Costantino, C.M., and Hafler, D.A. (2010). FOXP3+ regulatory T cells in the human immune system. Nat. Rev. Immunol. *10*, 490–500.
3. Ferreira, L.M.R., Muller, Y.D., Bluestone, J.A., and Tang, Q. (2019). Next-generation regulatory T cell therapy. Nat. Rev. Drug Discov. *18*, 749–769.
4. University of California, S.F., Foundation, J.D.R., Allergy, N.I.o., and Diseases, I. (2010). T1DM Immunotherapy Using CD4+CD127lo/-Cd25+ Polyclonal Tregs. https://classic.clinicaltrials.gov/show/NCT01210664.
5. Bacchetta, R., Medicine, C.I.f.R., Food Administration, D., and University, S. (2022). CD4^LVFOXP3 in Participants with IPEX. https://ClinicalTrials.gov/show/NCT05241444.
6. Therapeutics, S. (2021). Safety & Tolerability Study of Chimeric Antigen Receptor T-Reg Cell Therapy in Living Donor Renal Transplant Recipients. https://ClinicalTrials.gov/show/NCT04817774.
7. Santoni de Sio, F.R., Passerini, L., Valente, M.M., Russo, F., Naldini, L., Roncarolo, M.G., and Bacchetta, R. (2017). Ectopic FOXP3 Expression Preserves Primitive Features Of Human Hematopoietic Stem Cells While Impairing Functional T Cell Differentiation. Sci. Rep. *7*, 15820.
8. Sato, Y., Passerini, L., Piening, B.D., Uyeda, M.J., Goodwin, M., Gregori, S., Snyder, M.P., Bertaina, A., Roncarolo, M.G., and Bacchetta, R. (2020). Human-engineered Treg-like cells suppress FOXP3-deficient T cells but preserve adaptive immune responses in vivo. Clin. Transl. Immunol. *9*, e1214.
9. Allan, S.E., Alstad, A.N., Merindol, N., Crellin, N.K., Amendola, M., Bacchetta, R., Naldini, L., Roncarolo, M.G., Soudeyns, H., and Levings, M.K. (2008). Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. Mol. Ther. *16*, 194–202.
10. Passerini, L., Rossi Mel, E., Sartirana, C., Fousteri, G., Bondanza, A., Naldini, L., Roncarolo, M.G., and Bacchetta, R. (2013). CD4(+) T cells from IPEX patients convert into functional and stable regulatory T cells by FOXP3 gene transfer. Sci. Transl. Med. *5*, 215ra174.
11. Hausen, B., Gummert, J., Berry, G.J., Christians, U., Serkova, N., Ikonen, T., Hook, L., Legay, F., Schuler, W., Schreier, M.H., and Morris, R.E. (2000). Prevention of acute allograft rejection in nonhuman primate lung transplant recipients: induction with chimeric anti-interleukin-2 receptor monoclonal antibody improves the tolerability and potentiates the immunosuppressive activity of a regimen using low doses of both microemulsion cyclosporine and 40-O-(2-hydroxyethyl)-rapamycin. Transplantation *69*, 488–496. https://ClinicalTrials.gov/show/NCT05241444.
12. Bouvy, A.P., Klepper, M., Kho, M.M.L., Boer, K., Betjes, M.G.H., Weimar, W., and Baan, C.C. (2014). The impact of induction therapy on the homeostasis and function of regulatory T cells in kidney transplant patients. Nephrol. Dial. Transplant. *29*, 1587–1597.
13. Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. *4*, 330–336.
14. de Wilde, S., Guchelaar, H.J., Zandvliet, M.L., and Meij, P. (2016). Clinical development of gene- and cell-based therapies: overview of the European landscape. Mol. Ther. Methods Clin. Dev. *3*, 16073.
15. Lapteva, L., Purohit-Sheth, T., Serabian, M., and Puri, R.K. (2020). Clinical Development of Gene Therapies: The First Three Decades and Counting. Mol. Ther. Methods Clin. Dev. *19*, 387–397.
16. Shultz, L.D., Ishikawa, F., and Greiner, D.L. (2007). Humanized mice in translational biomedical research. Nat. Rev. Immunol. *7*, 118–130.
17. Mutis, T., van Rijn, R.S., Simonetti, E.R., Aarts-Riemens, T., Emmelot, M.E., van Bloois, L., Martens, A., Verdonck, L.F., and Ebeling, S.B. (2006). Human regulatory T cells control xenogeneic graft-versus-host disease induced by autologous T cells in RAG2-/-gammac-/- immunodeficient mice. Clin. Cancer Res. *12*, 5520–5525.

18. Bondanza, A., Valtolina, V., Magnani, Z., Ponzoni, M., Fleischhauer, K., Bonyhadi, M., Traversari, C., Sanvito, F., Toma, S., Radrizzani, M., et al. (2006). Suicide gene therapy of graft-versus-host disease induced by central memory human T lymphocytes. Blood *107*, 1828–1836.

19. Hannon, M., Lechanteur, C., Lucas, S., Somja, J., Seidel, L., Belle, L., Bruck, F., Baudoux, E., Giet, O., Chantillon, A.M., et al. (2014). Infusion of clinical-grade enriched regulatory T cells delays experimental xenogeneic graft-versus-host disease. Transfusion *54*, 353–363.

20. Santoni de Sio, F.R., Passerini, L., Restelli, S., Valente, M.M., Pramov, A., Maccari, M.E., Sanvito, F., Roncarolo, M.G., Porteus, M., and Bacchetta, R. (2018). Role of human forkhead box P3 in early thymic maturation and peripheral T-cell homeostasis. J. Allergy Clin. Immunol. *142*, 1909–1921.e9.

21. McMurchy, A.N., Gillies, J., Gizzi, M.C., Riba, M., Garcia-Manteiga, J.M., Cittaro, D., Lazarevic, D., Di Nunzio, S., Piras, I.S., Bulfone, A., et al. (2013). A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. Blood *121*, 1265–1275.

22. Kim, J.M., and Rudensky, A. (2006). The role of the transcription factor Foxp3 in the development of regulatory T cells. Immunol. Rev. *212*, 86–98.

23. Barzaghi, F., Amaya Hernandez, L.C., Neven, B., Ricci, S., Kucuk, Z.Y., Bleesing, J.J., Nademi, Z., Slatter, M.A., Ulloa, E.R., Shcherbina, A., et al. (2018). Long-term follow-up of IPEX syndrome patients after different therapeutic strategies: An international multicenter retrospective study. J. Allergy Clin. Immunol. *141*, 1036–1049.e5.

24. Ramsdell, F., and Ziegler, S.F. (2014). FOXP3 and scurfy: how it all began. Nat. Rev. Immunol. *14*, 343–349.

25. Goettel, J.A., Biswas, S., Lexmond, W.S., Yeste, A., Passerini, L., Patel, B., Yang, S., Sun, J., Ouahed, J., Shouval, D.S., et al. (2015). Fatal autoimmunity in mice reconstituted with human hematopoietic stem cells encoding defective FOXP3. Blood *125*, 3886–3895.

26. Locafaro, G., Andolfi, G., Russo, F., Cesana, L., Spinelli, A., Camisa, B., Ciceri, F., Lombardo, A., Bondanza, A., Roncarolo, M.G., and Gregori, S. (2017). IL-10-Engineered Human CD4(+) Tr1 Cells Eliminate Myeloid Leukemia in an HLA Class I-Dependent Mechanism. Mol. Ther. *25*, 2254–2269.

27. MacDonald, K.N., Ivison, S., Hippen, K.L., Hoeppli, R.E., Hall, M., Zheng, G., Dijke, I.E., Aklabi, M.A., Freed, D.H., Rebeyka, I., et al. (2019). Cryopreservation timing is a critical process parameter in a thymic regulatory T-cell therapy manufacturing protocol. Cytotherapy *21*, 1216–1233.

28. Bittner, S., Hehlgans, T., and Feuerer, M. (2023). Engineered Treg cells as putative therapeutics against inflammatory diseases and beyond. Trends Immunol. *44*, 468–483.

29. Tian, H., Lyu, Y., Yang, Y.G., and Hu, Z. (2020). Humanized Rodent Models for Cancer Research. Front. Oncol. *10*, 1696.

30. Cogels, M.M., Rouas, R., Ghanem, G.E., Martinive, P., Awada, A., Van Gestel, D., and Krayem, M. (2021). Humanized Mice as a Valuable Pre-Clinical Model for Cancer Immunotherapy Research. Front. Oncol. *11*, 784947.

31. Mian, S.A., Anjos-Afonso, F., and Bonnet, D. (2020). Advances in Human Immune System Mouse Models for Studying Human Hematopoiesis and Cancer Immunotherapy. Front. Immunol. *11*, 619236.

32. Cepika, A.M., Sato, Y., Liu, J.M.H., Uyeda, M.J., Bacchetta, R., and Roncarolo, M.G. (2018). Tregopathies: Monogenic diseases resulting in regulatory T-cell deficiency. J. Allergy Clin. Immunol. *142*, 1679–1695.

33. Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057–1061.

34. Goodwin, M., Lee, E., Lakshmanan, U., Shipp, S., Froessl, L., Barzaghi, F., Passerini, L., Narula, M., Sheikali, A., Lee, C.M., et al. (2020). CRISPR-based gene editing enables FOXP3gene repair in IPEX patient cells. Sci. Adv. *6*.

35. McGovern, J., Holler, A., Thomas, S., and Stauss, H.J. (2022). Forced Fox-P3 expression can improve the safety and antigen-specific function of engineered regulatory T cells. J. Autoimmun. *132*, 102888.

36. Henschel, P., Landwehr-Kenzel, S., Engels, N., Schienke, A., Kremer, J., Riet, T., Redel, N., Iordanidis, K., Saetzler, V., John, K., et al. (2023). Supraphysiological FOXP3 expression in human CAR-Tregs results in improved stability, efficacy, and safety of CAR-Treg products for clinical application. J. Autoimmun. *138*, 103057.

37. Mantri, S., Sheikali, A., Binns, C., Lyell, D.J., DiGiusto, D.L., Porteus, M.H., and Agarwal-Hashmi, R. (2021). The Binns Program for Cord Blood Research: A novel model of cord blood banking for academic biomedical research. Placenta *103*, 50–52.