

REVIEW**Towards gene therapy for IPEX syndrome***Simon Borna¹, Esmond Lee^{1,2}, Yohei Sato¹ and Rosa Bacchetta^{1,3}*¹ Department of Pediatrics, Division of Hematology, Oncology, Stem Cell Transplantation and Regenerative Medicine, Stanford University School of Medicine, Stanford, California, USA² Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California, USA³ Center for Definitive and Curative Medicine, Stanford University School of Medicine, Stanford, California, USA

Immune dysregulation polyendocrinopathy enteropathy X linked (IPEX) syndrome is an incurable disease of the immune system, with immune dysregulation that is caused by mutations in FOXP3. Current treatment options, such as pharmacological immune suppression and allogeneic hematopoietic stem cell transplantation, have been beneficial but present limitations, and their life-long consequences are ill-defined. Other similar blood monogenic diseases have been successfully treated using gene transfer in autologous patient cells, thus providing an effective and less invasive therapeutic. Development of gene therapy for patients with IPEX is particularly challenging because successful strategies must restore the complex expression profile of the transcription factor FOXP3, ensuring it is tightly regulated and its cell subset-specific roles are maintained. This review summarizes current efforts toward achieving gene therapy to treat immune dysregulation in IPEX patients.

Keywords: FOXP3 · IPEX · Gene therapy · DNA editing · Treg cells**Introduction**

The success of gene therapy to treat both genetic diseases and malignancies, including their subsequent translation to market-authorized therapies, has made genetic reprogramming of mammalian cells for clinical purposes a viable therapeutic option for patients [1].

The use of lentiviral (LV) vectors to safely deliver a transgene to hematopoietic stem and progenitor cells (HSPC) with modified promoters or with endogenous promoters was successfully brought to the clinic for several types of primary immunodeficiencies including SCID and Wiskott–Aldrich syndrome [2, 3]. In addition to the delivery of endogenous gene to HSPC allowing protein expression in multiple cell lineages, LV vectors have been used to deliver DNA constructs to T cells, whereby the exogenous

protein may lead to the acquisition of specific/desired T-cell properties. Pertinent examples are chimeric antigen receptors used in cancer therapy to instruct T cells to target and kill malignant cells [4, 5].

In contrast to ectopic LV-mediated random gene delivery, CRISPR/Cas9-based gene-editing technology enables single nucleotide corrections or site-specific insertion of coding sequence (cDNA) within or in front of a mutated gene, thus maintaining endogenous promoters and upstream regulatory sequences [6]. These strategies ensure the wildtype therapeutic gene expression in the right cell type during lineage differentiation, which is an essential requirement for functional repair of many disease-causing genes in HSPC. This feature is particularly relevant for functional restoration of the tightly regulated FOXP3 gene in patients with immune dysregulation polyendocrinopathy enteropathy X linked (IPEX) syndrome.

This review summarizes the two main gene-therapy-based approaches that are under development toward the clinical application for patients with IPEX, consisting of (1) engineered T cells

Correspondence: Dr. Rosa Bacchetta
e-mail: rosab@stanford.edu

that acquire regulatory function upon enforced FOXP3 expression and (2) HSPC-based gene delivery using lentiviral or CRISPR-based approaches.

Why gene therapy for IPEX syndrome?

IPEX syndrome is a deadly X-linked primary immunodeficiency, recognized as the prototype of primary immune regulatory disorders, and the first described tregopathy, a monogenic autoimmune disease caused by dysfunction of regulatory T (Treg) cells [7–9]. IPEX is caused by a mutation in the *FOXP3* gene, has early-onset, severe life-threatening clinical manifestations, and limited treatment options. Nonspecific pharmacological immunosuppressants are only partially effective and allogeneic hematopoietic stem cell transplantation (HSCT) is limited by a lack of suitable donors and transplant-related toxicities. As a monogenic disease of the blood immune system causing severe irreversible autoimmune-mediated organ damage, IPEX syndrome is an ideal disease candidate for gene therapy.

In its typical form, IPEX manifests with severe multiple autoimmunities, most notably in the skin (i.e. severe dermatitis), gastrointestinal tract (i.e. enteritis with refractory diarrhea), blood (autoimmune hemolytic anemia), and endocrine organs (i.e. type 1 diabetes). Our international retrospective natural history study of 96 patients showed that 95% of patients have disease onset during the first year of life, with 50% manifesting in the first month of life [9]. Of these patients, some will be refractory to available treatment options and remain with an uncontrolled disease, while others will partially respond to multiple immunosuppressive (IS) treatments, have some periods of clinical stability, evolving toward a more chronic form of IPEX. The current increased awareness of the disease has led to an increment in the overall diagnosis and revealed IPEX also among older children with atypical symptoms and later disease onset. New *FOXP3* mutations are being detected, and other presentations of the immune dysregulation are observed, including chronic or recurrent gut disease, recurrent autoimmune cytopenia, autoimmune hepatitis, subtle kidney pathology, various skin diseases, chronic muscle weakness, or clinical cases with solely type 1 diabetes. Therefore, in addition to the severe typical early-onset forms of IPEX, the atypical forms, comprising late-onset, mild-IPEX or unusual, sometime monosymptomatic disease, are being increasingly diagnosed [10]. Interestingly, despite the increased spectrum of phenotypes and new mutations described it is still very hard to establish a clear correlation between genotype and phenotype [10]. While the atypical forms may be less life-threatening, the affected patients still have significantly decreased quality of life relying on continuous medical assistance and constant or periodic pharmacological treatments with steroids and/or immune-suppressive agents that in the long term, bring systemic side effects. Indeed, our survey study on 96 patients with IPEX with a follow-up of 25 years, showed that the disease-free survival of patients under IS ($n = 31$) progressively declines with time [9].

Allogeneic-HSCT (allo-HSCT) is the only available cure for IPEX and has been successful for many children with IPEX who had a suitable donor. Data from our survey showed that the overall survival of patients with IPEX at 15 years after allo-HSCT ($n = 58$) is 73%, but disease-free survival is only 60%. An independent study by Chan et al. showed similar results for overall survival [11]. Further, pretransplant conditioning is not well tolerated in severely affected IPEX patients with advanced organ damage, leading to higher-than-expected peri-transplant complications [9]. Importantly, the degree of organ impairment at the time of allo-HSCT is the most important variable predicting outcome, independent of age, donor source, or conditioning regimen. This finding strongly supports the importance of early intervention prior to the onset of severe organ damage to stabilize the patient's clinical condition before allo-HSCT. This is currently achieved suboptimally, through the administration of IS drugs. Usually, steroids are the first line of immunosuppressive treatment. Calcineurin inhibitors, cyclosporin A and tacrolimus, have been widely used in the past. Recently, the use of mTOR inhibitor rapamycin has shown to be clinically beneficial, in some cases even as a single drug. Interestingly, rapamycin has been shown to improve the function of FOXP3-deficient Treg [9, 12].

Taken together, the development of a definitive approach that safely “fixes” the genetic defect in autologous cells could provide significant advantages as compared with current treatments for both severely affected patients as well as those with more moderate- or late-onset disease.

FOXP3 mutations lead to loss of suppressive function of CD4⁺CD25⁺-thymic-derived Treg cells. Treg cells play a crucial role in maintaining tolerance of immune cells to the host (self-tolerance). In a murine model of IPEX, the presence of normal Treg cells can rescue the disease manifestations, demonstrating that the lack of functional Treg cells is the main cause of the disease development [13]. In humans, the presence of the wild-type form of *FOXP3* in at least a fraction of immune cells is sufficient to maintain disease-free status in patients after HSCT [14] and prevent the onset of autoimmunity in carrier mothers [15, 16], suggesting that functional Treg cells can prevent the disease development also in humans. These observations provide a strong rationale for cell therapy based on infusion of functional Treg cells. However, the life span of the transferred Treg cells in patients remains to be determined, and in humans, *FOXP3* also has cell-intrinsic regulatory functions in effector T (Teff) cells [17, 18]. Therefore, a gene-transfer-based approach to correct/insert functional *FOXP3* gene in HSPCs may be necessary to fully cure the disease, guaranteeing its physiological presence during multilineage lymphoid development and life-long accrual of both Treg and Teff cells expressing normal *FOXP3*.

To assess the efficacy of strategies that involve either Treg replacement or restoration of *FOXP3* in HSPCs, it is critical to establish in vivo models to determine their ability to control immune dysregulation without impairing immune responses. Further, in the case of engineered HSPCs, it is essential to demonstrate full and safe differentiation into functionally

repaired Treg and Teff. Below we describe humanized mice (hu-mice) models designed for these goals.

Humanized mice as preclinical in vivo models to assess safety and efficacy of gene therapy for IPEX

Similar to IPEX syndrome in humans [19, 20], the loss of *Foxp3* in mice causes loss of Treg function, uncontrolled CD4⁺ T-cell expansion, and organ infiltration. The phenotype of FOXP3 deficient mice is very severe and leads to death in the first weeks of life. These mice develop a multiorgan autoimmunity characterized by inflammation of the skin, gastrointestinal tract, lungs, liver, skeletal muscles, and salivary glands. Biological findings include elevated proinflammatory cytokines TNF- α , INF- γ , and Th2 cytokines IL-4, IL-5, IL-13, and the presence of autoantibodies [13, 21–24]. However, although FOXP3 has a central role in both murine- and human Treg cell function [13, 25], there are key differences in murine and human FOXP3 biology which limits the use of murine *Foxp3* models in validating the translation of gene therapy approaches for the clinic.

In contrast to mice, where the *Foxp3* gene is expressed as a single protein, alternative splicing of the human *FOXP3* transcript gives rise to several different isoforms. The two predominant isoforms are full-length FOXP3, a homolog of murine *Foxp3*, and a $\Delta 2$ isoform, which skips exon 2. In addition, skipping of exon 7 gives rise to a less prominent isoform called $\Delta 7$ and another in which both exon 2 and 7 are excluded, $\Delta 2$, $\Delta 7$ having also been described [26–28]. Each of these isoforms likely has specific roles in human Treg- and/or Teff-cell biology. Notably, changes in the relative proportions of FOXP3 and $\Delta 2$ have been reported in various human diseases [27], and $\Delta 2$ and full-length isoforms were shown to have nonredundant roles in regulating the expression of genes associated with Treg suppressive function [29]. A detailed characterization of isotype-specific roles in human T-cell biology is summarized in two very comprehensive reviews by Mailer et al. [27, 28]. Another difference between human and murine FOXP3 is its expression pattern. In contrast to mice, human FOXP3 expression is not restricted to Treg cells but upon TCR stimulation, it is also expressed in Teff cells, where it negatively regulates Teff-cell proliferation, cytokine production [18], and ensures protection from restimulation-induced cell death [30]. These collective differences strongly support the need for hu-mice models to establish preclinical efficacy of IPEX gene therapies.

Hu-mice are generated by transplantation of human HSPC, obtained from cord blood or mobilized blood samples of healthy donors, to immunodeficient mice. Immunodeficient mice typically lack both T and B cells, and innate immune cells are severely compromised. The most common immunodeficient mice strain used as a recipient of human HSPCs is NOD-scid IL2R γ^{null} (NSG). The immune system of NSG mice, on the nonobese diabetic (NOD) genetic background, is severely compromised by dysfunction of *Prkdc* and the common γ chain signaling subunit of IL-2 [31].

Transplantation of human HSPC into NSG leads to development of all major human hematopoietic lineages [32]. Remarkably, both human Teff and Treg are allowed to develop in the murine thymic environment of NSG mice [32–35], which makes hu-mice very suitable preclinical models to study the effect of hematopoietic stem cell-based gene transfer methods for therapeutic purposes.

The NSG-based hu-mouse model was further improved by introducing the expression of human cytokines IL-3, SCF, and GM-CSF, resulting in transgenic NSG-SGM3 mice with increased multilineage HSPC engraftment [36]. NSG-SGM3 mice have increased numbers of peripheral Tregs as compared with NSG mice. As mature human Tregs do not express receptors for any of the human cytokines in NSG-SGM3 mice, and the number of human T cells and Tregs in the thymus are comparable to NSG mice, the increased number of Tregs in periphery of NSG-SGM3 mice probably results from an indirect mechanism mediated by the concomitant increase of the myeloid compartment [33, 36].

In addition to hu-mice generated by HSPC transplantation, suppressive function of human immune cells is frequently tested in the hu-mice model of xenogenic-graft versus host disease (xenoGVHD) [37, 38]. The xenoGVHD model is established by the transfer of human immunocompetent T cells into immunodeficient mice, which triggers a potent immune reaction against the xenogenic antigens. This immune stimulation leads to rapid engraftment, expansion of the activated human lymphocytes, and death of the mice in about 3 weeks. Importantly, co-transfer of Treg cells in this model can significantly prolong animal survival, confirming that this is a readably available in vivo system to assess a Treg cell product, including an engineered Treg-like product, in preventing and controlling xenoGVHD as well as to evaluate cell-dose effects [39, 40].

Goettel et al. were the first to reconstitute an immunodeficient mouse with HSPC from a patient with IPEX and also the first to model a monogenic autoimmune disease in hu-mice [41]. In the report, HSPC from an IPEX patient were transplanted into NSG mice or a variant of NSG mice – NSGAb1⁰DR1, which expresses human chimeric MHC II - HLA-DRA/B*0101 and is deficient for murine MHCII expression. IPEX-immune system reconstitution in NSG mice led to increased CD4⁺ and CD8⁺ T cells and the production of autoantibodies but was not sufficient to fully induce the phenotype of IPEX-like disease. In contrast, IPEX HSPC reconstituted NSGAb1⁰DR1 mice developed fatal IPEX-like autoimmunity characterized by immune cell infiltration in lungs, liver, and colon, increased production of proinflammatory cytokines and autoantibodies. The majority of the mice died between week 12 and 18 posttransplantation. Of note, the IPEX donor HSPC were negative for HLA-DRA/B*0101, which raises a question about the mechanism responsible for such a striking difference in development of IPEX disease between these two mice models [41]. Irrespective of the mechanism, these data are proof that it is possible to reproduce the IPEX disease in hu-mice, although the limited availability of patient HSPCs largely restricts the use of this model for preclinical studies.

An alternative model of human FOXP3 deficiency has been developed by Santoni et al. Here shRNA-mediated and TALEN-mediated FOXP3 knock-out (KO) technologies were used to downregulate the FOXP3 expression in HSPC which were transplanted to NSG mice (here termed FOXP3 KD/KO mice) [17]. Although residual FOXP3 expression was still relatively high, limiting its usage as a preclinical model, this hu-mouse model provided an interesting insight into the role of FOXP3 in human T cell biology. In line with the findings from Goettel et al., they showed expansion of peripheral CD4⁺ and CD8⁺ T cells with memory phenotype. Most strikingly, the report newly demonstrates the role of FOXP3 in Teff cell development. Thymocytes from the FOXP3 KD/KO had (1) reduced *nur-77* expression suggesting reduced TCR signaling strength, (2) reduced percentages of TCR β productive rearrangements, and (3) faster kinetics of thymus maturation/colonization and subsequently also involution. Given the large fraction of cells that remained unmodified Treg and the normal total frequencies of Treg in both spleen and thymus, the aberrant Teff cell development likely resulted from an intrinsic role for FOXP3 in Teff development rather than from the indirect effect of FOXP3 deficiency in Tregs. However, only a hu-mouse model in which FOXP3 KO cells could be distinguished apart from FOXP3 WT cells via a reporter gene expression (e.g. insertion of a reporter gene into FOXP3 locus to mark KO cells) would rule out any doubts about a direct effect of FOXP3 in Teff cell development.

More recently, we have developed a new FOXP3 deficient hu-mouse which we found useful for preclinical studies. Sato et al. used CRISPR-Cas9 to KO FOXP3 in human HSPC obtaining 30% FOXP3 KO and transplanted the modified cells into NSG mice [39]. In line with the previous two reports, the FOXP3 deficient hu-mice had expanded CD4⁺ and CD8⁺ T cells in periphery and a reduced naïve T cell compartment. Interestingly, and in contrast to IPEX patient HSPCs-transplanted NSG mice as reported by Goettel et al., CRISPR-Cas9 FOXP3 KO mice showed a mild but statistically significant increase in mortality. The discrepancy between the two reports may be connected to different effects of the patient's mutation and CRISPR-Cas9-introduced indels, respectively, on the FOXP3 function and/or to the animal housing conditions. In the preclinical setting, this FOXP3 KO hu-mouse model is an IPEX-like model that can be used to (1) assess the efficacy of preventing/controlling the pathologic expansion of FOXP3-deficient T cells by transfer of FOXP3-sufficient Treg cells; (2) determine the impact of Treg cell infusion on engraftment of other cell lineages; and (3) possibly, to assess the impact of Treg cell infusion on the immune response to pathogens [39]. Part of these applications is discussed in more detail in the following section describing the preclinical studies of Treg-gene therapy approach. However, further work to improve the IPEX disease model made by transplantation of healthy donor FOXP3 KO HSPC is still desired.

Overall, the hu-mice model of FOXP3 deficiency largely recapitulates the phenotype observed in humans, allowing us to monitor the effects of a treatment on immune system dysregulation and study the molecular mechanism of the disease (Table 1).

Treg cell-based gene-therapy: FOXP3 engineered CD4⁺ T cells converted into Treg-like cells

As discussed above, multiple evidence from FOXP3-deficient mice, carrier mothers, and IPEX patients with partial chimerism after HSCT strongly suggests that restoration of Treg compartment in IPEX could be curative. Treg cell therapy, using either freshly isolated or in vitro expanded cells, has demonstrated safety and a level of efficacy in early phase clinical trials for GVHD prevention following allo-HSCT, or in type 1 diabetes [42–44]. For IPEX patients, administration of an allogeneic Treg cell product could increase the risk of rejection since they have preserved immune responses, and, on the other hand, infusion of autologous expanded Treg cells would not be feasible because of their genetic dysfunction. These observations prompted us, several years ago, to investigate the possibility of using LV to constitutively express wild-type FOXP3 (LVFOXP3) in autologous CD4⁺ T cells, with the aim of converting them to Treg-like cells (CD4^{LVFOXP3}).

Conversion by lentiviral-mediated and constitutive FOXP3 expression

Ectopic expression of *Foxp3* to generate Treg-like functional cells has been previously performed in murine cells. Fontenot et al. showed that retroviral mediated ectopic expression of *Foxp3* can confer suppressive function to Teffs and injection of these engineered ectopically expressing FOXP3 cells can rescue the fatal autoimmunity in *Foxp3* deficient mice [13]. More recently, Delville et al. used LV-delivered human FOXP3 transgene to generate Treg-like cells from CD4⁺ T cells isolated from *Foxp3* deficient mice [45]. Mice that received the combination of cyclophosphamide and interleukin-2 with either WT Treg cells or the LV-converted Treg-like cells gained weight, had improved disease scores for eczema on the tail, blepharitis, and survival, supporting the engineered Treg-like therapeutic approach to control the IPEX pathology.

In human cells, however, the expression pattern and function of FOXP3 are more complex and retrovirus-mediated overexpression of FOXP3 did not consistently result in the generation of potent suppressive T cells, mainly due to the unstable and activation-dependent retroviral-mediated transgene expression [46]. We subsequently found that LV-mediated expression of FOXP3 under the control of the human elongation factor EF1 α promoter (LVFOXP3) can efficiently convert human CD4⁺ T cells into Treg-like cells demonstrating that high and stable FOXP3 expression is required to convert human Teffs into fully functional Treg-like cells [47]. LVFOXP3, also referred to in our previous publications as pCCLFP3, also expresses Δ NGFR (i.e. CD271), a truncated nerve growth factor receptor expressed under control of a minimal CMV promoter as a cell-surface marker for the selection of transduced cells (Fig. 1). Δ NGFR expression enables purification during cell manufacture, and in vivo tracking is clinically

Table 1. Overview of humanized (hu)-mice models of polyendocrinopathy enteropathy X linked disease

Murine Model	Human HSPC origin and genetic modification	Mice Phenotype	Phenotype of Teff cells	Phenotype of Treg cells	Major model limitations	Publication	Year
NSG, NSG-AB ⁰ DR1	HD and IPEX patient HSPC	NSG: Splenomegaly, autoantibodies NSG-AB ⁰ DR1: Splenomegaly, autoantibodies with broad autoreactivity, T-cell infiltration in lung, liver, colon and intestine, inflammatory cytokines in lung and liver, high mortality rate	Expansion of CD4+ and CD8+ T cells	Graft derived Treg: reduced suppressive function	Availability of IPEX HSPC	Goettel et al. "Fatal autoimmunity in mice reconstituted with human hematopoietic stem cells encoding defective FOXP3" [41]	2015
NSG	In HD HSPC, FOXP3 KD using shRNA and partial KO using TALEN technology	Accelerated thymus involution and thymocyte maturation, T-cell infiltration in liver.	Reduced TCR signaling during T-cell development, expansion of CD4+ and CD8+ T cells, Th2 polarization, increased effector and central memory CD4+ and CD8+ T-cell compartments, increased clonality and reduced percentages of TCR β productive rearrangements in splenic T cells.	Normal numbers in thymus and periphery, TSDR demethylated, normal suppressive function	Low KD/KO efficiency, not possible to distinguish KO and WT cells	Santoni et al., "Role of human forkhead box P3 in early thymic maturation and peripheral T-cell homeostasis" [17]	2018
NSG	In HD, FOXP3 KO using CRISPR/Cas9	T-cell infiltration in colon, slight but significantly increased mortality	CD4+ and CD8+ T cells expansion, increased CD4/CD8 ratio, reduced percentage of naive T cells.	Normal to low numbers in spleen, reduced FOXP3 expression in splenocytes	Low KO efficiency, not possible to distinguish KO and WT cells	Sato et al. "Human-engineered Treg-like cells suppress FOXP3-deficient T cells but preserve adaptive immune responses in vivo" [39]	2020

HSPC: hematopoietic stem and precursor cells; HD: Healthy Donor; KD: Knock-down; KO: Knock-out.

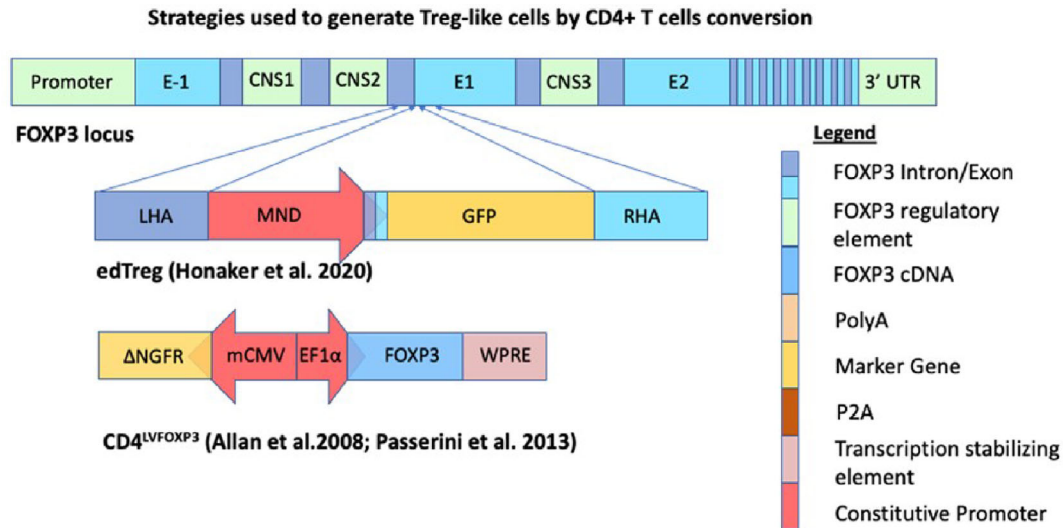


Figure 1. Schematic representation of DNA-editing strategy to generate edTreg and lentiviral vectors used for the generation of CD4^{LVFOXP3} cells. DNA-editing strategy to generate edTreg cells from CD4⁺ Teff cells by insertion of DNA construct containing MND promoter in front of exon 1 of FOXP3 gene (top) and lentiviral-based gene addition strategy to generate CD4^{LVFOXP3} using bidirectional vector coding for full-length FOXP3 and ΔNGFR reporter gene (bottom).

safe and non-immunogenic in humans [48]. In preclinical studies, transduction of peripheral CD4⁺ T cells with LVFOXP3 and in vitro expansion of transduced cells led to the generation of a homogeneous pool of T cells constitutively expressing FOXP3 [49]

The resulting CD4^{LVFOXP3} have similar in vitro and in vivo functional properties as naturally occurring Treg cells. Briefly, CD4^{LVFOXP3} are CD25^{high} CD127^{low} CTLA-4⁺ ICOS⁺ Helios⁺; in vitro, they show reduced proliferative capacity and cytokine production and suppress activated effector T-cell functions. Most importantly, CD4^{LVFOXP3} from HD and patients with IPEX prevent and control xenoGVHD reactions in hu-mice [39, 47, 49]. In addition, we observed that mice treated with CD4^{LVFOXP3} were able to better tolerate a second injection of Teff cells, suggesting a lasting immunomodulatory effect exerted by the initial CD4^{LVFOXP3}. In the IPEX hu-mouse model described in the prior section, CD4^{LVFOXP3} can prevent abnormal CD4⁺ memory T cell proliferation and tissue infiltration of the FOXP3KO cells [39]. These data strongly support the ability of the CD4^{LVFOXP3} to provide immune regulation of hyperproliferative activated T cells, such as those that are likely pathogenic in patients with IPEX. Moreover, we demonstrated phenotypic stability of the CD4^{LVFOXP3} in vitro and in vivo and the in vitro persistence of a polyclonal TCR-repertoire in the transduced cells. These observations (1) support the possibility that the CD4^{LVFOXP3} are stable, maintaining their suppressive function once injected into the inflammatory environment of an IPEX patient and (2) allow us to speculate that pathogenic autoreactive T cells present in the original CD4⁺ T cells collected from the patient, will undergo conversion into Treg-like cells, thereby preserving their TCR, and this could provide antigen-specific immune regulation after infusion and prevent further immune-mediated organ damage. Whether a sufficient number of autoreactive T cells are present in the blood or are exclusively localized in the site of the autoimmune reaction remains to be determined. The

safety of the CD4^{LVFOXP3} cells was further demonstrated in three different hu-mice models by showing that infusion of CD4^{LVFOXP3}: (1) does not block an early ongoing immune response towards pathogens, as evidenced by the presence of Ag-specific T cells able to normally respond to rechallenge with the same antigen (we tested *Candida*, *tetanus*, adenovirus, and CMV) in the spleens of CD4^{LVFOXP3} treated mice and (2) does not impair immune surveillance and tumor antigen clearance by autologous responder PBMC co-injected with CD4^{LVFOXP3}, in a skin sarcoma model; and does not alter immune reconstitution and survival of myeloid and lymphoid subsets including B and NK cells [39].

CD4^{LVFOXP3} are obtained from total CD4⁺T cells, which facilitates the manufacturing process from both the peripheral blood of healthy donors and even more so in IPEX patients. The current clinical manufacturing protocol for the generation of CD4^{LVFOXP3} is 18 days long and is aimed at achieving clinical feasibility for infusion into patients of different ages. In October 2020, CD4^{LVFOXP3} Treg-like cells were granted Orphan Drug designation by the FDA for the treatment of IPEX syndrome, designated as a rare pediatric disease confirming the compelling therapeutic potential of autologous CD4^{LVFOXP3} engineered Treg-like cells. A First-time in Human, Phase 1 dose-escalation clinical trial is now open at Stanford (NCT05241444), to establish the safety and feasibility of administering autologous CD4^{LVFOXP3} in patients with IPEX syndrome. In line with the cell dose used in previous Treg cell therapy clinical trials, the starting dose of CD4^{LVFOXP3} will be 10⁶ cells/kg. The cell administration will be in the absence of any conditioning. The participants enrolled in the study will be pediatric or young adults with progressive history of IPEX diseases and in need of immunosuppression. This study aims also to assess, in a preliminary fashion, the impact of these cells on clinical manifestations of IPEX as well as the pharmacokinetics and pharmacodynamics of CD4^{LVFOXP3} in humans. Since the CD4^{LVFOXP3} are traceable

using Δ *NGFR* reporter gene, we will be able to assess the survival of these engineered Treg-like cells, correlate their proportion/presence to the safety and potential impact on the disease manifestations. This cell product may become a long-term cure, a bridging therapy for patients waiting for suitable allo-HSCT donor, or be used as the only treatment for patients with mild IPEX without need for additional immunosuppression.

Conversion by FOXP3 locus editing

As it was observed for LVFOXP3 gene transfer approach, gene editing has also been effective in the generation of Treg-like cells. Honaker et al. used TALEN and CRISPR/Cas9-mediated gene editing to insert a constitutive promoter between the endogenous FOXP3 promoter and *FOXP3* coding sequence (Fig. 1) forcing expression of FOXP3 in Teff cells and converting them to Treg-like cells (edTregs) [50]. Consistent with our previous work on LVFOXP3 converted Treg-like cells, edTregs cells showed suppressive capacity in vitro and in vivo in a xeno-GvHD model. The edTregs could only treat patients with autoimmune diseases not caused by mutant FOXP3; however, the authors speculate that insertion of constitutive promoter together with wild-type FOXP3 cDNA may be performed and used for treatment of IPEX syndrome.

Indeed, using CRISPR/Cas9-mediated gene editing, we have recently shown that insertion of a FOXP3 cDNA coding for full length FOXP3 delivered by AAV6, at the *FOXP3* gene locus, in IPEX Treg can restore FOXP3 expression under physiological regulation of the endogenous promoter/enhancer and reestablish suppressive function [51], supporting the molecular feasibility of the editing approach. However, given the difficulties in isolating peripheral Treg from IPEX patients, this approach might not reach clinical feasibility, unless applied to HSPC, as described in the next section.

In conclusion, engineered Treg-like cells can be generated starting from CD4⁺ T cells, through either LVFOXP3 transfer or gene editing approaches, both of which are supported by robust preclinical data.

HSPC-based gene therapy strategies for IPEX syndrome

Primary CD4⁺ T cells modified to express FOXP3 provide a valuable source of functional Treg-like cells able to suppress undesirable immune response in preclinical hu-mice models; however, the open question remains how long these cells survive in a patient and whether repeated infusions will be necessary to sustain peripheral tolerance. Plausibly, the only long-lasting therapeutic approach allowing for correction of FOXP3 expression in all the target-cell subsets, while preserving the highly selective spatiotemporal regulation of FOXP3 expression, involves correction of a patient's own HSPCs. In addition, data from patients post-HSCT with low donor chimerism (less than 30%) and high

chimerism in the Treg lineage suggest that healthy donor Tregs have selective advantage over IPEX Treg [14]. Therefore, a low threshold of corrected stem cells may be sufficient to cure IPEX, supporting feasibility of HSPC-based gene therapy.

Lentiviral-mediated recombinant FOXP3 gene addition in HSPC

The need to preserve the endogenous regulation of *FOXP3* for its normal function is evident from the pioneering work of Santoni et al. [52]. The authors used the same LV construct as used to make CD4^{LVFOXP3} and tested it in HSPCs. While FOXP3-expressing HSPCs showed improved engraftment in NSG mice, the mRNA expression of key genes was altered. TGF β 1 and p21, both involved in controlling human HSC quiescence and self-renewal [53, 54], were upregulated. Matrix metalloproteinase 9, associated with HSPC mobilization in mice was also upregulated [55]. Interestingly, the authors hypothesized that by altering the molecular pathways controlling primitive features of HSPCs, ectopic FOXP3 expression improves the maintenance of more primitive HSCs in the bulk HSPC population. Analyzing lymphocyte populations in LVFOXP3 HSPC-engrafted mice, a decrease in mature thymocytes and splenic CD4⁺ and CD8⁺ T cells was observed. Upon challenge with breast-cancer-tumor cells, inflammatory cytokines such as IL1 β , IL5, CCL4, IL10, and IL17 were significantly reduced in the serum of LVFOXP3 HSPC-engrafted NSG mice, indicating that constitutive expression of FOXP3 in HSPCs altered the ability of differentiated T cells to respond to external cues. CD4⁺ cells from spleens of these challenged mice were stimulated and reduced proliferation was observed in LVFOXP3 cells compared with control cells. Taken together, these results show that ectopic constitutive-FOXP3 expression in HSPCs alters the engraftment and T-cell lineage differentiation, demonstrating that it is not a suitable approach for autologous stem cell therapy for IPEX, highlighting the need to preserve regulated gene expression.

The regulatory regions of *FOXP3* comprise a specific promoter and 3 conserved *FOXP3*-specific regulatory elements (CNS1-3). CNS1 plays a role in peripheral Treg induction, as shown in CNS1 deficient mice who displayed unaltered thymic Treg differentiation but a decrease in Tregs in gut-associated lymphoid tissues as well as mesenteric lymph nodes [56]. CNS3 is a pioneer element containing a c-Rel transcription factor binding site. C-Rel binds to CNS3 downstream of TCR activation, facilitating *Foxp3* expression and Treg differentiation. CNS3 deficient mice had five-fold less *Foxp3*⁺ CD4 single-positive thymic Tregs. Finally, CNS2 contains a CpG island that is exclusively demethylated in mature Tregs, an event that maintains constant *Foxp3* expression [56]. Consequently, CNS2-deficient Treg cells lose *Foxp3* expression over time.

To pursue preserved gene regulation, Masiuk et al. designed an LV construct containing the sequence of the endogenous *FOXP3* promoter together with all the three CNS elements [57]. The three CNS sequences were added upstream of the *FOXP3*

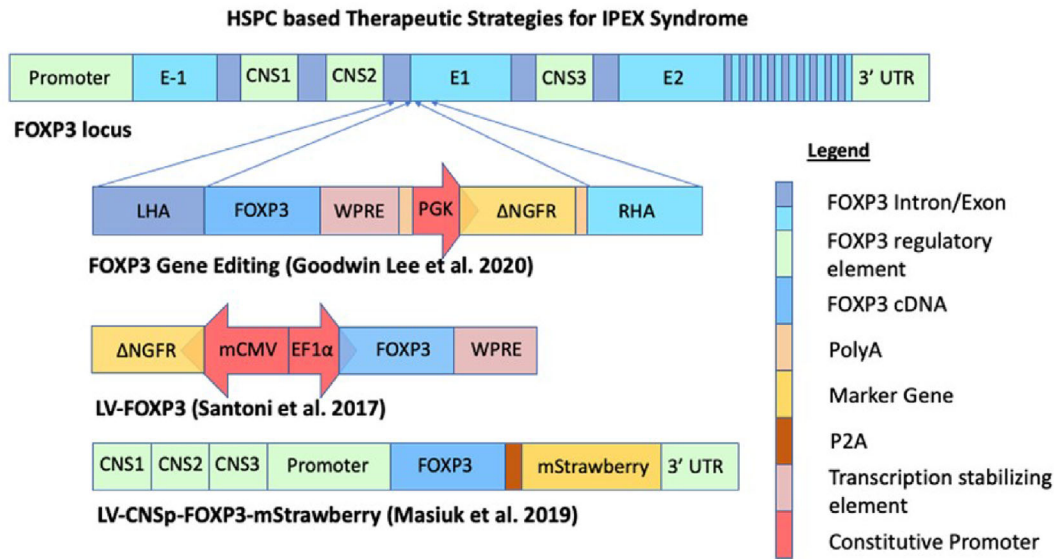


Figure 2. Schematic representation of lentiviral vectors and DNA editing strategies used to add or correct FOXP3 in HSPC. Strategies which deliver therapeutic FOXP3 to HSPCs for the differentiation of immune cells that express therapeutic FOXP3. HSPC gene-editing strategy using construct coding for full length FOXP3 and reporter gene Δ NGFR under control of PGK promoter (top), bidirectional lentiviral vector used to constitutively express full length FOXP3 in HSPC (middle), lentiviral vector containing regulatory elements CNS 1–3, and endogenous promoter design to achieve lineage-specific FOXP3 expression from HSPC-derived lymphocytes (bottom). HSPCs, hematopoietic stem and precursor cells.

promoter followed by *FOXP3* cDNA and an mStrawberry reporter linked by a P2A sequence (Fig. 2). The *FOXP3* 3'-UTR was added to the end of the expression cassette (LV-CNSp-FOXP3). HSPCs from mice that expressed GFP at the endogenous *FOXP3* promoter were transduced with LV-CNSp-FOXP3 and engrafted in congenic recipients. Tregs generated from the transplanted HSPCs (CD4⁺ GFP⁺) co-expressed mStrawberry, confirming the activity of the expression cassette. Within the hematopoietic subsets from the spleen, almost no mStrawberry was detected in HSPCs, myeloid cells, or B cells but some CD8⁺ and CD4⁺GFP-T cells were mStrawberry⁺, suggesting some leakiness of expression. Remarkably, CD4⁺ T cells derived from mice engrafted with LV-CNS123p-FOXP3 transduced *Foxp3*-deficient HSPCs were able to rescue recipient neonatal scurfy mice from the typical autoimmune phenotype of scaly, thickened ear skin, splenomegaly, a higher percentage of activated CD62L CD44⁺ CD4 T cells and elevated levels of inflammatory serum cytokines. When engrafted in NSG-SGM3 mice, human cord blood HSPCs transduced with LV-CNS123p-mStrawberry reporter generated mStrawberry⁺ CD4 T cells that were also FOXP3⁺. However, 19% of mStrawberry⁺ cells were not FOXP3⁺, suggesting that despite the presence of key *FOXP3* regulatory elements, there are some differences in how the LV construct regulates FOXP3 expression compared with the endogenous locus, and there is likely a certain level of leakiness of lentiviral FOXP3 expression. At the *FOXP3* locus in humans, the CNS sites are interspersed between the exon E-1 and exon 2 (Fig. 2), and thus it is possible that their spatial distribution is important for assembly of transcription factor complexes that regulate FOXP3 expression. In addition, the viral CNS2 remained demethylated in both sorted CD4⁺ Teff and Tregs, whereas the endogenous CNS2 of CD4⁺ Teffs, but not Tregs,

are methylated, as it is expected physiologically [58]. Therefore, CNS2 demethylation may have contributed to partial leakiness of FOXP3 expression in Teff cells, which raises concerns in terms of safety. Although promising, this approach needs further optimization to address the abnormal distribution of the FOXP3-specific demethylation and expression in Treg and Teff cells.

CRIPR/Cas9-mediated FOXP3 gene editing in HSPC

While LV approaches lead to semirandom insertion of variable copies of the LV template per cell, gene editing with CRISPR/Cas9 allows genetic manipulation at specific loci. To maintain FOXP3 gene expression under the control of the endogenous promoter and CNS regulatory elements, our group successfully developed a strategy for FOXP3 gene correction utilizing CRISPR/Cas9 and AAV6 virus for homologous template delivery, containing full-length FOXP3 cDNA, that can restore FOXP3 expression independent of the patient mutations, which are located throughout the gene [51]. In addition, the construct contains the Δ NGFR surface marker gene under a constitutive promoter, PGK, which marks edited cells, enabling their purification and monitoring. This strategy allows the inserted FOXP3 sequence to be under the control of the regulatory elements at the FOXP3 locus. The DNA editing was first validated in T cells. We showed that ex vivo edited normal donor and IPEX Teff cells have, upon TCR stimulation, a normal transient FOXP3 expression profile, cytokine production, and proliferation rates. In contrast, ex vivo edited normal donor Tregs reached about half of the normal FOXP3 expression, and their ability to suppress activated CD4⁺ T responder cells was toward the lower range of wild-type Tregs. FOXP3 gene-edited

Table 2. Different gene-therapy approaches aiming to treat IPEX syndrome, with possible advantages and disadvantages, as suggested by the authors of this review

Approach	Targeted cell type	Description	Advantages	Disadvantages/pitfalls	First references
Conversion to Treg-like cells by LV-FOXP3 gene delivery	CD4+ T cell (HD and IPEX patients)	Lentiviral delivery of exogenous FOXP3 cDNA under a constitutive promoter and codelivery of the surface marker Δ NGFR (CD271) (bidirectional vector).	(1) Effective conversion of both FOXP3 mutated or FOXP3 wild type Teff cells. (2) Stable expression. (3) Traceability <i>in vivo</i>	(1) Unknown lifespan of Treg-like cells in patient, (2) nontargeted integration into genome	Sarah Allan et al., Molec Ther. 2008 [47]; Passerini et al., Sci Transl Med. 2013 [49]
Conversion to Treg-like cells by gene editing-based FOXP3 forced expression	CD4+ T cell	Insertion of an exogenous constitutive promoter into FOXP3 locus to enforce expression of the endogenous gene	(1) Locus specific integration, (2) stable expression, (3) preservation of alternative splicing	(1) Unknown lifespan of Treg-like cells in patient; (2) not suitable to rescue expression of a mutated FOXP3 gene	Honaker et al., Sci Transl Med. 2020 [50]
LV-FOXP3 delivery of FOXP3 cassette containing regulatory elements	HSPC	Delivery of LV-FOXP3 cassette containing regulatory elements and endogenous FOXP3 promoter	(1) High FOXP3 expression, (2) one-time therapeutic intervention	(1) Possible leakiness of FOXP3 expression in Teff cells, (2) expression of only full-length isoform of FOXP3, (3) nontargeted integration into genome	Masiuk et al., Cell Stem Cell. 2019 [57]
FOXP3 gene correction	HSPC	Insertion of FOXP3 cDNA into FOXP3 locus with preservation of endogenous regulatory elements and promoter and co-insertion of the surface marker Δ NGFR (CD271)	(1) Locus specific integration, (2) preservation of endogenous gene regulation and lineage specific expression, (3) one-time therapeutic intervention	(1) expression of only FOXP3 full-length isoform, (2) suboptimal expression	Goodwin & Lee et al., Sci Adv. 2020 [51]

Tregs from IPEX patients showed restoration of FOXP3 expression and suppressive capacity up to the lower level of normal. Importantly, *FOXP3*-edited cord blood HSPCs transplanted into NSG-SGM3 neonates, engrafted, and were capable of multilineage hematopoietic reconstitution. However, Tregs derived from edited HSPCs were fewer in number and showed reduced FOXP3 expression compared with wild-type controls. This result suggests that a better level of FOXP3 expression may be necessary for optimal Treg commitment. It is also possible that the presence of only full-length isoform may not be sufficient to fully support Treg development and or stability. In addition, the induction and stability of FOXP3 expression may be affected by the site of the construct insertion or the loss of introns and 3'-UTR sequences [59, 60]. In our editing strategy, the donor template is inserted after exon 1 and thus CNS3 is moved downstream of the inserted gene cassette (Fig. 2). The shift of the CNS3 may spatially interfere with the assembly of transcriptional complexes associated with induction of FOXP3 expression during Treg development.

Altogether, although further testing and optimization of the current HSPC-therapeutic approaches are necessary to ensure clinical safety and efficacy, the work that is in progress represents a big step toward the definitive cure of IPEX by a single therapeutic intervention.

Conclusion

IPEX syndrome is a prototype autoimmune disease enabling the identification of the critical role of FOXP3 in human Treg cell biology as well as the crucial role of Treg cells in preventing autoimmunity. Despite important progress that has been made in the diagnosis, disease management and treatment, IPEX remains an incurable disease with severe impact on the quality of life of the patients and their families. The recent developments of gene transfer and correction strategies, the availability of human models to investigate human immune disease, and the improved understanding of the basic molecular mechanisms leading to the IPEX pathology, enabled us and others to design and test new therapeutic approaches aiming to cure IPEX (Table 2). To our knowledge, the first gene therapy-based treatment to be tested in IPEX patients is with the FOXP3 engineered Treg-like cells, CD4^{LVFOXP3} (NCT05241444). However, even if CD4^{LVFOXP3} proves effective in the treatment of IPEX, it should not discourage further development of gene-based therapies aiming to correct or add the FOXP3 cDNA in HSPCs. It is conceivable that CD4^{LVFOXP3} may represent a substantial improvement as compared to IS and could be complementary to HSCT now in use, or to HSPC based gene therapy approaches when they become available. In addition, Treg cell dysfunction or quantitative deficiency contributes to a large variety of autoimmune diseases, which offer an opportunity to subsequently extend the application of FOXP3-engineered Treg-like cells, suggesting that IPEX disease may become a prototype disease also from the perspective of gene transfer-based therapy for more common autoimmune diseases.

There is no doubt that the large scientific effort in gene therapy will continue and will eventually provide solutions for rare as well as more common autoimmune diseases. The remaining challenges to be prepared for will be to overcome the financial burden and allow accessibility to the technology for all patients in need [61].

Acknowledgments: We would like to thank Rhonda Perriman for helping with editing and formatting. Dr. R. Bacchetta, MD, is an endowed Anne T. and Robert M. Bass Faculty Scholar in Pediatric Cancer and Blood Diseases, Stanford University School of Medicine. S. Borna, PhD, is supported by Stanford Maternal and Child Health Research Institute Postdoctoral support Grant. This work was supported by the Stanford Maternal and Child Health Research Institute, the Center of Genetic Immune Diseases (CGID) and the Center for Definitive and Curative Medicine (CDCM) at Stanford University, and by the California Institute for Regenerative Medicine (CIRM) through several grants to R. Bacchetta.

Conflict of Interest: The authors declare no commercial or financial conflict of interest.

Author contributions: SB and RB with help of EL wrote the manuscript. EL wrote section HSPC-based gene therapy strategies for IPEX and made the figures. YS contributed to the section: FOXP3 engineered CD4+ T cells converted into Treg-like cells.

References

- 1 High, K. A., Roncarolo, M. G., Gene Therapy. *N Engl J Med* 2019. **381**: 455–464.
- 2 Aiuti, A., Biasco, L., Scaramuzza, S., Ferrua, F., Cicalese, M. P., Baricordi, C., Dionisio, F., et al., Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* 2013. **341**: 1233151.
- 3 Kohn, D. B., Booth, C., Shaw, K. L., Xu-Bayford, J., Garabedian, E., Trevisan, V., Carbonaro-Sarracino, D. A., et al., Autologous Ex Vivo lentiviral gene therapy for adenosine deaminase deficiency. *N Engl J Med* 2021. **384**: 2002–2013.
- 4 Milone, M. C., O'Doherty, U., Clinical use of lentiviral vectors. *Leukemia* 2018. **32**: 1529–1541.
- 5 Weber, E. W., Maus, M. V., Mackall, C. L., The emerging landscape of immune cell therapies. *Cell* 2020. **181**: 46–62.
- 6 Porteus, M. H., A new class of medicines through DNA editing. *N Engl J Med* 2019. **380**: 947–959.
- 7 Bacchetta, R., Barzaghi, F., Roncarolo, M. G., From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. *Ann N Y Acad Sci* 2018. **1417**: 5–22.
- 8 Cepika, A. M., Sato, Y., Liu, J. M., Uyeda, M. J., Bacchetta, R., Roncarolo, M. G., Tregopathies: Monogenic diseases resulting in regulatory T-cell deficiency. *J Allergy Clin Immunol* 2018. **142**: 1679–1695.

- 9 Barzaghi, F., Amaya Hernandez, L. C., Neven, B., Ricci, S., Kucuk, Z. Y., Bleesing, J. J., Nademi, Z., et al., Long-term follow-up of IPEX syndrome patients after different therapeutic strategies: an international multicenter retrospective study. *J Allergy Clin Immunol* 2018. **141**: 1036–1049 e1035.
- 10 Consonni, F., Ciullini Mannurita, S., Gambineri, E., Atypical presentations of IPEX: expect the unexpected. *Front Pediatr* 2021. **9**: 643094.
- 11 Chan, A. Y., Leiding, J. W., Liu, X., Logan, B. R., Burroughs, L. M., Allenspach, E. J., Skoda-Smith, S., et al., Hematopoietic cell transplantation in patients with primary immune regulatory disorders (PIRD): a primary immune deficiency treatment consortium (PIDTC) survey. *Front Immunol* 2020. **11**: 239.
- 12 Passerini, L., Barzaghi, F., Curto, R., Sartirana, C., Barera, G., Tucci, F., Albarello, L., et al. Treatment with rapamycin can restore regulatory T-cell function in IPEX patients. *J Allergy Clin Immunol* 2020. **145**: 1262–1271 e1213.
- 13 Fontenot, J. D., Gavin, M. A., Rudensky, A. Y., Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003. **4**: 330–336.
- 14 Seidel, M. G., Fritsch, G., Lion, T., Jurgens, B., Heitger, A., Bacchetta, R., Lawitschka, A., Peters, C., Gadner, H., Matthes-Martin, S., Selective engraftment of donor CD4+25high FOXP3-positive T cells in IPEX syndrome after nonmyeloablative hematopoietic stem cell transplantation. *Blood* 2009. **113**: 5689–5691.
- 15 Di Nunzio, S., Cecconi, M., Passerini, L., McMurphy, A. N., Baron, U., Turbachova, I., Vignola, S., Valencic, E., Tommasini, A., Junker, A., Gazzola, G., Olek, S., Levings, M. K., Perroni, L., Roncarolo, M. G., Bacchetta, R., Wild-type FOXP3 is selectively active in CD4+CD25(hi) regulatory T cells of healthy female carriers of different FOXP3 mutations. *Blood* 2009. **114**: 4138–4141.
- 16 Zemmour, D., Charbonnier, L. M., Leon, J., Six, E., Keles, S., Delville, M., Benamar, M., Baris, S., Zuber, J., Chen, K., Neven, B., Garcia-Lloret, M. I., Ruemmele, F. M., Brugnara, C., Cerf-Bensussan, N., Rieux-Laucat, F., Cavazzana, M., Andre, I., Chatila, T. A., Mathis, D., Benoist, C., Single-cell analysis of FOXP3 deficiencies in humans and mice unmasks intrinsic and extrinsic CD4(+) T cell perturbations. *Nat Immunol* 2021. **22**: 607–619.
- 17 Santoni de Sio, F. R., Passerini, L., Restelli, S., Valente, M. M., Pramov, A., Maccari, M. E., Sanvito, F., Roncarolo, M. G., Porteus, M., Bacchetta, R., Role of human forkhead box P3 in early thymic maturation and peripheral T-cell homeostasis. *J Allergy Clin Immunol* 2018. **142**: 1909–1921 e1909.
- 18 McMurphy, 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., Roncarolo, M. G., Stupka, E., Bacchetta, R., Levings, M. K., A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. *Blood* 2013. **121**: 1265–1275.
- 19 Wildin, R. S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J. L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricarelli, F. D., Byrne, G., McEuen, M., Proll, S., Appleby, M., Brunkow, M. E., X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 2001. **27**: 18–20.
- 20 Bennett, C. L., Christie, J., Ramsdell, F., Brunkow, M. E., Ferguson, P. J., Whitesell, L., Kelly, T. E., Saulsbury, F. T., Chance, P. F., Ochs, H. D., The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 2001. **27**: 20–21.
- 21 Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paepfer, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F., Ramsdell, F., Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 2001. **27**: 68–73.
- 22 Sharma, R., Sung, S. S., Fu, S. M., Ju, S. T., Regulation of multi-organ inflammation in the regulatory T cell-deficient scurfy mice. *J Biomed Sci* 2009. **16**: 20.
- 23 Sharma, R., Sharma, P. R., Kim, Y. C., Leitinger, N., Lee, J. K., Fu, S. M., Ju, S. T., IL-2-controlled expression of multiple T cell trafficking genes and Th2 cytokines in the regulatory T cell-deficient scurfy mice: implication to multiorgan inflammation and control of skin and lung inflammation. *J Immunol* 2011. **186**: 1268–1278.
- 24 Aschermann, S., Lehmann, C. H., Mihai, S., Schett, G., Dudziak, D., Nimmerjahn, F., B cells are critical for autoimmune pathology in Scurfy mice. *Proc Natl Acad Sci U S A* 2013. **110**: 19042–19047.
- 25 Bacchetta, R., Passerini, L., Gambineri, E., Dai, M., Allan, S. E., Perroni, L., Dagna-Bricarelli, F., Sartirana, C., Matthes-Martin, S., Lawitschka, A., Azzari, C., Ziegler, S. F., Levings, M. K., Roncarolo, M. G., Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J Clin Invest* 2006. **116**: 1713–1722.
- 26 Kaur, G., Goodall, J. C., Jarvis, L. B., Hill Gaston, J. S., Characterisation of Foxp3 splice variants in human CD4+ and CD8+ T cells—identification of Foxp3Delta7 in human regulatory T cells. *Mol Immunol* 2010. **48**: 321–332.
- 27 Mailer, R. K. W., Alternative Splicing of FOXP3-Virtue and Vice. *Front Immunol* 2018. **9**: 530.
- 28 Mailer, R. K., IPEX as a consequence of alternatively spliced FOXP3. *Front Pediatr* 2020. **8**: 594375.
- 29 Sato, Y., Liu, J., Lee, E., Perriman, R., Roncarolo, M. G., Bacchetta, R., Co-expression of FOXP3FL and FOXP3Δ2 isoforms is required for optimal treg-like cell phenotypes and suppressive function. *Frontiers in Immunology* 2021. **12**.
- 30 Voss, K., Lake, C., Luthers, C. R., Lott, N. M., Dorjbal, B., Arjunaraja, S., Bauman, B. M., Soltis, A. R., Sukumar, G., Dalgard, C. L., Snow, A. L., FOXP3 protects conventional human T cells from premature restimulation-induced cell death. *Cell Mol Immunol* 2021. **18**: 194–205.
- 31 Shultz, L. D., Lyons, B. L., Burzenski, L. M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S. D., King, M., Mangada, J., Greiner, D. L., Handgretinger, R., Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* 2005. **174**: 6477–6489.
- 32 Yahata, T., Ando, K., Nakamura, Y., Ueyama, Y., Shimamura, K., Tamaoki, N., Kato, S., Hotta, T., Functional human T lymphocyte development from cord blood CD34+ cells in nonobese diabetic/Shi-scid, IL-2 receptor gamma null mice. *J Immunol* 2002. **169**: 204–209.
- 33 Billerbeck, E., Barry, W. T., Mu, K., Dorner, M., Rice, C. M., Ploss, A., Development of human CD4+FoxP3+ regulatory T cells in human stem cell factor-, granulocyte-macrophage colony-stimulating factor-, and interleukin-3-expressing NOD-SCID IL2Rgamma(null) humanized mice. *Blood* 2011. **117**: 3076–3086.
- 34 Khosravi-Maharlooei, M., Hoelzl, M., Li, H. W., Madley, R. C., Waffarn, E. E., Danzl, N. M., Sykes, M., Rapid thymectomy of NSG mice to analyze the role of native and grafted thymi in humanized mice. *Eur J Immunol* 2020. **50**: 138–141.
- 35 Halkias, J., Yen, B., Taylor, K. T., Reinhartz, O., Winoto, A., Robey, E. A. and Melichar, H. J., Conserved and divergent aspects of human T-cell development and migration in humanized mice. *Immunol Cell Biol* 2015. **93**: 716–726.
- 36 Hahn, S. A., Bellinghausen, I., Trinschek, B., Becker, C., Translating treg therapy in humanized mice. *Front Immunol* 2015. **6**: 623.
- 37 van Rijn, R. S., Simonetti, E. R., Hagenbeek, A., Hogenes, M. C., de Weger, R. A., Canninga-van Dijk, M. R., Weijer, K., Spits, H., Storm, G., van Bloois, L., Rijkers, G., Martens, A. C., Ebeling, S. B., A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/- gammac-/- double-mutant mice. *Blood* 2003. **102**: 2522–2531.
- 38 King, M. A., Covassin, L., Brehm, M. A., Racki, W., Pearson, T., Leif, J., Laning, J., Fodor, W., Foreman, O., Burzenski, L., Chase, T. H., Gott, B.,

- Rossini, A. A., Bortell, R., Shultz, L. D., Greiner, D. L., Human peripheral blood leucocyte non-obese diabetic-severe combined immunodeficiency interleukin-2 receptor gamma chain gene mouse model of xenogeneic graft-versus-host-like disease and the role of host major histocompatibility complex. *Clin Exp Immunol* 2009. 157: 104–118.
- 39 Sato, Y., Passerini, L., Piening, B. D., Uyeda, M. J., Goodwin, M., Gregori, S., Snyder, M. P., Bertaina, A., Roncarolo, M. G., Bacchetta, R., Human-engineered Treg-like cells suppress FOXP3-deficient T cells but preserve adaptive immune responses in vivo. *Clin Transl Immunology* 2020. 9: e1214.
- 40 Schumann, K., Raju, S. S., Lauber, M., Kolb, S., Shifrut, E., Cortez, J. T., Skartsis, N., et al., Functional CRISPR dissection of gene networks controlling human regulatory T cell identity. *Nat Immunol* 2020. 21: 1456–1466.
- 41 Goettel, J. A., Biswas, S., Lexmond, W. S., Yeste, A., Passerini, L., Patel, B., Yang, S., et al., Fatal autoimmunity in mice reconstituted with human hematopoietic stem cells encoding defective FOXP3. *Blood* 2015. 125: 3886–3895.
- 42 Bluestone, J. A., Buckner, J. H., Fitch, M., Gitelman, S. E., Gupta, S., Hellerstein, M. K., Herold, K. C., et al., Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med* 2015. 7: 315ra189.
- 43 Bluestone, J. A. and Tang, Q., Treg cells-the next frontier of cell therapy. *Science* 2018. 362: 154–155.
- 44 Brunstein, C. G., Miller, J. S., McKenna, D. H., Hippen, K. L., DeFor, T. E., Sumstad, D., Curtsinger, J., et al., Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect. *Blood* 2016. 127: 1044–1051.
- 45 Delville, M., Bellier, F., Leon, J., Klifa, R., Lizot, S., Vincon, H., Sobrino, S., et al., A combination of cyclophosphamide and interleukin-2 allows CD4+ T cells converted to Tregs to control scurfy syndrome. *Blood* 2021. 137: 2326–2336.
- 46 Allan, S. E., Passerini, L., Bacchetta, R., Crellin, N., Dai, M., Orban, P. C., Ziegler, S. F., Roncarolo, M. G., Levings, M. K., The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest* 2005. 115: 3276–3284.
- 47 Allan, S. E., Alstad, A. N., Merindol, N., Crellin, N. K., Amendola, M., Bacchetta, R., Naldini, L., Roncarolo, M. G., Soudeyans, H., Levings, M. K., Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. *Mol Ther* 2008. 16: 194–202.
- 48 Bonini, C., Grez, M., Traversari, C., Ciceri, F., Marktel, S., Ferrari, G., Din-aer, M., et al. Safety of retroviral gene marking with a truncated NGF receptor. *Nat Med* 2003. 9: 367–369.
- 49 Passerini, L., Rossi Mel, E., Sartirana, C., Foustari, G., Bondanza, A., Naldini, L., Roncarolo, M. G., Bacchetta, R., CD4(+) T cells from IPEX patients convert into functional and stable regulatory T cells by FOXP3 gene transfer. *Sci Transl Med* 2013. 5: 215ra174.
- 50 Honaker, Y., Hubbard, N., Xiang, Y., Fisher, L., Hagin, D., Sommer, K., Song, Y., Yang, S. J., Lopez, C., Tappen, T., Dam, E. M., Khan, I., Hale, M., Buckner, J. H., Scharenberg, A. M., Torgerson, T. R., Rawlings, D. J., Gene editing to induce FOXP3 expression in human CD4(+) T cells leads to a stable regulatory phenotype and function. *Sci Transl Med* 2020. 12.
- 51 Goodwin, M., Lee, E., Lakshmanan, U., Shipp, S., Froessler, L., Barzaghi, F., Passerini, L., et al., CRISPR-based gene editing enables FOXP3 gene repair in IPEX patient cells. *Sci Adv* 2020. 6: eaaz0571.
- 52 Santoni de Sio, F. R., Passerini, L., Valente, M. M., Russo, F., Naldini, L., Roncarolo, M. G., Bacchetta, R., Ectopic FOXP3 Expression Preserves Primitive Features Of Human Hematopoietic Stem Cells While Impairing Functional T Cell Differentiation. *Sci Rep* 2017. 7: 15820.
- 53 Blank, U. and Karlsson, S., TGF-beta signaling in the control of hematopoietic stem cells. *Blood* 2015. 125: 3542–3550.
- 54 Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M., Scadden, D. T., Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 2000. 287: 1804–1808.
- 55 Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N. R., Crystal, R. G., Besmer, P., Lyden, D., Moore, M. A., Werb, Z., Rafii, S., Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 2002. 109: 625–637.
- 56 Zheng, Y., Josefowicz, S., Chaudhry, A., Peng, X. P., Forbush, K., Rudensky, A. Y., Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* 2010. 463: 808–812.
- 57 Masiuk, K. E., Laborada, J., Roncarolo, M. G., Hollis, R. P., Kohn, D. B., Lentiviral gene therapy in HSCs restores lineage-specific Foxp3 expression and suppresses autoimmunity in a mouse model of IPEX syndrome. *Cell Stem Cell* 2019. 24: 309–317 e307.
- 58 Baron, U., Floess, S., Wiczorek, G., Baumann, K., Grutzkau, A., Dong, J., Thiel, A., Boeld, T. J., Hoffmann, P., Edinger, M., Turbachova, I., Hamann, A., Olek, S., Huehn, J., DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol* 2007. 37: 2378–2389.
- 59 Gray, D. H., Villegas, I., Long, J., Santos, J., Keir, A., Abele, A., Kuo, C. Y., Kohn, D. B., Optimizing integration and expression of transgenic Bruton's tyrosine kinase for CRISPR-Cas9-mediated gene editing of X-Linked agammaglobulinemia. *CRISPR J* 2021. 4: 191–206.
- 60 Nott, A., Le Hir, H., Moore, M. J., Splicing enhances translation in mammalian cells: an additional function of the exon junction complex. *Genes Dev* 2004. 18: 210–222.
- 61 Gene therapies should be for all. *Nature Medicine* 2021. 27: 1311–1311. <https://www.nature.com/articles/s41591-021-01481-9>

Abbreviations: **AAV6:** Adeno-Associated Virus Type 6 · **ADA:** adenosine deaminase · **allo-HSCT:** allogenic HSCT · **CCL, FDA:** U.S. Food and drug administration · **cdNA:** coding DNA · **CNS:** conserved noncoding sequence · **DNA-PKcs:** the DNA-dependent protein kinase · **FOXP3:** forkhead box P3 · **GM-CSF:** granulocyte macrophage colony stimulating factor · **HCS:** Hematopoietic stem cell · **HLA:** human leukocyte antigen · **HSCT:** hematopoietic stem cell transplantation · **HSPC:** hematopoietic stem and progenitor cell · **hu-mice:** humanized mice · **IL:** interleukin · **INF:** interferon · **IPEX:** immune dysregulation polyendocrinopathy enteropathy X linked · **IS:** immunosuppression · **IV:** lentiviral vector · **MHC II:** major histocompatibility complex II · **NOD:** non-obese diabetic · **NSG:** NOD-scid IL2Rγ^{null} · **SCF:** stem cell factor · **SCID:** severe combined immunodeficiency · **TCR:** T cell receptor · **TCRβ:** TCR receptor beta chain · **Teff:** effector T cells · **TGF:** transforming growth factor · **TNF:** tumor necrosis factor · **Treg:** regulatory T cells · **WAS:** Wiskott–Aldrich syndrome · **WT:** wild type · **xenoGVHD:** chemogenic graft versus host disease · **ΔNGFR:** truncated form of the Nerve Growth Factor Receptor

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

Received: 5/11/2021

Revised: 29/1/2022

Accepted: 29/3/2022

Accepted article online: 30/3/2022