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Gene/Cell Therapy Approaches for Immune Dysregulation Polyendocrinopathy Enteropathy X-Linked Syndrome

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Abstract: Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome is a rare autoimmune disease due to mutations in the gene encoding for Forkhead box P3 (FOXP3), a transcription factor fundamental for the function of thymus-derived (t) regulatory T (Treg) cells. The dysfunction of Treg cells results in the development of devastating autoimmune manifestations affecting multiple organs, eventually leading to premature death in infants, if not promptly treated by hematopoietic stem cell transplantation (HSCT). Novel gene therapy strategies can be developed for IPEX syndrome as more definitive cure than allogeneic HSCT. Here we describe the therapeutic approaches, alternative to HSCT, currently under development. We described that effector T cells can be converted in regulatory T cells by LV-mediated FOXP3-gene transfer in differentiated T lymphocytes. Despite *FOXP3* mutations mainly affect a highly specific T cell subset, manipulation of stem cells could be required for long-term remission of the disease. Therefore, we believe that a more comprehensive strategy should aim at correcting *FOXP3*-mutated stem cells. Potentials and hurdles of both strategies will be highlighted here.

Keywords: Autoimmunity, cell therapy, Forkhead box P3, gene correction, Immune dysregulation Polyendocrinopathy Enteropathy X-linked syndrome, lentiviral vector, regulatory T cells.

1. INTRODUCTION

Primary immunodeficiency disorders (PIDs) are a heterogeneous group of rare genetic diseases affecting different arms of the immune system. PIDs are mostly characterized by increased susceptibility to infections, although 20% of patients with PIDs also manifest with autoimmune symptoms, which can be the predominant feature in some patients [1]. Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome is the prototype PID with prevailing autoimmunity [2]. The disease is caused by mutations in the gene encoding for the transcription factor *forkhead box p3* (*FOXP3*) [3], which lead to the loss of function of thymus-derived $CD4^+CD25^+$ regulatory T (tTreg) cells [4], a small subset of circulating $CD4^+$ T lymphocytes devoted to control immune responses to self and foreign antigens (Ags) [5]. In IPEX patients the absence of a functional Treg cell compartment leads to the development of multiple autoimmune manifestations, including severe enteropathy, Type 1 diabetes (T1D), and eczema [6, 7].

Notably, in about one third of patients with clinical symptoms resembling IPEX syndrome, *FOXP3* is not mutated. In these patients, referred to as "IPEX-like", the underlying genetic defect is unknown, with the exception of few cases with identified causative mutations in *IL2RA* [8-10], *STAT5b* [11-14] and *ITCH* [15]. While in IPEX patients tTreg cells are present but dysfunctional [16-19], in at least a subset of IPEX-like patients the disease is associated with a reduction in the relative amount of circulating tTreg cells, with consequent imbalance between the regulatory and effector T (Teff) cell compartments [20].

Autoimmune symptoms associated with quantitative or functional Treg cell defects have also been described in well defined PIDs of different genetic origin, such as Di George syndrome [21] and Wiscott Aldrich syndrome (WAS) [22, 23].

In this review we will give an overview of the current knowledge of IPEX pathogenesis, disease manifestation, and therapy, and discuss the innovative therapeutic approaches that we are currently developing for the treatment of IPEX and IPEX-like syndromes, with focus on Treg-based cell therapy, whose application could potentially extend to several autoimmune diseases of different origin.

2. IPEX SYNDROME: CLINICAL MANIFESTATIONS AND DISEASE PATHOGENESIS

IPEX is a rare disease, with less than 160 cases described worldwide in the last ten years [6]. Patients display life-threatening multi-organ autoimmunity, which manifests in males usually in the first months or years of life. The distinctive features of the disease comprise severe enteropathy with

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refractory diarrhea, usually associated with villous atrophy, T1D, and dermatitis. Additional autoimmune manifestations comprise autoimmune endocrinopathies, i.e. thyroiditis, cytopenias, including hemolytic anemia, thrombocytopenia, and neutropenia, and hepatitis with positive auto-antibodies (Abs). Infections are also often reported, although they may be the consequence of immunosuppressive (IS) therapy.

Beside the severely affected patients with early onset and complete clinical manifestation, many patients manifest with a milder form of the disease, which is often mis-diagnosed or diagnosed later in young boys. Specific anti-enterocyte auto-Abs, such as anti-harmonin and anti-villin auto-Abs are highly specific for IPEX syndrome, regardless of disease severity [24-26]. Therefore, prompt recognition of atypical forms of IPEX could be facilitated in patients manifesting with gastro-intestinal symptoms by screening for these auto-Abs . No clear genotype-phenotype correlation, which would help to predict the patient-specific course of the disease, has been so far identified [27, 28], although the increasing number of cases recently described will help in addressing it.

Regardless of the type and site of the mutation, autoimmune manifestations result from partial or total loss of function by Treg cells, which is considered the primary cause of disease. *FOXP3*-mutated Treg cells display defective *in vitro* suppressive function [16, 19, 29] and unstable behaviour in inflammatory conditions, with putative conversion from a regulatory to an effector (i.e. IL-17-producing) phenotype [17].

Additional defects in the Teff cell and in the B cell compartments have also been described. Peripheral T cells from IPEX patients have altered cytokine production, with impairment of Th1 related cytokines and relative skew to a Th2 profile [16, 30, 31], and an increased proportion of IL-17-producing cells in PBMC [17] and gut infiltrates (Bacchetta, unpublished data). While there are evidences that the Teff cell involvement is directly dependent on mutant FOXP3 expression in activated Teff cells [32], B cell defects are likely to be an indirect consequence of Treg cell dysfunction [33]. Indeed, autoreactive mature naïve B cells accumulate in the peripheral blood of IPEX patients, implicating alterations of the peripheral B-cell tolerance checkpoint [33]. In addition, multiple tissue-specific auto-Abs, other than auto-Abs to enterocyte Ags [10, 24, 25] are often detected in IPEX sera.

Based on this knowledge, in IPEX syndrome the impairment of Treg cell function is crucial for disease pathogenesis, suggesting that therapies aimed at improving and/or restoring a functional Treg compartment should be beneficial to IPEX patients.

3. CURRENT THERAPEUTIC APPROACHES

IPEX syndrome is often fatal early in infancy, therefore a prompt diagnosis is essential to start treatment as soon as possible, before tissue damage spreads to multiple organs. The current treatments available for IPEX patients include supportive therapy, IS therapy, and hematopoietic stem cell transplantation (HSCT). Allogeneic HSCT is the best treatment so far available, with better success reported for reduced-intensity conditioning regimens, based on the experiences so far reported in literature ([34] and reviewed in [6]).

For patients who do not undergo HSCT, the treatment is limited to supportive therapies, including nutritional support and replacement therapy for endocrine diseases, and to combination of multiple IS drugs, without permanent control of autoimmunity in most of the patients. Notably, the drug rapamycin has been reported to be a successful alternative to calcineurin inhibitors, with clinical remission described in four IPEX patients with long-term follow-up [27, 35, 36]. Despite these latter promising results, HSCT still remains the only curative treatment currently available [27], although suitable donors for HSCT are not available for all patients and the poor clinical conditions of these patients make them particularly susceptible to the toxicity of conditioning regimens and post-transplant complications. Therefore, the need of effective therapeutic approaches is still unmet for patients with IPEX syndrome.

Based on HSCT outcome in the context of IPEX syndrome, we learned that partial donor chimerism is sufficient for complete disease remission, provided that full engraftment is achieved in the Treg compartment, suggesting that few functional Treg cells could be sufficient to control autoimmunity in IPEX syndrome [34, 37, 38]. Similarly, partial bone marrow transplant or adoptive Treg cell transfer in *scurfy* mice, the natural animal model for FOXP3 deficiency, is sufficient to control the disease [39], confirming the generally accepted idea that *FOXP3*-mutated Treg cells are the snipers in IPEX syndrome. In addition, in female healthy carriers of *FOXP3* mutations only the wild type *FOXP3* allele is active in Treg cells, giving rise to a functional Treg compartment, with no signs of disease, despite mixed population of *FOXP3*-mutated and -wild-type expressing Teff and B cells are circulating in peripheral blood [40].

Based on the latter observations, we reasoned that restoration of a functional Treg cell pool could be an opportunity to control the disease. We therefore designed cell/gene therapy approaches to selectively restore the Treg compartment in patients with IPEX syndrome, as described below.

4. FUTURE THERAPEUTIC PERSPECTIVE: TREG-BASED IMMUNOTHERAPY AND STEM-CELL BASED THERAPIES

In the recent years the technology for genetic reprogramming of mammalian cells have remarkably improved, allowing translation of gene therapy-based approaches in clinical trials for the cure of several genetic diseases and cancer [41]. Gene therapy with genetically modified hematopoietic stem cells has proven to be safe and effective when applied not only to monogenic PIDs [42-44], but also to lysosomal storage diseases [45]. Similarly, gene therapy with peripheral T lymphocyte has been applied to Adenosine Deaminase deficient Severe Combined Immune Deficiency (ADA-SCID) [44] and for the treatment of cancer [46]. Therefore, both T-cell- and stem-cell-based gene therapy has become reality in the clinical practice.

Using *in vitro* and pre-clinical models we are currently investigating the feasibility and efficacy of multiple gene-therapy-based strategies to restore a functional Treg com-

partment in patients with IPEX syndrome, with the ultimate goal of controlling the devastating autoimmunity resulting from mutations of the *FOXP3* gene. These include i) adoptive transfer of autologous Treg cells generated *in vitro* by lentiviral vector- (LV-) mediated ectopic overexpression of FOXP3 in conventional $CD4^+$ T cells; ii) infusion of autologous hematopoietic precursors engineered to express wild-type FOXP3, or iii) administration of autologous gene-corrected hematopoietic precursors (Fig. **1**).

4.1. Treg-Based Immunotherapy

Treg cells are critically involved in the control of immune reactions to self and non-self Ags, including allo-Ags, tumor-Ags, and allergens, and they can possibly induce long-term Ag-specific tolerance, without limiting protective responses to pathogens. Due to their power as specific modulators of immunity, investigators have proposed their use in clinically relevant settings to dampen undesirable immune responses, as in the case of tolerance promotion after solid organ or bone marrow transplant, in inflammatory diseases, and autoimmune diabetes (reviewed in [47]).

Treg cells, including both $CD25^+$ Treg cells and T regulatory type 1 (Tr1) cells, have been applied in several clinical trials with the aim of preventing Graft-versus-host disease (GvHD) after allogeneic HSCT. The results of the concluded trials agree on the feasibility and overall safety of the procedure [48-52], and in some of them also a certain efficacy has been showed [49, 50]. Clinical application of Treg cells has been extended to inflammatory diseases in a recently completed trial in which Ag-specific Tr1 cell clones were used to treat patients with Crohn's Disease (CD), resulting in a significant reduction in CD Activity Index in responder patients [53]. These encouraging results paved the way for a wider application of Treg cells in therapeutic settings. Ongoing trials comprise i) application of several immunoregulatory cell types, including freshly isolated and *in vitro* expanded $CD25^+$ Treg cells, after solid organ transplantation, with the aim of inducing tolerance to allo-Ags and avoiding the life-long IS treatment that kidney transplant recipients usually undergo [54]; ii) use of *in vitro* expanded polyclonal $CD25^+$ Treg cells in patients with recent onset T1D [55].

The gene transfer technology has been also applied to the Treg field with the aim of generating homogeneous and functional Treg cell populations by ectopic overexpression of FOXP3 in conventional $CD4^+$ T cells from healthy donors [4, 56-58] or by ectopic expression of genes encoding for T cell receptors with known specificity in polyclonal Treg cells [59-61].

Based on these promising pre-clinical and clinical results, we envisaged the possibility to restore immune regulation in IPEX patients by administration of autologous Treg cells generated *in vitro* by LV-mediated overexpression of wild-type FOXP3 in conventional $CD4^+$ T cells (Fig. **1**). We recently demonstrated that IPEX *FOXP3*-mutated $CD4^+$ T cells could be efficiently converted into functional Treg-like cells, regardless of the type or site of mutation. Constitutive over-expression of FOXP3 generates functional and stable $FOXP3^+$ Treg-like cells ($CD4^{FOXP3}$ T cells). The latter display potent suppressive activity both *in vitro* and *in vivo,* in a model of xeno-GvHD, reduced proliferative capacity and cytokine production [62]. Furthermore, $CD4^{FOXP3}$ T cells generated from naïve $CD4^+$ T cells have stable expression of FOXP3 in steady state and inflammatory conditions, in which they maintain stable suppressive function and cytokine production profile [62]. In terms of safety, $CD4^{FOXP3}$ T cells appear to be unable to expand when injected *in vivo*, even in inflammatory conditions, making them safe for clinical application [62]. We therefore propose that, when a HLA-compatible donor is not available or the patient does not meet the conditions for transplantation, treatment with engineered T cells could be beneficial to control the severity of the disease, alone or in combination with IS.

One of the main open issues of such a treatment is related to the lifespan of $CD4^{FOXP3}$ T cells, which is of difficult assessment in pre-clinical studies. The other possible drawback is a generalized effect of immunosuppression that these potent $CD4^{FOXP3}$ T cells may cause once *in vivo*. In order to address this, we are currently establishing a protocol to generate $CD4^{FOXP3}$ T cells from Ag-experienced T cells with known specificity, which should restrict their suppressive effect to the target Ag (Bacchetta, unpublished results). This approach could extend the application of the $CD4^{FOXP3}$ T-cell product beyond IPEX syndrome, to treat any autoimmune disease with known target auto-Ags.

4.2. Stem-Cell-Based Gene Therapy

Although our studies provided evidences that $CD4^{FOXP3}$ T cells have the potential to control immune dysregulation in IPEX patients, such an approach would not correct the defects affecting the Teff compartment, in which the role of FOXP3 is only partially understood [32], and may require multiple infusions for permanent control of the disease. A more rationale and definitive approach would imply engineering hematopoietic stem cells. Since the constitutive overexpression of FOXP3, could be detrimental in hematopoietic precursors, which physiologically do not express FOXP3, we are exploring different strategies to modulate FOXP3 expression in HSC, for example using a T cell specific promoter (i.e. CD4) with addition of cis-regulatory elements, such as microRNA (miR) target sequences and $CD8^+$ T cell specific silencer sequence, to prevent transgene expression in HSC precursor, antigen presenting cells and $CD8^+$ T lymphocytes [63]. The capacity of such engineered HSC to generate functional Treg cells, without impairment of the Teff cell compartment, will be evaluated *in vivo* in pre-clinical humanized murine models, using immunodeficient mice as transplant recipient.

Although transplantation of engineered HSC has proven to be effective in other PIDs, such as ADA-SCID and WAS [42, 44], gene therapy for IPEX syndrome is more challenging, because of the specific expression of FOXP3 in a limited cell subset in physiological conditions. Furthermore, studies on the role of FOXP3 during thymic T-cell development and T-cell repertoire selection are still incomplete [64], making it difficult to predict the outcome of ectopic FOXP3 expression in developing thymocytes.

Therefore, the development of gene transfer- based stem cell therapy for IPEX syndrome remains challenging (Fig. **1**).

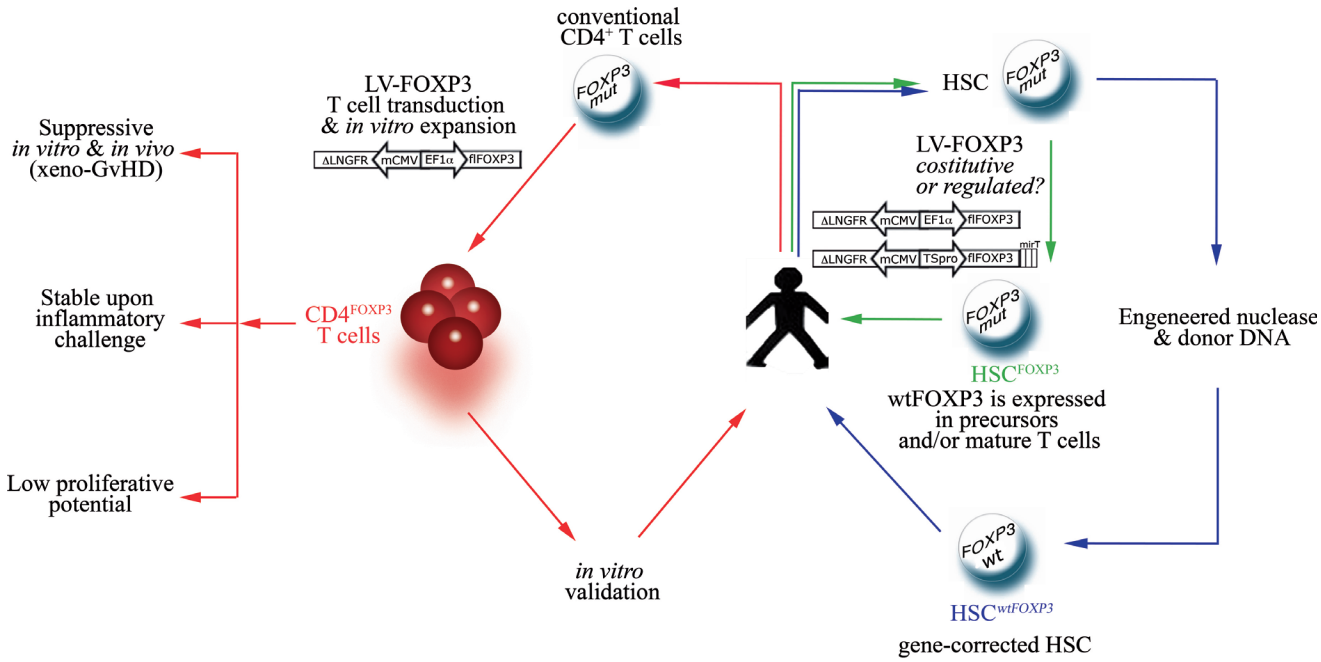

Fig. (1). The scheme summarizes the alternative therapeutic approaches for IPEX syndrome proposed in the present review. Xeno-GvHD: xenogeneic Graft *versus* host disease; mut: mutated; wt: wild type; LV: lentiviral vector; HSC: hematopoietic stem cells; EF1α: elongation factor 1-α promoter; ΔLNGFR: complementary DNA of the truncated low affinity nerve growth factor; mCMV: minimal core promoter derived from the human cytomegalovirus; flFOXP3: complementary DNA of the full length isoform of FOXP3; mirT: micro-RNA target sequences; TSpro: tissue-specific promoter; HSC^{FOXP3}: hematopoietic stem cells transduced with lentiviral vector encoding for FOXP3; $HSC^{wtFOXP3}$: hematopoietic stem cells after target insertion of a wtFOXP3-encoding DNA fragment.

4.3. Genome Editing Based Gene Therapy

While pre-clinical results using a gene transfer lentiviral approach for gene therapy of IPEX are promising, the achievement of specific expression in the regulatory T-cell lineage is still questionable and the risk of insertional oncogenesis is a real limitation, suggesting that genome editing/gene correction may be the best long-term gene therapy strategy (Fig. **1**). The general gene correction approach would be to deliver an engineered nuclease and donor DNA gene correction fragment simultaneously to either hematopoietic stem, progenitor cells or to T-cell precursors. The engineered nucleases would be designed to create a site-specific DNA double strand break (DSB) in the *FOXP3* gene. This DSB would then activate the cellular homologous recombination machinery that would utilize the DNA donor correction fragment as a template for repair thereby both healing the DSB and correcting the gene mutation. There are now several different platforms for the design of engineered nucleases including homing endonucleases/meganucleases, zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs) and RNA guided endonucleases (RGENs of the CRISPR/Cas9 family) (reviewed in [65]). Each of these platforms have different advantages and disadvantages but in the end a clinical grade nuclease would have both high on-target activity to simulate the gene correction in a large fraction of cells and a high specificity such that few genomic mutations at other sites in the genome were created in the gene correction process. Natural meganucleases are the most specific but have been challenging to re-engineer to novel disease-related target sites. ZFNs are currently the most clinically advanced having already entered clinical trials, as an approach to create autologous cells that are resistant to HIV by disrupting the CCR5 gene [66]. In addition, the use of ZFNs targeting *IL2RG* for the correction of hematopoietic stem cells from X-linked SCID patients has been recently reported [67]. TALENs are simpler to engineer than ZFNs, show improved specificity over ZFNs and have been utilized in a number of pre-clinical studies for genetic diseases ([68-70], and Porteus, unpublished data). Finally, CRISPR /Cas9 RGENs are the simplest to engineer, have shown tremendous activity in a range of different cell types [71] but because they have only been used for genome editing since 2013, issues about specificity and activity in primary somatic cells (including HSC) remain. Because mutations that cause IPEX are scattered throughout the gene, the most efficient gene editing strategy would probably be a functional gene correction approach. In this approach a wild-type FOXP3 cDNA would be precisely inserted such that it utilized the endogenous initiation codon. In this way the wild-type cDNA would be expressed and regulated from the endogenous regulatory elements and with the correct design of the donor construct, any splicing regulation could also be maintained. By targeting the wild-type cDNA to functionally correct the endogenous gene, one would solve the potential drawbacks of insertional oncogenesis and mis-regulated gene expression that may occur in a lentiviral strategy. Current experiments are ongoing to test the advantage and feasibility of gene correction for IPEX.

CONCLUDING REMARKS

Knowledge and biotechnology have tremendously empowered the field of gene manipulation and gene correction

for multiple diseases. Physicians and researchers will have to continue working to confirm the benefit of such an approach and to make gene therapy not only feasible, but also affordable for a large number of centers and for patients all around the world.

LIST OF ABBREVIATIONS

Ab	=	Antibody
ADA-SCID	=	Adenosine Deaminase deficient Severe Combined Immune Deficiency
Ag	=	Antigen
CD	=	Crohn's Disease
DSB	=	DNA double strand break
FOXP3	=	Forkhead box p3
GvHD	=	Graft-versus-host disease
HSCT	=	Hematopoietic stem cell transplantationì
IPEX	=	Immune Dysregulation Polyendocrinopathy Enteropathy X-linked
IS	=	Immunosuppressive
LV	=	Lentiviral-vector
miR	=	micro-RNA
PBMC	=	Peripheral blood mononuclear cells
PID	=	Primary immunodeficiency
RGENs	=	RNA guided endonucleases
(t)Treg cell	=	(thymic-derived) regulatory T cell
T1D	=	Type 1 diabetes
TALENs	=	Transcription activator-like effector nucleases
Teff cell	=	Effector T cell
Tr1 cell	=	T regulatory type 1 cell
WAS	=	Wiscott Aldrich syndrome
ZFNs	=	Zinc finger nucleases

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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PATIENT CONSENT

Declared none.

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