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Regulatory T Cells: The Many Faces of Foxp3

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Abstract

Regulatory T (Treg) cells expressing the transcription factor forkhead box P3 (Foxp3) play a requisite role in the maintenance of immunological homeostasis and prevention of peripheral self-tolerance breakdown. Although Foxp3 by itself is neither necessary nor sufficient to specify many aspects of the Treg cell phenotype, its sustained expression in Treg cells is indispensable for their phenotypic stability, metabolic fitness, and regulatory function. In this Review, we summarize recent advances in Treg cell biology, with a particular emphasis on the role of Foxp3 as a transcriptional modulator and metabolic gatekeeper essential to an effective immune regulatory response. We discuss these findings in the context of human inborn errors of immune dysregulation, with a focus on *FOXP3* mutations, leading to Treg cell deficiency. We also highlight emerging concepts of therapeutic Treg cell reprogramming to restore tolerance in the settings of immune dysregulatory disorders.

Keywords

Autoimmunity; Foxp3; immune dysregulation; immune tolerance; immunometabolism; Interleukin 2; IPEX; Rapamycin; Regulatory T cells; Regulatory T cell Reprogramming

Introduction

Since its discovery close to two decades ago, Foxp3 has emerged as a key regulator of immune tolerance by virtue of its function as a master switch factor involved in the differentiation of regulatory T (Treg) cells. Its deficiency in humans gives rise to an autoimmune lymphoproliferative disease: immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX), and to a homologous *scurfy* phenotype in mice. In the interim, much has been learnt about how Foxp3 orchestrates Treg cell responses, and

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how mutations subvert its functions to promote disease. In this review, we survey novel insights into mechanisms of Foxp3 action including its versatility in directing tissue and immune response-specific outcomes by co-opting different transcriptional programs, the vulnerability of such co-option to dysregulation leading to reprogramming of Treg cells towards T effector cell phenotypes, and the emerging role of Foxp3 as a metabolic gatekeeper that maintains the identity and regulatory functions of Treg cells. We also focus on the mechanisms by which gene mutations selectively impair distinct aspects of Foxp3 function, and therapeutic interventions aimed at restoring Treg cell function in the context of Foxp3 deficiency.

Historic perspective

Treg cells were originally described as a subpopulation of $CD4^+$ T cells characterized by high expression of the IL-2 receptor (IL-2R) alpha chain (CD25) and ability to control autoimmunity in mice elicited by thymic manipulation or lymphopenic complementation [1-5]. In 2000, Chatila et al described mutations in the gene encoding the transcription factor forkhead (FKH) box (Fox) P3 (Foxp3), originally called *JM2,* as the cause of an autoimmune lymphoproliferative disorder in human subjects termed X-linked autoimmunity-allergic dysregulation syndrome (XLAAD) and later codified as IPEX [6]. IPEX and scurfy-causing mutations in *FOXP3* and its orthologous mouse gene, respectively, were also described shortly thereafter [7, 8]. The identification of Foxp3 as essential for controlling Treg cell function was established by seminal studies demonstrating that the lymphoproliferative disease in *scurfy* mice results from lack of functional Treg cells [9, 10]. Enforced expression of Foxp3 in conventional murine $CD4^+CD25^-$ T (T_{conv}) cells led to the acquisition of a regulatory phenotype, while adoptive transfer of $CD4^+CD25^+Foxp3^+$ Treg cells into neonatal *scurfy* mice prevented autoimmune disease development [9]. Subsequently, studies in mice using *Foxp3* reporter alleles demonstrated that thymic development of Treg cells proceeds uninterrupted in the absence of functional Foxp3 but leads to the generation of aberrant effector memory-like Treg cells that lack regulatory function [11, 12]. Similarly, $CD4^+CD25^{high}$ Treg-like cells from human subjects with loss of function *FOXP3* mutations failed to suppress autologous effector T cell responses despite being comparable in number and phenotype to those of healthy donors [13]. In addition to their essential role in the maintenance of peripheral tolerance to self-tissues, it is now appreciated that Treg cells play a critical role in enforcing tolerance to the “extended self’, including the commensal flora and innocuous environmental antigens, as well as mediating broad homeostatic and tissue repair functions [14].

Natural and induced Foxp3+ Treg cells

Treg cells represent 5 to 10 % of the total $CD4^+$ T cell pool and express αβ T cell receptors (TCR) with a broad repertoire that is largely distinct from that of T_{conv} cells [15, 16]. Treg cells derive from two distinct populations that act synergistically to enforce peripheral tolerance (Fig. 1) [17]. $CD4^+CD25^+Foxp3^+$ natural regulatory T (nTreg) cells differentiate in the thymus from immature precursors and play a critical role in enforcing tolerance to self-antigens (Fig. 1). Induced regulatory T (iTreg) cells are generated *de novo* extrathymically from naive T_{conv} cells in select niches, especially those at the mucosal

interfaces including the gastrointestinal and respiratory tracts, that offer specialized antigen-presenting cells producing transforming growth factor beta (TGF-β) and retinoic acid, as well as the availability of conducive commensal metabolites (Fig. 1) [18]. The generation of iTreg cells in the gastrointestinal tract is facilitated by mucosal $CD103^{+}CD11c^{+}$ dendritic cells (DCs), while the same role is played by the alveolar macrophages in the lungs [19, 20] (Fig. 1). iTreg cells can also be generated *in-vitro* following TCR activation of naïve T_{conv} cells in the presence of IL-2 and TGF-β [21]. As a function of their distinct developmental ontology, the TCR repertoires of nTreg and iTreg cells are largely non-overlapping [22]. While the TCR repertoire of iTreg cells is directed towards commensal antigens and environmental allergens, nTreg cells express an anti-self-biased TCR repertoire [23, 24] (Fig. 1). This minimal TCR repertoire overlap enables the specification of complimentary antigen coverage in the maintenance of peripheral tolerance, with the presence of both cell types required for optimal tolerance induction [22].

Epigenetic mechanisms regulate Treg cell lineage stability and phenotypic identity [25-28]. For example, the Treg-specific demethylation region (TSDR) within the conserved non-coding sequences 2 (CNS2) of the *Foxp3* locus is mostly demethylated in nTreg cells, whereas it is only partially demethylated in iTreg cells or T_{conv} cells that transiently express Foxp3. nTreg cells also exhibit a specific hypomethylation pattern in genes such as *Il2ra, Ctla4, Tnfrsf18,* and *Ikzf2* as well as histone modifications including trimethylation of histone H3 lysine 4 (H3K4me) [29]. In the context of an inflammatory environment, iTreg cells are particularly prone to destabilization, may lose Foxp3 expression and degenerate into effector cells (ex-Treg) that contribute to diseases pathogenesis [30, 31]. nTreg cells may also destabilize in the context of chronic inflammatory/autoimmune processes and acquire effector-like phenotypes [32, 33].

Phenotypic classification of Foxp3⁺ Treg cells

In addition to their constitutively high CD25 cell surface expression, Treg cells express low levels of the IL-7 receptor alpha chain (CD127), which inversely correlates with Foxp3 expression [34]. Treg cells also express high levels of the cytotoxic T-lymphocyte antigen 4 (CTLA-4), as well as molecules that constitute a canonical Treg cell surface signature, including the glucocorticoid-induced tumor necrosis factor receptor (TNFR) family related protein (GITR) and inducible co-stimulator (ICOS) [reviewed in 35]. However, the phenotypic demarcation of Treg cells using these markers is complicated by their frequent upregulation in T_{conv} cells following TCR engagement. In human $CD4^{+}$ T_{conv} cells, Foxp3 can be transiently induced at low levels in response to TCR stimulation, and activation inducible upregulation of other “canonical” Treg cell markers (e.g CTLA-4) in T_{conv} cells can further stymie the identification of bone fide Treg cell populations. With that in mind, careful gating on $CD4^{+}CD25^{hi}CD127^{low}$ T cells in human peripheral blood can identify a population that is > 90 % Treg cells as confirmed by high Foxp3 expression and demethylation at the *Foxp3* locus (34, 36, 37).

Human peripheral blood Treg cells have been functionally subdivided into distinct subsets based on their expression of the common leukocyte antigen isoform RA (CD45RA) and Foxp3 [38]. While $CD45RA^{+}Foxp3^{low}$ cells represent a resting population of Treg cells

(rTreg), CD45RA^{-} Foxp3^{high} cells constitute an activated Treg cell (aTreg) fraction that is derived from rTregs *in-vitro* and *in-vivo* [38]. This fractionation approach has been leveraged clinically to demonstrate a decrease in the proportion of aTreg cells in human subjects with autoimmune conditions such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [38, 39]. In mice, resting and activated Treg cell populations are functionally delineated by expression of the markers Ly-6C, CD44 and CCR7 [40, 41]. Additional markers have been identified that help segregate nTreg from iTreg cells [42-45]. Helios, a member of the Ikaros transcription factor family, is highly expressed in nTreg cells, but not in iTreg cells (Fig. 1) [42, 43]. Similarly, neuropilin-1 (Nrp-1) may also serve as an additional marker that is expressed at high levels on nTreg cells, although its expression may be upregulated on iTreg cells in the context of inflammation (Fig. 1) [44, 45].

Treg cell lineage specification and Foxp3

The initial discovery of Foxp3 as a “master switch gene” of the Treg cell lineage being absolutely required for Treg cell phenotypic and functional specification has evolved into a view of Foxp3 as a molecular “orchestrator” of the Treg cell program (Fig. 2). Previously we demonstrated that development of nTreg cells in the thymi of mice with a mutant *Foxp3* allele proceeds uninterrupted, and that both Foxp3-deficient and sufficient Treg cells share a core Treg cell transcriptome with intact transcriptional activity at the *Foxp3* locus [11]. A study by Gavin et al similarly demonstrated that *Foxp3* largely consolidates pre-existing features of Treg cell precursors to solidify rather than establish the Treg cell lineage [12]. Moreover, cross sectional analysis of the Treg cell signature revealed that *Foxp3* plays a relatively minor role in specifying the transcriptional landscape of Treg cells [46, 47]. Nevertheless, Foxp3 activity was critical for conferring Treg cell suppressor function by stabilizing and amplifying Treg cell expression of genes such as *Il2ra, Ctla4* and *Tnfrsf18* [11, 12]. In addition, Foxp3 dependent repression of effector cytokine gene expression (e.g IL-4, IFN-γ, IL-17 and IL-21) and downregulation of the cyclic nucleotide phosphodiesterase 3B (PDE3B) activity is essential for the maintenance of Treg cell homeostasis [11]. Collectively these studies demonstrate that additional elements upstream of *Foxp3* specify the Treg lineage as part of a higher order regulatory network and that *Foxp3* is absolutely required for Treg cell suppressor function.

Other Fox transcription factors also contribute to Treg cell lineage specification. Foxp1 promotes Foxp3 occupancy of several Treg cell signature genes, and its deficiency results in diminished expression of key molecules implicated in Treg cell suppressor function including CTLA-4 and CD25 [48]. Similarly, Foxo transcription factors play a critical role Treg cell lineage specification through direct control of *Foxp3* and *Ctla4* activity [49, 50]. In particular, Foxo1 represses Treg cell IFN-γ expression, and its deficiency in Treg cells precipitates a fatal inflammatory disorder similar to that of Foxp3 deficiency [51]. In turn, repression of Foxo1 is critical for the differentiation of activated Treg cells as evidenced by dysregulated Treg cell homing to non-lymphoid tissues and impaired suppression of CD8^{+} T cell dependent inflammation in mice expressing a constitutively active Foxo1 in the Treg cell compartment [52]. Although Foxo3 deficient mice do not exhibit manifestations of spontaneous T cell activation, T cell specific deletion of both genes encoding the Foxo1 and Foxo3 transcription factors precipitates a multifocal inflammatory disease due to

compromised Treg cell differentiation and function [50, 53]. Collectively, these studies illustrate the critical, non-redundant roles played by different Fox transcription factors in the Treg cell lineage.

The modular nature of Foxp3$^+$ Treg cell suppressor functions

Treg cells target a broad array of innate and adaptive immune cells, including CD4$^+$ and CD8$^+$ T lymphocytes, B lymphocytes, monocytes/macrophages, DCs, mast cells and innate lymphoid cells (ILC) [reviewed in 54]. Although multiple immunoregulatory mechanisms are utilized by Treg cells to limit inflammatory immune responses, individual mechanisms may be operative in a dedicated modular fashion that is restricted to particular tissue sites or distinct inflammatory settings. Treg cells can enforce suppressor function by diverse mechanisms that target specific effector pathways and/or responses, including contact-dependent mechanisms, immunomodulatory cytokines such as IL-10, TGF-β and IL-35, as well as through metabolic perturbation of target cells (Fig. 3). Mutations in human subjects and mouse models have clarified the mechanisms by which these pathways contribute to peripheral tolerance maintenance and the consequences of their disruption in promoting disease.

A prime example of contact-dependent Treg cell-mediated suppression involves the CTLA-4/lipopolysaccharide-responsive beige-like anchor (LRBA) axis. CTLA-4 downregulates major costimulatory molecule expression on antigen presenting cells (APCs) and limits costimulatory signals by cell extrinsic depletion of its ligands CD80 and CD86 (Fig. 3) [55, 56]. Moreover, CTLA-4 may additionally stimulate indoleamine 2,3-dioxygenase (IDO) expression in human and murine DC subsets, which induces catabolism of the essential amino acid tryptophan to starve T cells [57]. In mice, Treg cell specific deletion of CTLA-4 results in a fatal autoimmune lymphoproliferative disease [58]. Human subjects with heterozygous mutations in CTLA-4 also present with an autosomal dominant autoimmune lymphoproliferative disease, while autosomal recessive mutations in LRBA, which regulates CTLA-4 cell surface expression, result in a phenotypically similar disease [59, 60]. CTLA-4 antagonism using monoclonal anti-CTLA-4 antibodies (mAbs) reverses Treg suppressor function *in-vivo,* while its germline deficiency in mice leads to a fatal lymphoproliferative disorder within the first month of life [61, 62].

Additional mechanisms by which Treg cells may act to control the immune response through cell contact involve the interaction of LAG-3 on Treg cells with MHC-II on immature DCs, leading to suppression of DC maturation (Fig. 3) [63]. Antigen specific Tregs may also physically remove cognate peptide MHC-II complexes from the surface of DC's *in-vivo* in a CTLA-4 independent manner (Fig. 3) [64]. A different suppressive mechanism involves Treg cell-mediated cytolysis of target cells, which is dependent on the activity of granzyme B (Fig. 3) [65]. Similarly, human Treg cells expressing granzyme A have been shown to induce target cell killing in a perforin dependent manner *in-vitro* (Fig. 3) [66]. This mechanism may be relevant to the profound immune dysregulation in patients with hemophagocytic lymphohistiocytosis (HLH) disorders, the causative mutations of which, including those in the perforin gene, may result in defective Treg cell cytolytic function [67, 68].

Treg cells also exert regulation through soluble intermediates. The ecto-enzymes ATP apyrase (CD39) and ecto-5'-AMP-nucleotidase (CD73), which in tandem generate the immunosuppressive purine nucleoside adenosine (Fig. 3) [69]. Adenosine can signal via the high affinity G protein-coupled (GPRC) adenosine A2A receptor (A2AR) expressed on T cells to mediate inhibitory signaling in a cAMP dependent manner [70]. Moreover, Treg cells have been proposed to act as an IL-2 sink through their constitutive CD25 expression and IL-2 signals are required for optimal Treg suppressor function through STAT5b activation (Fig. 3) [71, 72].

The immunoregulatory cytokines IL-10 and TGF-β represent two key Treg cell effector modules with specialized functions. Treg cell derived IL-10 is particularly important in restraining immunological hyperreactivity at environmental interfaces including the colon, skin, and lung [73, 74]. Mice harboring IL-10 deficient Treg cells develop spontaneous colitis and exhibit heightened immune-mediated reactivity in the airways [73]. Similarly, ablation of the interleukin 10 receptor (IL-10R) in Treg cells impairs STAT3 phosphorylation and results in selective dysregulation of T_H17 immune responses accompanied by development of severe immune-mediated colitis [74]. Moreover, IL-10 may promote the expansion and function of $Foxp3^+$ iTreg cells by enhancing Foxo1 nuclear localization and augmenting STAT3 signaling [75]. In human subjects, IL-10/IL10R gene mutations are associated with chronic gastrointestinal inflammatory disorders such as ulcerative colitis and Crohn's disease (reviewed in 76, 77].

TFG-β represents another dedicated module of Treg suppression that plays a privileged role at mucosal interfaces. For example, Treg cells require TFG-β signals to appropriately limit IL-17 production and dampen T_H17 cell responses in the gastrointestinal tract [78]. Bi-allelic Treg cell-specific deletion of *Tgfb1* in mice results in a scurfy mouse like phenotype, while monoallelic deletion results in allergic dysregulation, reflecting a non-redundant privileged role for Treg cell-derived TGF-β1 in controlling the immune response (manuscript in preparation). In humans, heterozygous loss of function mutations in *TGFBR1* and *TGFBR2* gives rise to the Loeys Dietz syndrome, associated with allergic dysregulation in addition to non-immune somatic phenotypes [79]. Biallelic *TGFB1* loss of function mutations results in severe inflammatory bowel disease and encephalopathy [80]. Together these findings emphasize the critical role of the TGF-β module in regulating both allergic and autoimmune processes.

Tissue and functional adaptation of $Foxp3^+$ Treg cells

The suppressive function of Treg cells is critical for the prevention of autoimmunity and collateral damage to healthy tissues and organs resulting from inflammatory immune responses. Treg cells also play an important role in enforcing homeostasis in non-lymphoid tissues including skeletal muscle, visceral adipose tissue (VAT), and the colonic lamina propria. The unique phenotype and function of Treg cells at these sites is largely dictated by the local environment as well as tissue specific physiological cues [reviewed in 81]. For example, Treg cells enriched in VAT express the peroxisome proliferator-activated receptor gamma (PPARγ) and are critically involved in the control of VAT inflammation and insulin sensitivity [82, 83]. Similarly, expression of the retinoid-related orphan receptor alpha

(RORα) in skin resident Treg cells drives expression of the tumor necrosis factor receptor superfamily member 25 (TNFRSF25, also known as death receptor 3 or DR3) which promotes Treg cell mediated suppression of ILC-2 activation and leads to restraint of allergic skin inflammation [84]. More recently, skin resident Treg cells expressing high levels of the Notch ligand family member Jagged 1 (Jag1) were shown to facilitate the differentiation of hair follicle stem cells (HFSC) and promote hair follicle regeneration [85]. In skeletal muscle, a distinct population of Treg cells producing the epidermal growth factor amphiregulin promoted repair of damaged muscle following acute muscle injury [86]. Similarly, in a mouse model of muscular dystrophy, Treg cell depletion was associated with exacerbation of muscle injury [87].

Interestingly, selective deficiency of amphiregulin in Treg cells did not affect their suppressor function but lead to severe lung damage during influenza viral infection [88]. In the central nervous system, Treg cells were shown to exhibit regenerative properties, including the capacity to promote oligodendrocyte progenitor cell differentiation and myelination [89]. Collectively, these studies highlight the importance of phenotypic and functional adaptation of Treg cells in diverse tissue environments that shape distinct non-immunomodulatory functions of Treg cells.

It is now appreciated that Treg cells show a functional plasticity that results in the acquisition of distinct helper T (T_H) cell transcriptional machinery leading to superior control of the corresponding T_H cell immune response (Fig. 4) [33, 90-94]. For example, STAT3 activation in Treg cells is required for optimal suppression of T_H17 immune responses and results in the acquisition by Treg cells of T_H17 specific molecules, such as the interleukin 6 receptor (IL-6R) [90] (Fig. 4). Similarly, expression of the T_H1 transcriptional master regulator T-bet in Treg cells is critical for efficient control of T_H1 immune responses and prevention of T_H1 mediated autoimmunity [91]. Foxp3 dependent expression of the interferon regulatory factor-4 (IRF4) endows Treg cells with a preferential capacity to suppress T_H2 immune responses while IRF4 deficiency in Treg cells promotes dysregulation of T_H2 immune responses [92]. Similarly, the T_H2 master regulator GATA-3 binds to the CNS2 of the *Foxp3* locus to promote its activity [93]. Furthermore, follicular regulatory T (T_{FR}) cells expressing the chemokine receptor CXCR5 are critically dependent on the transcriptional repressor Bcl6 for efficient control of the germinal center reaction as well as plasma cell differentiation [94]. Collectively these studies underscore the importance of molecular co-option by Treg cells of diverse T_H cell transcriptional machinery as mechanism for optimal control of T_H specific immune responses. The susceptibility of such co-option to pathological Treg cell reprogramming and dysregulation is discussed below.

Pathological reprogramming of Foxp3$^+$ Treg cells

Treg cells exhibit a wide versatility in directing immune response-specific outcomes by their ability to hijack distinct molecular modules directing specialized T_H cell responses (Fig. 4) [33, 90-94]. For example, Treg cells undergo “abortive” Th1 reprogramming by upregulating *Tbx21* expression in an IFN-γ STAT1 dependent manner that results in their acquisition of a partial Th1 program characterized by CXCR3 expression (Fig. 4) [33]. However, in the context of this appropriation, Treg cells fail to upregulate expression of the interleukin

receptor 12 beta 2 subunit (IL12Rβ2), which is required for completion of T_H1 differentiation. Failure of such restraint in the context of aberrant inflammation may lead to redirection toward T_H like phenotypes resulting in the dysfunctional reprogramming of Treg cells. For example, our laboratory recently demonstrated that constitutive Notch signaling destabilizes Treg cells by promoting nTreg cell T_H1 like skewing [32].

Recent studies have elucidated mechanisms by which Treg cells may acquire effector T cell attributes that contribute to their pathogenic reprogramming in disease states characterized by persistent inflammation (Fig. 5). Mice carrying a tyrosine (Y) to phenylalanine (F) substitution at position 709 of the murine IL-4Rα (*Il4ra*^{F709}), which inactivates the receptor immunotyrosine inhibitory motif (ITIM) [95, 96], model human polymorphisms in the interleukin-4 receptor alpha chain (IL-4Rα) that promote enhanced STAT6 activation. The *Il4ra*^{F709} mice exhibit exaggerated T_H2 polarization, augmented antigen induced IgE responses, and exacerbated airway inflammation and hyperreactivity. Critically, Treg cells from *Il4ra*^{F709} mice acquired a T_H2-like phenotype characterized by augmented STAT6 signaling and heightened expression of canonical T_H2 transcription factors including IRF-4 and GATA-3 (Fig. 5) [97]. This T_H2 reprogramming was also evident in peripheral antigen specific Treg cells of milk allergic children [97]. Importantly, Treg lineage specific ablation of *Il13, Il14* or *Stat6* in *Il4ra*^{F709} mice reversed T_H2 reprogramming and restored Treg cell regulatory function.

Another example of pathological reprogramming of iTreg cells in the context of allergic airway inflammation was provided in studies on a human IL-4R variant with a glutamine (Q) to arginine (R) substitution at position 576. This variant acts as an allergic asthma susceptibility gene and strongly correlates with the prevalence and clinical severity of asthma [98]. Mice homozygous for the *Il4ra*R^{576} allele exhibited exaggerated allergic airway inflammation following sensitization and challenge with allergens, driven by the destabilization of allergen specific iTreg cells towards a pathogenic T_H17 like phenotype (Fig. 5) [99]. Treg specific deletion of *Il6ra* and *Rorc* or neutralization with an anti-IL-6 mAb prevented airway inflammation pursuant to T_H17 Treg cell reprogramming.

In the gut, microbiota induced expression of the transcriptional factor ROR-γt in Treg cells is indispensable for regulation of T_H17 colonic inflammation as well as restriction of T_H2 immune mediated pathology [100, 101]. More recently, we demonstrated that dysbiosis promotes the breakdown of oral tolerance as a result of a failure to induce protective ROR-γt dependent iTreg responses in food allergic children and mice, allowing instead for the emergence of pathogenic Th2 [102].

Collectively, these studies reveal an important role for pathological Treg cell reprogramming in sustaining chronic inflammatory allergic and autoimmune diseases, and the potential for interventions aimed at resetting such reprogramming as a novel therapeutic approach.

Foxp3 as a metabolic gatekeeper

Treg cells exhibit a unique metabolic signature in comparison to other T_H cell subsets, which sustains their proliferative and regulatory functions while constraining the

development of effector programs normally associated with Th cells [103, 104] (Fig. 6). While T_H1, T_H2, and T_H17 cells express high levels of the glucose transporter 1 (GLUT1) and display a heightened glycolytic rate, Treg cells at steady state preferentially utilize fatty acid oxidation (FAO) and pyruvate dependent oxidative phosphorylation (OXPHOS) to satisfy their energetic demands [reviewed in 105] (Fig. 6). Foxp3 confers a metabolic advantage on Treg cells in low-glucose lactate rich environments by suppressing *Myc* gene expression and Treg cell glycolysis in order to maintain regulatory function [103]. In contrast, inflammatory triggers acting via toll like receptors (TLR) promote Treg cell proliferation by engaging the PI(3)K-AKT-mTORC1 pathway, which leads to GLUT1 upregulation and augmentation of glycolysis [106] (Fig. 6). Although upregulation of GLUT1 promotes Treg cell expansion, it dampens Treg cell regulatory function and reduces expression levels of Foxp3. Furthermore, expression of Foxp3 results in upregulation of several mitochondrial protein-encoding genes, including components of the electron transport chain [107]. This in turn drives an increase in the respiratory capacity of Treg cells and augments their ability to utilize fatty acids in order to fuel OXPHOS. More recently, it was shown that loss of the mitochondrial complex III in Treg cells promotes systemic autoimmunity by impairing Treg cell suppressor function without altering expression of Foxp3 [108]. Collectively, these studies suggest that Treg cell metabolic pathways can be therapeutically exploited to enhance or brake peripheral tolerance.

We have further explored the role of Foxp3 as a metabolic regulator in Foxp3-deficient mice in which a mutant *Foxp3* allele directs the expression of a humanized Cre recombinase (iCre) fused with EGFP ($Foxp3^{\Delta EGFPiCre}$) [109]. Foxp3 deficiency dysregulated mTORC2 signaling and induced metabolic reprogramming of the mutant Treg cells characterized by augmented glycolysis and OXPHOS (Fig. 6). Mutant Treg cell specific deletion of the mTORC2 adaptor gene *rictor* ameliorated disease in $Foxp3^{\Delta EGFPiCre}$ mice, reversed augmentation of glycolytic and respiratory activity, and partially restored mutant Treg cell suppressor function [109]. Similarly, Foxp3-deficient Treg cells of IPEX patients exhibited dysregulated mTORC2 signaling and augmented glycolysis, both of which were reversed upon treatment with mTOR inhibitors such as rapamycin to reestablish regulatory function [109]. These data demonstrate that Foxp3 plays a critical role in orchestrating Treg cell metabolism and suggests that therapeutic targeting of metabolic pathways of Foxp3-deficeint Treg cells may provide an important conceptual advance in the restoration of immune tolerance.

Foxp3 and Treg cell defects in human autoimmune disease

A number of mendelian disorders of immune dysregulation have been identified as a consequence of impaired Treg cell function and development [110, 111]. Loss of function mutations affecting *FOXP3* lead to the generation of defective Treg cells and give rise to the IPEX syndrome in human subjects. This syndrome exclusively affects males and is frequently lethal in the absence of Treg cell reconstitution by hematopoietic stem cell (HSC) transplantation. Consistent with its X-linked mode of inheritance, female carriers are asymptomatic. The immunopathology results from unrestrained T cell activation, with an additional contribution by autoantibodies. The hallmarks of this syndrome include autoimmune enteropathy, autoimmune endocrinopathy, eczematous dermatitis, and immune

mediated cytopenia's. The autoimmune endocrinopathy is characterized by the early onset (1st year of life) of insulin-dependent type 1 diabetes (T1D) with islet cell destruction. Additionally, patients present with allergic dysregulation that manifests as food allergy, increased serum IgE levels, peripheral eosinophilia, and prominent Th2 skewing that collectively indicate the breakdown of oral tolerance.

FOXP3 is comprised of 12 exons encoding a 431 amino acid Foxp3 protein that consists of a C-terminal FKH domain, a proline rich N-terminal (PRR) domain, a C2H2 zinc finger (ZF), and a central leucine zipper (LZ) domain (Fig. 7). The FHK domain is required for DNA binding activity, nuclear localization, and interacts with several transcription factors including the nuclear factor of activated T cells (NFAT), leading to expression of canonical Treg cell components such as CD25, GITR and CTLA-4 [112]. The LZ and ZF domains are involved in protein to protein interactions with the latter implicated in assembly of higher order complexes of Foxp3 [reviewed in 111]. Several proteins interact with FOXP3 at its N-terminus, including the transcription factor hypoxia inducible factor 1 alpha (HIF1α) and the histone acetyltransferase p300 [reviewed in 111].

Studies in mice revealed that Foxp3 can bind to thousands of genomic sites and interact with diverse binding partners to orchestrate the development and function of Treg cells (Fig. 2) [113-115]. It is thought that Foxp3 interacts with its binding partners in a modular fashion, where distinct functional domains of Foxp3 complexes act as simple repressors or activators in a dominant manner to drive the Foxp3 program [113-115]. Conversely, evidence for a "collaborative" view for distinct Foxp3 domains operating as a flexible molecular machine is now emerging [116]. For example, to elucidate function-structure relationships within Foxp3 and assess its ability to bind DNA and regulate transcriptional activity, Kwon et al performed a systematic alanine scan mutagenesis of Foxp3 [116]. Interestingly, the mutations affected transcriptional activity in a largely integrative manner, often implicating the entire molecule rather than ascribing distinct functions to individual Foxp3 domains. Moreover, missense mutations in Foxp3 with no overt autoimmune manifestations revealed Treg cell defects only in the context of systemic stress, implicating such mutations in non-classical IPEX like pathologies.

The spectrum of Foxp3 mutations in IPEX have been extensively documented and reviewed and are summarized in Fig. 7 [117-119]. Although IPEX is associated with both missense and nonsense mutations of *FOXP3,* the majority fall within the former group and affect all domains, but disproportionately the FKH DNA binding domain (Fig. 7). Several of these mutations have been studied at great detail, notably A384T [120,121]. Mouse models of these mutations have also clarified the mechanisms by which they act to promote disease. Emerging genotype/phenotype relationships in IPEX suggest that similar genotypes do not necessarily result in similar phenotypes with respect to disease severity and clinical presentation [122]. However, mutations within the functional domains of *FOXP3,* particularly those affecting the FKH and leucine zipper domains, are generally associated with more severe clinical phenotypes while missense mutations, small in-frame deletions, and mutations within the promoter and 5' untranslated region of *FOXP3* are associated with a milder clinical phenotype [reviewed in 111].

A common IPEX missense mutation (A384T) (Fig. 7) in the FKH domain of Foxp3 disrupts its binding to the histone acetyltransferase Tat-interaction protein 60 (TIP60) and abolishes the suppressor function of Treg cells [120]. The same mutation was reported in another study to broaden the DNA binding specificity of Foxp3 and results in tissue-specific autoimmunity and impaired Treg cell fitness through enhanced repression of BATF activity [121]. A mutation (M370I) in the domain swap interface of Foxp3, which is required for dimerization through the FKH domain, resulted in deregulation of the T_H2 locus and generation of T_H2-like Treg cells expressing the transcription factor GATA-3 [123]. Moreover, expression of this Foxp3 variant in mice promoted the development of unrestrained T_H2 immune responses independent of IL4/IL-13 production by Teff cells [123]. A recent report demonstrated that expression of an alternatively spliced isoform of *FOXP3,* which excludes exon 2 (FOXP3Δ2), was sufficient to support Treg cell development and mitigate some distinct features of classical IPEX, even in the absence of full length FOXP3 [124]. Overall, these findings emphasize distinct actions by which *FOXP3* mutations may act to subvert Treg cell regulatory function in order to promote immune dysregulation and autoimmunity.

IPEX-like and other Treg cell-related disorders with functional Foxp3

A number of inborn errors of immune dysregulation that affect Treg cell development have been described in patients who lack detectable mutations in *FOXP3* but have overlapping features with IPEX syndrome [111]. Loss of function mutations in genes along the IL-2 receptor axis, including *IL2RA/B* and *STAT5B,* are well studied examples [reviewed in 111]. CD25 deficiency due to *IL2RA* mutations mimics classical IPEX in presenting with eczema, enteropathy, and autoimmunity and lymphoproliferation, reflecting both the failure of Treg cells to regulate effector cells by means of IL-2 deprivation (the IL-2 sink effect) and also the role of IL-2 in mediating Treg cell fitness [125-128]. However, patients with CD25 deficiency are distinguished by a susceptibility to chronic infections, particularly to herpes family viruses [125-128]. More recently, homozygous mutations in *IL2RB* were shown to result in severe early onset multi-system autoimmunity, characterized by diminished frequencies of Treg cells with similar features to *IL2RA* deficiency, including a susceptibility to viral infections [129, 130]. Consistent with an absolute requirement for STAT5b in IL-2R signaling, several features of *STAT5B* deficiency are shared with *IL2RA* deficiency and include decreased numbers of Treg cells characterized by reduced Foxp3 expression and impaired regulatory function [131, 132]. More recently, dominant negative heterozygous mutations in *STAT5B* have been described that give rise to growth hormone insensitivity and immune dysregulation with eczema and elevated IgE but in the absence of frank immunodeficiency [133].

A prominent cause of IPEX-like disorders are mutations affecting the *CTLA4-LRBA* axis [134-135]. Homozygous loss of function mutations in *LRBA,* which result in the near total loss of CTLA4 expression, result in profound Treg cell dysregulation characterized by impaired Treg cell-mediated suppression, marked T follicular helper (TFH) cell expansion, contraction of the Treg cell compartment in general and the T follicular regulatory compartment in particular and increased autoantibody production [60, 134]. Mutations in *CTLA4,* the overwhelming majority of which are heterozygous in nature, largely phenocopy

the presentation of LRBA deficiency, albeit with variable penetrance and more indolent disease onset and course [135].

Many primary immunodeficiencies, some of which are listed below, are associated with Treg cell dysfunction, reflecting the role of those pathways impacted by the respective deficiency in Treg cell development and differentiated functions. Hypomorphic mutations affecting the recombination-activating gene 1 (*RAG1*) and *RAG2* give rise to severe restrictions on the Treg cell repertoire, leading to a phenotype of immune dysregulation and autoimmunity [136, 137]. Examples ranging across a spectrum of decreasing disease severity include Omenn's syndrome, atypical SCID, and delayed onset combined immunodeficiency with granulomas and/or autoimmunity [138]. All of these disorders manifest immune dysregulation reflective of Treg cell deficiency, characterized by restricted repertoire diversity [138]. Mutations in other genes such as those involved in the store-operated calcium entry, including STIM1 and ORAI 1, encoding stromal interaction molecule 1 and calcium release activated modulator 1 respectively, give rise to a disorder of immunodeficiency and autoimmunity with the latter related to impaired T follicular and tissue resident Treg cell differentiation [139]. Other immunodeficiency-causing gene defects, including those affecting the actin cytoskeleton such as the dedicator of cytokinesis 8 (DOCK8) and Wiskott-Aldrich protein (WASP) deficiencies, may also present with an IPEX-like phenotype or more typically immune dysregulation due to the profound impairment of Treg cell function [140, 141]. Similarly, homozygous loss of function mutations in the *CARMIL2* (*RLTPR*) gene, encoding the capping protein regulator and myosin 1 linker 2, which is critical for CD28 co-stimulatory signaling and cytoskeletal organization, results in the profound depletion of Treg cells and severe Th2 cell skewing [142-144]. Gain of function mutations in *STAT1* may result in an IPEX like phenotype despite normal Treg cell frequencies, possibly reflecting altered transcriptional programs in these cells [145].

Therapeutic manipulation of Foxp3 Treg cells

The critical role of Foxp3$^+$ Treg cells in the maintenance of immunological homeostasis makes them an attractive immunotherapeutic target for the induction or restoration of tolerance. Decreased Treg cell function as well as numerical Treg cell deficiencies have been described in several autoimmune diseases in human subjects and experimental models in mice [reviewed in 146]. Several clinical studies using already approved drugs (e.g IL-2, rapamycin) have shown that Treg cells can be successfully expanded *in-vivo* with clinically meaningful benefits [reviewed in 147]. Low dose IL-2 therapy, which preferentially activates Treg cells as a consequence of their constitutive CD25 expression, was effective in the treatment of graft versus host disease (GvHD) as well as hepatitis C virus- induced vasculitis [148-150]. Robust expansion of Treg cells following low dose IL-2 therapy has also been observed in patients with type 1 diabetes (T1D) and alopecia areata, where Treg cell recruitment in lesional skin was accompanied by partial regrowth of hair scalp and reduced effector CD8$^+$ T cell infiltration [151-153]. Promising results have also emerged from clinical trials in systemic lupus erythematosus (SLE)[154]. Moreover, low dose IL-2 was shown to selectively increase the function and abundance of Treg cells in patients with SLE without affecting the frequencies of T_H1 and T_H2 cells [155]. Remarkably, all patients in

this trial exhibited decreased disease activity at the end of the study, accompanied by decreased T_H17 and TFH cell numbers [155].

In addition to low dose IL-2, other therapeutic approaches include the development of IL-2 Fc fusion proteins or biochemically coupling IL-2 to molecular carriers such as polyethylene glycol (PEG) for the generation of novel IL-2 muteins that preferentially stimulate Treg cell activity [156-158] (Fig. 8). Furthermore, the generation of orthogonal IL-2 cytokine receptor complexes as well as anti-IL-2 antibodies, which when complexed with IL-2 can preferentially stimulate the expansion of Treg or T_{conv} cells, is also underway [156-158]. This concept is based on mouse studies demonstrating that IL-2/antibody complexes elicit preferential expansion of Treg cells *in-vivo* [159], leading to protection from experimental autoimmune encephalomyelitis (EAE) and prevention of pancreatic islet allograft rejection [160]. Alternatively, massive *ex-vivo* expansion of patient derived polyclonal nTreg cells for the purposes of adoptive cell transfer (ACT) therapy in autoimmune disease settings is already under exploration (Fig. 8). The first clinical studies utilizing *ex-vivo* expanded polyclonal nTreg cells for ACT in GvHD and T1D demonstrated the feasibility and safety of this approach [161, 162]. A more recent escalation phase 1 clinical trial in T1D with polyclonal Treg cells was also shown to be well tolerated [163]. Interestingly, these studies revealed long-term retention of a subset of the *ex-vivo* transferred polyclonal Treg cells with a broad regulatory phenotype. *ex-vivo* transferred polyclonal Treg cells with a broad regulatory phenotype.

A conceptually novel approach to restoring immune tolerance involves the metabolic reprogramming of Treg cells, including the inhibition of dysregulated mTORC2 activity in Foxp3-deficient Treg cells, including those of IPEX subjects, by using the dual mTOR inhibitor rapamycin [109]. Such an approach is consistent with the observation that rapamycin provides superior immunosuppression in patients with IPEX [164]. Combinatorial metabolic reprogramming approaches in which mTORC2 inhibition is allied with other interventions that suppress the exuberant oxidative phosphorylation and glutaminolysis found in Foxp3-deficient Treg cells and restores their defective fatty acid oxidation may further potentiate the regulatory functions of mutant Treg cells in IPEX subjects [109]. Finally, emerging genetic approaches including gene editing of mutant *FOXP3* and related genes involved in monogenic disorders of immune dysregulation as well as engineering antigen-specific Treg cells for the purpose of treating autoimmune diseases promise novel approaches to therapy in the forthcoming years.

Conclusions

Since the discovery of Foxp3, a veritable revolution in our understanding of immune regulation and its disruption in a variety of human genetic and acquired disorders has emerged. Future research promises to deliver selective manipulation of the Treg cell responses as means of delivering precision immunotherapy. There is also the emerging role of Treg cells in the maintenance of tissue homeostasis, relevant to a variety of non-immune disorders, including metabolic and degenerative disorders.

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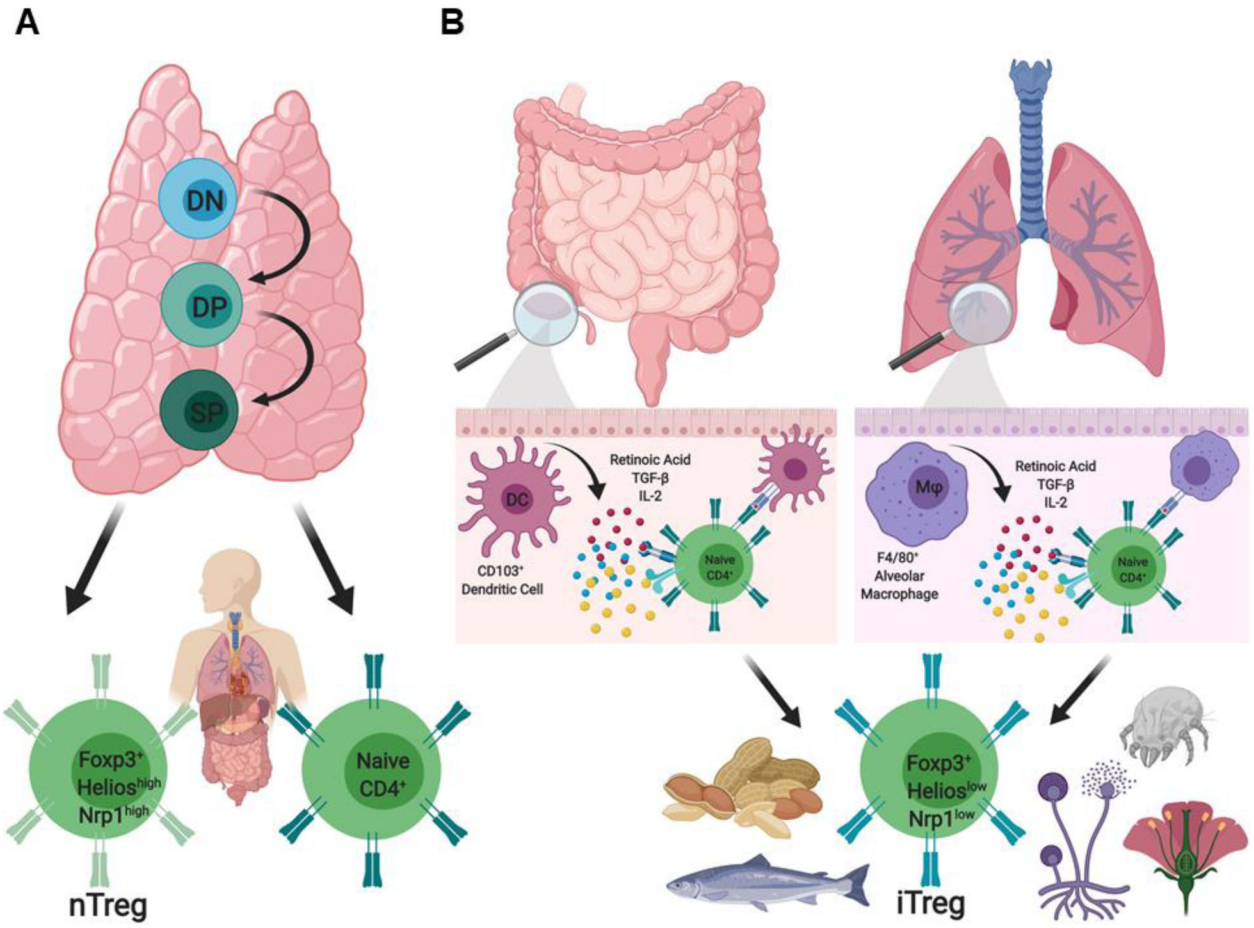

Figure 1. Natural and induced Treg cell subsets.

The peripheral Treg cell pool is composed of 2 distinct populations, nTreg and iTreg cells, which express similar levels of the transcription factor Foxp3 but have non-overlapping TCR repertoires. **(A)** $Foxp3^{+}Helios^{high}Neuropilin^{high}$nTreg cells differentiate in the thymus and play a critical role in enforcing tolerance to self-antigens. **(B)** $Foxp3^{+}Helios^{low}Neuropilin^{low}$ iTreg cells are generated *de novo* extrathymically in peripheral lymphoid tissue from naïve $CD4^{+}CD25^{-}$ Tconv cells in the presence of retinoic acid, TGF-β, and IL-2 following TCR engagement by $CD103^{+}$ dendritic cells or $F4/80^{+}$ macrophages in the intestinal mucosa and alveola inerstitia, respectively. Unlike nTreg cells, iTreg cells are skewed in favor of recognizing non-self antigens, including the commensal flora and infectious agents, and innocuous antigens such as allergens and foods.

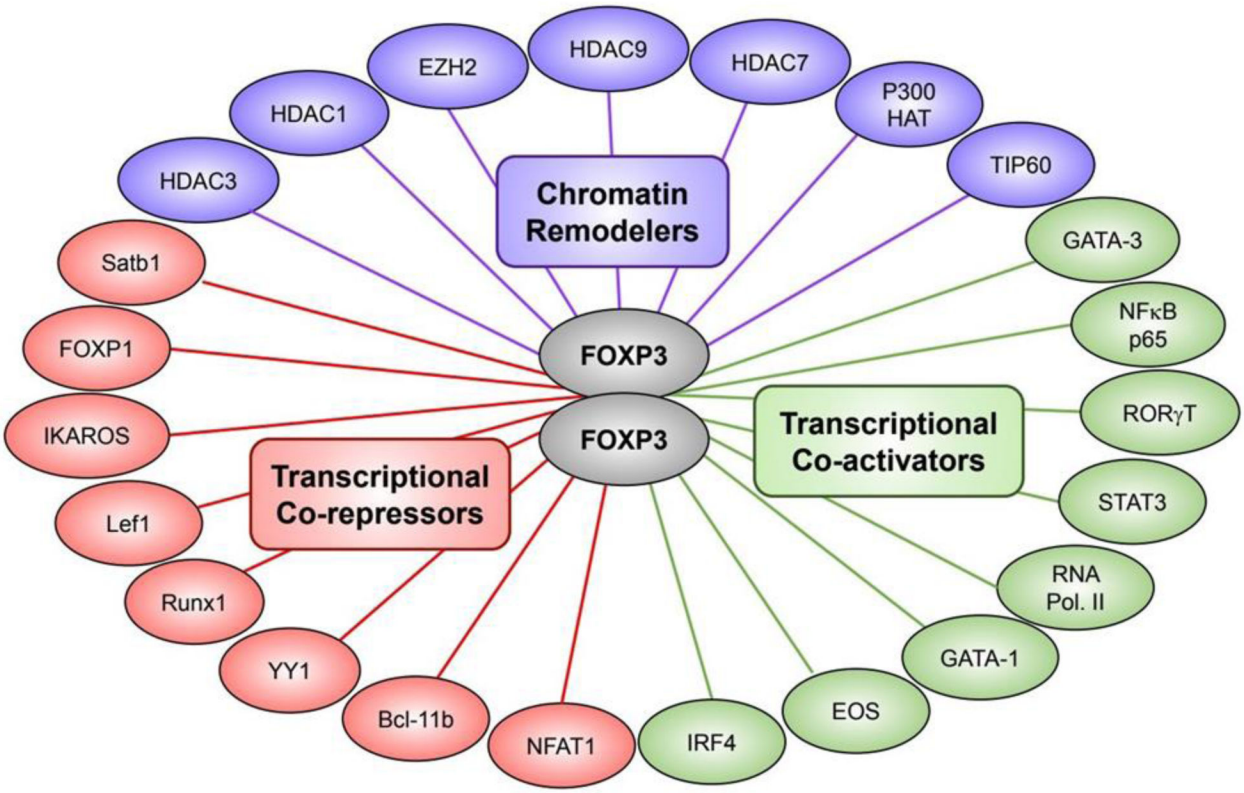

Figure 2. The Foxp3 interactome.

Foxp3 can modulate the transcriptome of Treg cells by distinct mechanisms depending on its interaction with diverse binding partners. Foxp3 can modulate the accessibility of genes to several transcription factors through its interaction with chromatin remodelers including the histone acetyltransferases P300 and TIP60. Additionally, Foxp3 may interact with various transcriptional co-activators or co-repressors, leading to the upregulation or downregulation of gene expression, respectively.

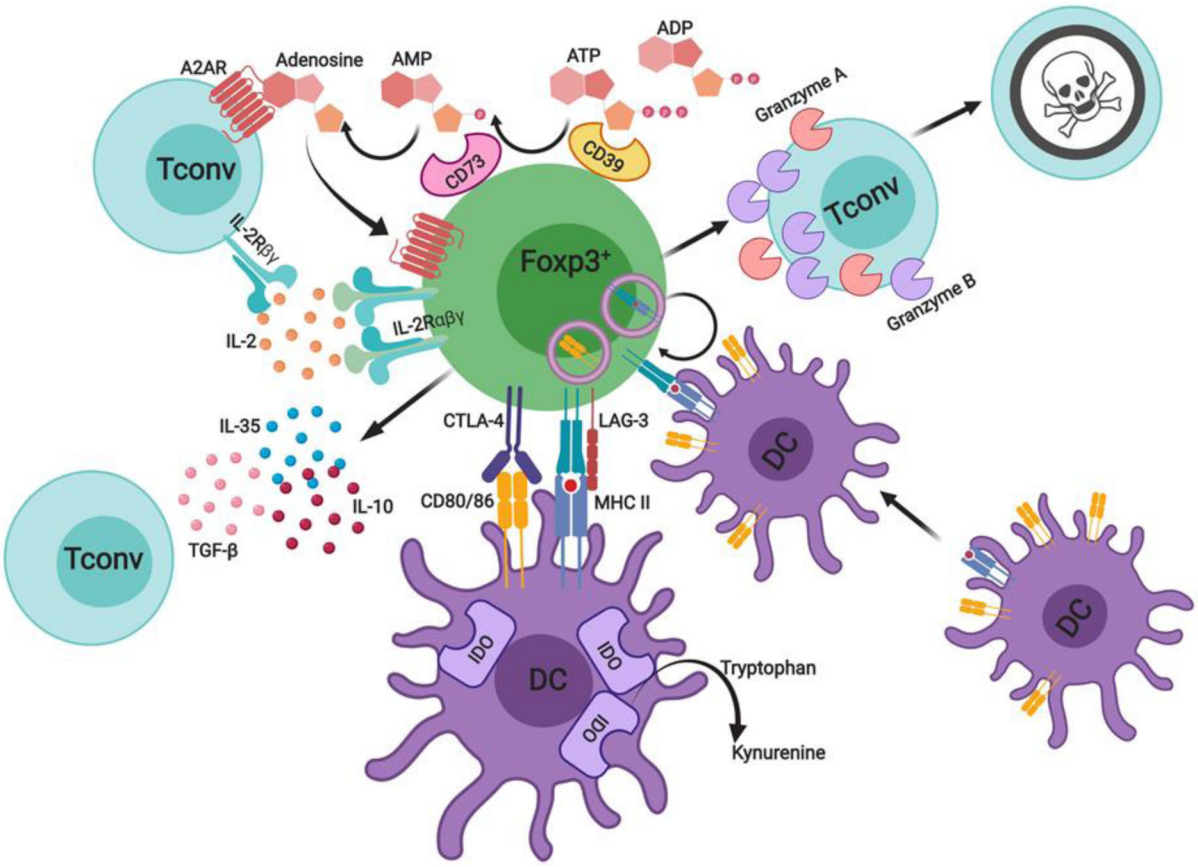

Figure 3. The modular nature of Treg cell suppression.

Treg cells suppress innate and adaptive immune responses through multiple mechanisms in order to enforce immunological tolerance. These include inhibitory cytokines such as IL-10, TGF-β1 and IL-35, suppression of antigen presentation by professional antigen presenting cells (CTLA-4, LAG-3), granzyme and perforin dependent target cell cytolysis. Additional mechanisms include the generation of immunosuppressive adenosine by Treg cell ectoenzymes CD39 and CD73, and competition for endogenous IL-2 through expression of the high affinity IL-2 receptor alpha chain (CD25). Importantly, individual modules (e.g IL-10, TGF-β1) operate in a non-redundant manner to prevent peripheral tolerance breakdown, while mutations affecting the respective modules (e.g. CTLA-4, IL-2Rα and β subunits, TGF-β1 etc), give rise to distinct immune dysregulatory human diseases.

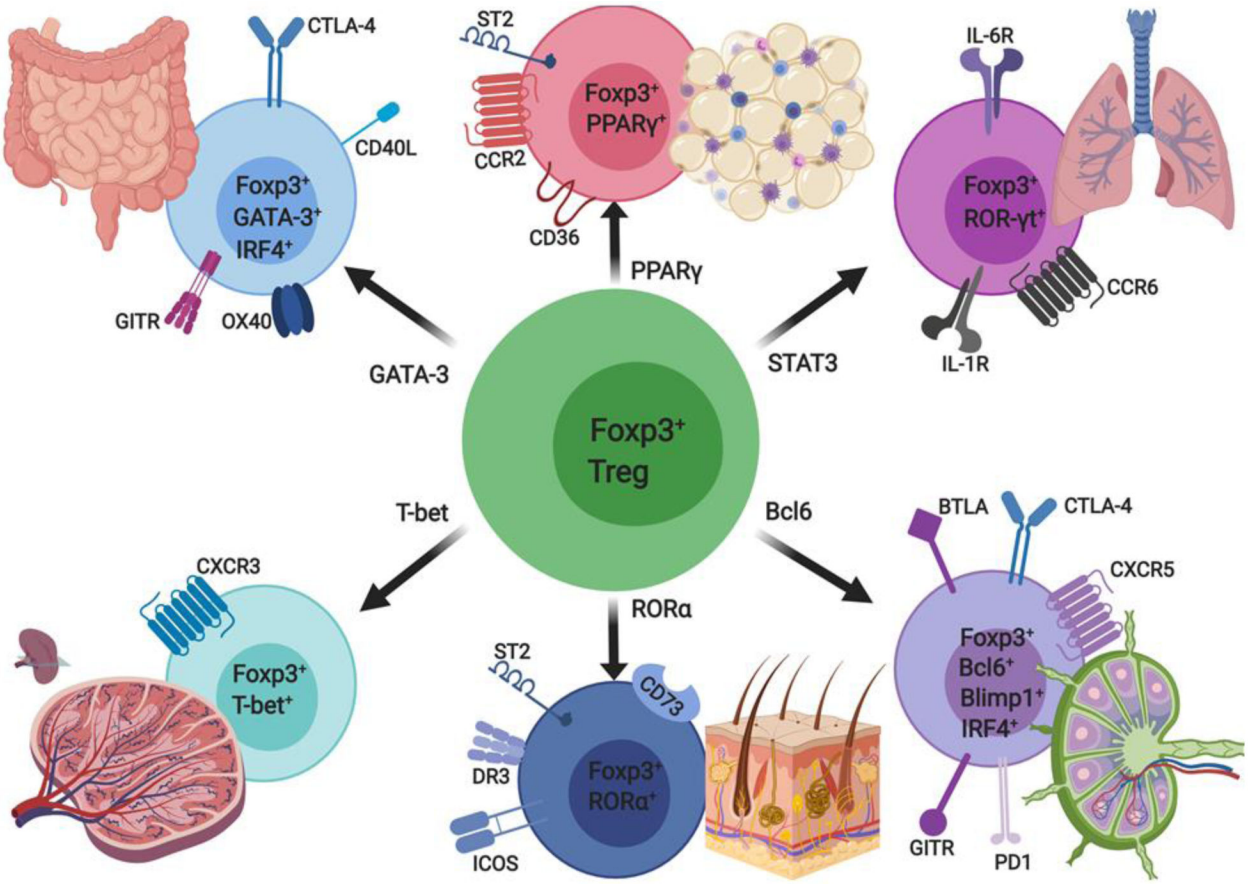

Figure 4. Functional adaptation of Treg cells.

Treg cells regulate T_{conv} cell responses in an individually T_H cell lineage dependent manner. To suppress T_H1 responses Treg cells express the T_H1 associated transcription factor T-bet and upregulate CXCR3 expression to transiently localize with expanded effector T_H1 cells. Similarly, Treg cells partially acquire GATA-3 and ROR-γt transcriptional programs to enforce control of T_H2 and T_H17 cell responses respectively. Expression of RORα in skin resident Treg cells is critical for expression of death receptor 3 (DR3) which in turn promotes ILC2 activation and control of allergic skin inflammation. $CXCR5^+$ follicular regulatory T (T_{FR}) cells expressing the transcription factor Bcl6 control the germinal center reaction. In visceral adipose tissue (VAT), Treg cells express the peroxisome proliferator-activated receptor (PPAR)-γ, which plays an important role in restoration of insulin sensitivity and maintenance of VAT Treg cell function and phenotype.

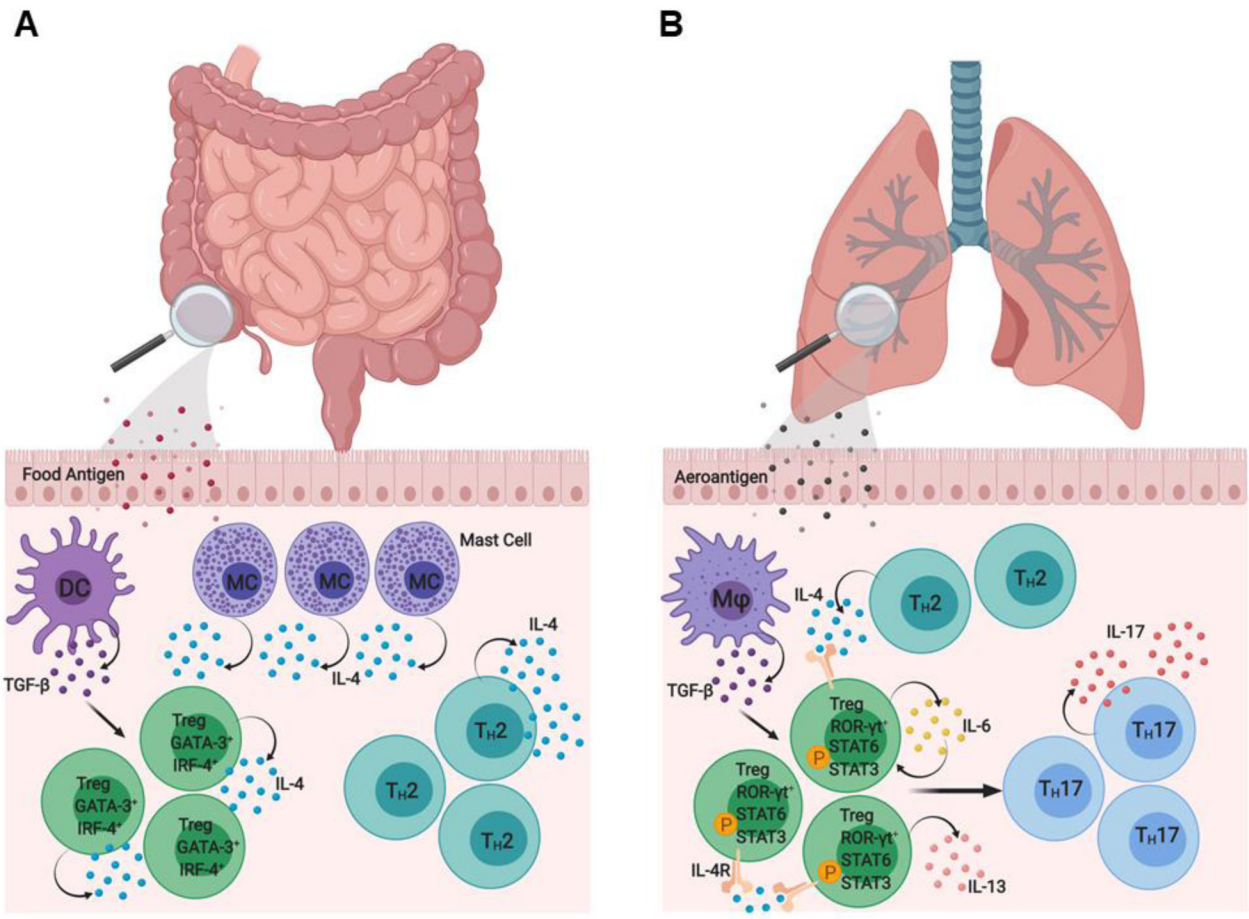

Figure 5. Pathogenic Treg cell reprogramming.

Two illustrative examples of pathogenic Treg cell reprogramming in human diseases. **(A)** In food allergy, allergen-specific Treg cells in the intestinal mucosa can acquire a pathogenic T_H2 cell-like phenotype characterized by increased GATA-3 expression and enhanced IL-4 production. This pathological T_H2 cell-like reprogramming results in the accumulation of dysfunctional antigen-specific Treg cells which fail to control effector T_H2 and mast cell responses to promote allergic disease. **(B)** In asthma, a human IL-4Rα allele bearing a glutamine to arginine substitution at position 576 (IL-4Rα-Q576R) promotes asthma severity by driving mixed T_H2-T_H17 inflammation. In addition to activating STAT6, IL-4/IL-13 signaling via IL-4/IL-4R-R576 variant activates downstream MAPKs, which in turn drive an autocrine IL-6/ STAT3 activation loop. This activation results in a pathological T_H17 cell-like reprogramming of nascent allergen-specific iTreg cells in the lung, characterized by increased ROR-γt expression and elevated IL-17 production.

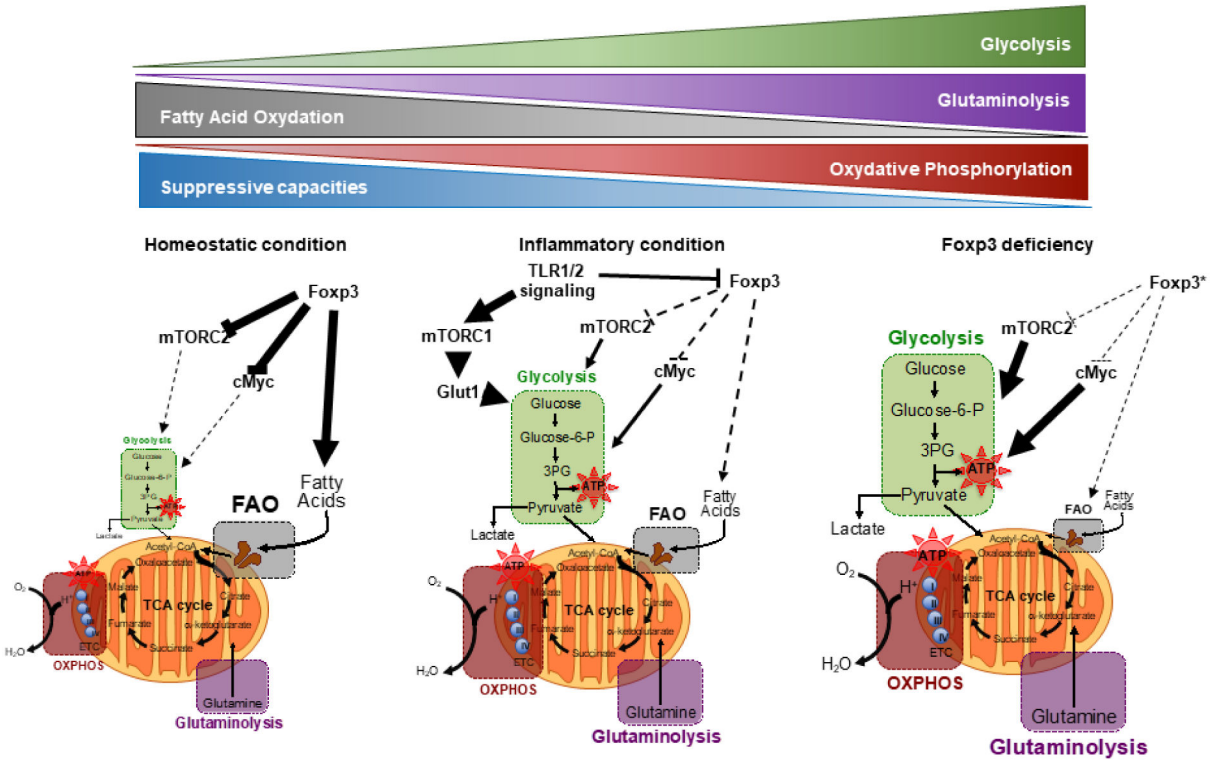

Figure 6. Metabolic states of Treg cells in health and disease.

Under homeostatic condition, Foxp3 controls Treg cell metabolism by promoting fatty acid oxidation (FAO) and by limiting glycolysis through the inhibition of c-Myc and mTORC2 pathways. Under inflammatory condition, signals such as those via TLR1/2 promote glycolysis by inducing Glut1 expression in an mTORC1-dependent manner and by modulating Foxp3 expression in Treg cells. In IPEX patients and Foxp3-deficient mice, the Foxp3-deficient Treg cells undergo metabolic reprogramming characteristic of an effector memory cell-like phenotype, involving heightened aerobic glycolysis, tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation (OXPHOS), driven to large extent by mTORC2 dysregulation. Other changes include loss of fatty acid oxidation and increased glutaminolysis.

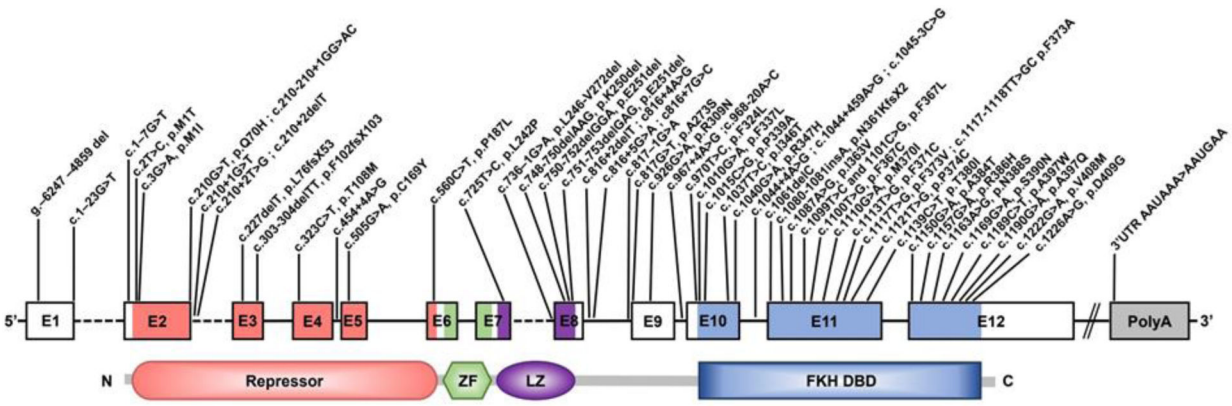

Figure 7. The spectrum of Human Foxp3 mutations.

Schematic representation of *FOXP3* illustrating the exons, the protein domains and mapped mutations of described IPEX patients. Amino acid changes are referred to by their single letter code. The N-terminal proline rich repressor domain (Repressor), zinc finger (ZF) motif, leucine zipper domain (LZ) and the forkhead DNA-binding domain (FKH DBD) are indicated.

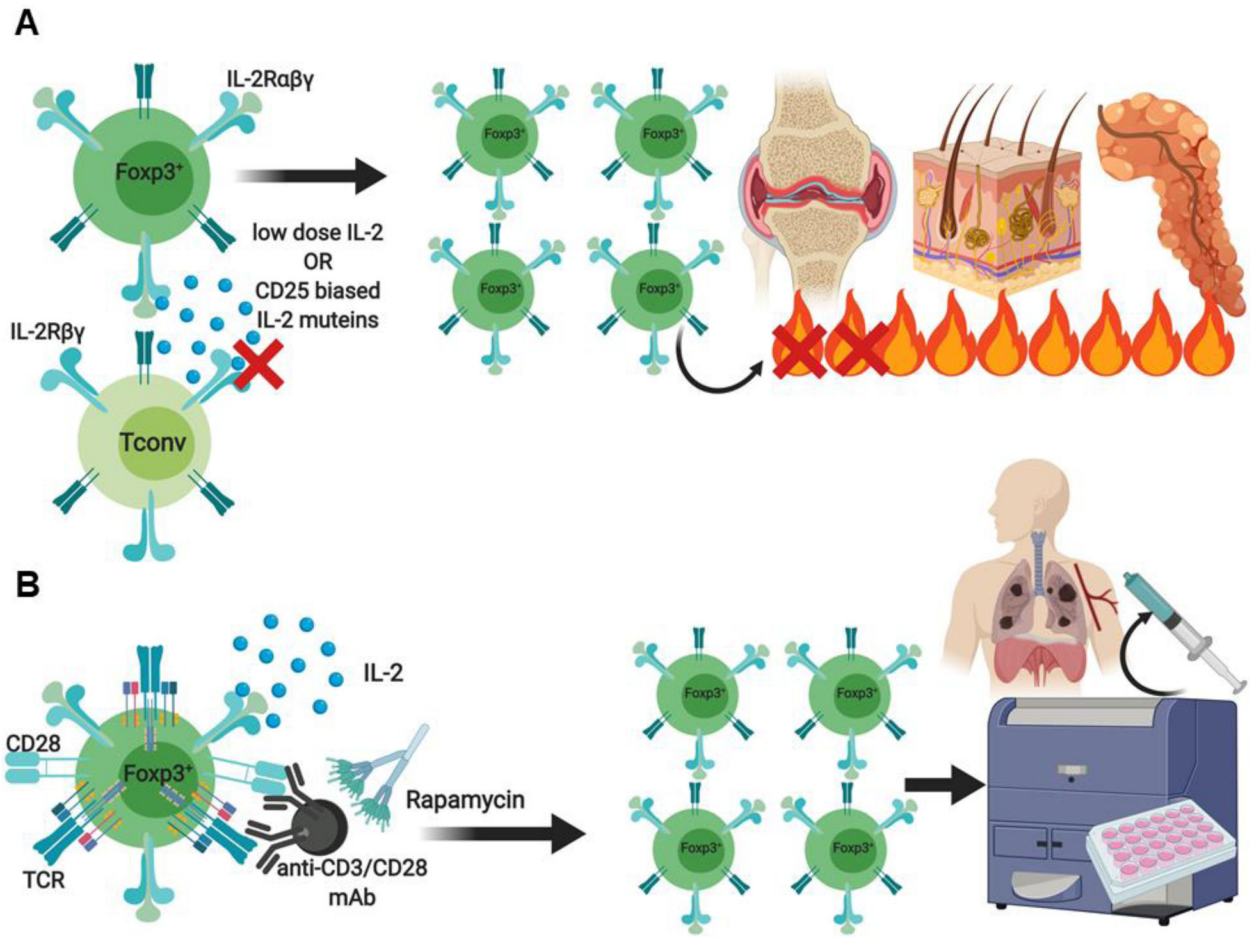

Figure 8. Therapeutic manipulation of Foxp3$^+$ Treg cells.

Examples of Treg cell-based therapy approaches. **(A)** Treg cells can be selectively expanded *in-vivo* with low dose IL-2 therapy or by IL-2 muteins engineered to preferentially interact with the high affinity IL-2 receptor alpha chain (CD25) expressed by Treg cells. **(B)** Alternatively, autologous $CD4^+CD25^+CD127^-$Treg cells can be massively expanded *ex vivo* following stimulation with anti-CD3/CD28 mAbs in the presence of IL-2, rapamycin, or Treg cell-biased IL-2 muteins to generate a clinical grade adoptive Treg cell transfer therapy product.