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***In vivo* Induction of Regulatory T Cells for Immune Tolerance in Hemophilia**

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Abstract

Current therapy for the X-linked coagulation disorder hemophilia is based on intravenous infusion of the specifically deficient coagulation factor. However, 20–30% of hemophilia A patients (factor VIII, FVIII, deficiency) generate inhibitory antibodies against FVIII. Whilst formation of inhibitors directed against factor IX, FIX, resulting from hemophilia B treatment is comparatively rare, a serious complication that is often associated with additional immunotoxicities, *e.g.* anaphylaxis, occurs. Current immune tolerance protocols to eradicate inhibitors are lengthy, expensive, not effective in all patients, and there are no prophylactic tolerance regimens to prevent inhibitor formation. The outcomes of recent experiments in animal models of hemophilia demonstrate that regulatory CD4⁺ T cells (Treg) are of paramount importance in controlling B cell responses to FVIII and FIX. This article reviews several novel strategies designed to *in vivo* induce coagulation factor-specific Treg cells and discusses the subsets of Treg that may promote immune tolerance in hemophilia. Among others, drug- and gene transfer-based protocols, lymphocyte transplant, and oral tolerance are reviewed.

Keywords

Hemophilia; Treg; FoxP3; IL-10; TGF- β ; Tr1; LAP

1. Introduction

Hemophilia A and B are the X-linked bleeding disorders caused by a deficiency of coagulation factor VIII or IX (FVIII or FIX), respectively. This disease occurs approximately 1 in 5,000 male births for hemophilia A, and 1 in 30,000 for hemophilia B, worldwide. Presently, treatment is based on periodical intravenous administration of the deficient coagulation factor. The currently most problematic complication is the development of neutralizing antibodies (inhibitors), which compromise therapy, may create immune toxicity, and increase the cost of treatment [1]. The incidence of inhibitor formation

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is about 20–30% in hemophilia A, and further elevated in African-American and Latino patients [1, 2]. Although inhibitor formation occurs only in approximately 1.5–3% of the hemophilia B cases, it is more prevalent in severe hemophilia B patients, often with additional consequences. It is estimated that 25–50% of the patients with inhibitors to FIX develop anaphylactic reactions, which further increase risks of morbidity and mortality [2]. There are no prophylactic protocols available for prevention of these pathogenic antibody responses in patients. Furthermore, agents that promote coagulation via alternative pathways, are often expensive, short acting, and have to be carefully dosed to avoid increased risks for thrombosis [3].

Clinical immune tolerance induction (ITI) protocols, which aim to reduce or eradicate inhibitors, are based on frequent high-dose factor administration for a long period of time (months to > 1 years), are expensive (often exceeding 1 million dollars). These approaches may have to be terminated in the case of FIX inhibitors, because they induce a nephrotic syndrome or anaphylactic reactions [1, 4]. Therefore, a widely accepted goal for hemophilia care is to develop effective alternative approaches to control inhibitors.

A novel approach employs the induction and/or recruitment of CD4⁺ regulatory T cells (Treg). Treg are critical in controlling responses to therapeutic proteins introduced via protein or gene replacement therapy for genetic diseases including hemophilia [5]. Antibody formation to therapeutic proteins is typically T helper cell-dependent, while a shift from activation of T help to induction of Treg may promote tolerance through active suppression. Treg-based therapies possess multiple advantages, such as limited global immune suppression, antigen-specificity, and potential for lasting tolerance [6–8]. During the past decade, many studies targeting Treg achieved immune tolerance in a variety of diseases models including autoimmune diseases, transplantation, autoinflammation, and other immune-related diseases [9].

Treg represent a diverse population of lymphocytes that regulate humoral and cell mediated responses by ways of removing autoreactive T cells, inducing tolerance, and dampening inflammation [8, 10]. Treg, which have an indispensable role in maintaining the homeostasis of the immune system, can be broadly divided into two main subsets: naturally occurring Treg (nTreg) and peripherally induced Treg (iTreg). CD4⁺CD25⁺Foxp3⁺ nTreg are also called thymus-derived Treg (tTreg), as they develop in the thymus of naïve animals [11]. By contrast, iTreg, which are also called pTreg, are induced in the periphery by stimulation of naïve T cells with antigen under specific tolerogenic conditions, involving repeated antigen presentation by immature or semi-mature dendritic cells, which express low levels or median levels of co-stimulatory molecules and MHC class II [12, 13]. Stimulation of the T cells in the presence of immunosuppressive cytokines, such as IL-10 and TGF- β , or immunosuppressive small molecules, such as retinoic acid or immunosuppressive neuropeptide, accelerates the induction of iTreg [14]. In addition to a large set of data in animal models, there is also a limited amount of data in humans with hemophilia that support control of inhibitor formation by FoxP3⁺ Treg (reviewed elsewhere) [15–17]. Depending on the local microenvironments, specificity to certain antigens and expression of different markers characteristic for Treg may emerge [14]. Other populations of iTreg,

which include Foxp3⁺ Treg, LAP⁺ Treg, Th3 cells, and CD49d⁺ LAG⁺ Tr1 cells, may also cooperate to generate immune tolerance.

2 Subsets of CD4⁺ regulatory T cells

In 1995, Sakaguchi *et al.* described a subset of suppressive CD4⁺ T cells expressing high levels of CD25 (IL-2 receptor α chain) and called these Treg [18]. Subsequently, the transcription factor forkhead box P3 (Foxp3) was identified as a key marker of and crucial for the development and function of Treg [19–21]. Since then, the field of Treg biology has evolved rapidly. Several phenotypically distinct Treg populations have been characterized, such as CD4⁺CD25⁺Foxp3⁺ Treg, Tr1 cells, LAP⁺ cells/Th3 cells (Fig. 1), regulatory subsets of CD8⁺ T cells, and CD3⁺CD4⁺CD8⁺ regulatory cells [22, 23]. In addition, certain B lymphocytes may also possess immunosuppressive function [24, 25]. The present review will focus on CD4⁺ regulatory T cells.

Currently, CD4⁺ T cells are commonly divided into several distinct lineages, conventional T helper (Th) cell subsets [Th1, Th2, Th17] and regulatory T cells [7]. Conventional Th cells shape the adaptive immunity by activating other effector cells such as CD8⁺ cytotoxic T lymphocytes (CTL) or B cells in an antigen-dependent manner. Treg are defined as T cells diminishing potentially harmful immune responses, including inhibition of T cell proliferation and blockade of inflammatory cytokines release [26]. Treg are functionally specialized to their local environment. They are able to sense cytokines, metabolites, or catabolites on the local milieu and consequently adjust the expression of genes, which is essential for their proper function [7]. Treg are pivotal in supporting central and peripheral tolerance. Their perturbation may lead to autoimmune diseases or other immunopathology.

2.1 CD4⁺CD25⁺Foxp3⁺ Treg

CD4⁺CD25⁺Foxp3⁺ Treg have been documented to have immunoregulatory activity in mice, rats, and humans by numerous studies [27, 28]. Normally, about 5 – 10% of peripheral CD4⁺ T cells in mice and humans are CD4⁺CD25⁺Foxp3⁺ Treg [6]. Besides CD25, other surface markers were also reported such as cytotoxic T lymphocyte antigen 4 (CTLA-4) [29], glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) [30], low levels of CD45RB [31], integrin α 4 β 7 (CD103) [32], and other adhesion molecules like CD11a, CD44, and CD54 [33]. However, those markers are also expressed in conventional T cells upon activation. A more specific and unambiguous marker to distinguish Tregs from other Th lineages is the transcription factor Foxp3 (Fig. 1A). Foxp3 is predominantly expressed in CD4⁺CD25⁺ Tregs, whereas not in murine effector T cells upon activation, and expressed only weakly in activated human effector T cells [34].

Intestinal Treg express T cell receptors that recognize antigens that are specific for the gut microbiota. However, not all intestinal CD4⁺ Treg express FoxP3, *e.g.* Treg cells that respond to the *Clostridium* species or to the polysaccharide of *Bacteroides fragilis* [35, 36]. The data indicate that in the mouse the intestinal microbial milieu facilitates Treg development by itself or in cooperation with the host tissue to promote systemic homeostasis. Which subsets of intestinal bacteria-induced Treg cells control intestinal inflammation in humans needs further investigations.

2.1.1 The Transcription factor Foxp3 is a master regulator for CD4⁺CD25⁺ Treg differentiation, maintenance, and function. Germline deletion of the Foxp3 gene in mice resulted in the absence of CD4⁺CD25⁺ Treg and the development of a lethal autoimmune syndrome, which can be rescued by transfer of Treg derived from wild-type (WT) mice [19, 21]. Mutation of Foxp3 gene in human causes severe autoimmune disease – IPEX syndrome [37]. Ablation of Foxp3 in differentiated mature Treg led to loss of its suppressive function and the up-regulation of effector cytokines characteristic of other CD4 helper lineages, including IL-4, IL-17, IFN- γ , TNF- α , and IL-2 [38]. Moreover, retroviral gene transfer of Foxp3 gene into naïve T cells of mice and humans converted those T cells towards a phenotype and function similar to those of naturally occurring Treg [20, 39].

However, Foxp3 is not the sole gene to establish the full CD4⁺CD25⁺ Tregs transcriptional program, as revealed by the comparison studies of the transcriptional profiles between Treg and conventional CD4⁺ T cells with retroviral transduction of Foxp3. To establish a more complete signature genes expression profile of Treg, additional genes are required, including but not limited to IKAROS family zinc finger 4 (IKZF4), interferon regulatory factor 4 (IRF4), SATB homeobox 1 (SATB1), lymphoid enhancer-binding factor 1 (LEF1), and GATA binding protein 3 (GATA3) [40]. Interestingly, Treg development involves a specific CpG hypomethylation pattern in Treg-expressed genes, which is established independently of FoxP3 expression but equally required for Treg function [41]. It is therefore possible that Foxp3 activity requires a Treg-specific epigenetic landscape, and cooperates with other genes to establish full Treg identity.

The Foxp3 promoter is highly conserved. The human Foxp3 promoter contains 6 NFAT and AP-1 binding sites. Additionally, the Foxp3 gene holds 3 proximal intronic conserved non-coding DNA sequence (CNS). CNS1 includes a TGF- β responsive element, and NFAT and Smad binding sites [42]. It is involved in TGF- β induced Foxp3 expression of pTregs in gut-associated lymphoid tissues [43]. CNS2, corresponding to the TCR-responsive enhancer, contains a CpG island, and binding sites of CREB and STAT5 [44]. It is required for Foxp3 expression in mature tTregs [43]. CNS3, contains binding sites for c-Rel and has a prominent role in Treg generation in both thymus and periphery [43].

2.1.2 Mechanisms of Treg-mediated suppression appear remarkably diverse and are therefore not always clear. Depending on the localization and the developmental stage of Treg, as well as the strength or stage of the immune reactions, suppressive mechanisms might be different, involving a dynamic interplay between T cells and antigen presenting cells (APCs) rather than a simple on / off suppressor function [9]. Thus, the studies of Treg suppression sometimes yielded controversial results in different experimental models. Some of the proposed mechanisms are summarized in Fig.1A.

In vitro studies have accumulated several clues for Treg function. When co-cultured, CD4⁺CD25⁺ Treg are able to suppress the proliferation of responder CD4⁺CD25⁻ T cells. The suppression is abrogated by a membrane that physically separates Tregs from the responders [45], suggesting a cell contact dependent mechanism. Another possibility is that some cytokines secreted by Tregs function in a gradient fashion and require proximity between suppressor and responder. For example, IL-35 was shown to be an inhibitory

cytokine contributing substantially to the function of Treg [46]. Another secreted molecule potentially critical is galectin-1, which preferentially expressed in Treg and upregulated upon T cell receptor (TCR) activation. Galectin-1 deficient mice have reduced Treg activity. Blocking galectin-1 markedly decreases the inhibitory effects of Treg [47]. The role of IL-2 had been under dispute. Many studies showed that Treg inhibits the expression of IL-2 in responder T cells. However, blocking the response to IL-2 with anti-CD25 had no effect on the function of human Treg [48]. Certainly, IL-2 is an important growth factor for Treg. One cell-contact mechanism for Treg-mediated suppression is cytotoxicity of target cells. Activation of mouse or human Treg can upregulate expression of granzyme B [49, 50]. Granzyme B-deficient Treg have decreased suppressive activity [49]. Human Treg can kill CD8⁺ T cells and other cell types in a perforin-dependent, Fas-FasL independent manner [50].

In vivo experiments revealed critical roles of suppressive cytokines IL-10 and TGF- β . IL-10 deficient Tregs cannot suppress inflammatory colitis induced by transfer of naïve CD45RB^{high} T cells into SCID mice [51]. In consistent, blocking IL-10 or TGF- β signaling with antibodies abolished the suppressive effects of Treg on colitis [52]. Impaired TGF- β signaling in the CD8 effectors with a dominant-negative TGF- β receptor conferred resistance to Treg-mediated suppression [53]. It is also possible that Treg physically interact directly with effector T cells or indirectly via the APCs and home to different parts of the body. One proposed mechanism is that Tregs might trap effector cells at the site of immunization and prevents their egress to the target organ [45]. Another potential mechanism is that Treg exert their effects by reducing the stimulatory capacity of DCs, or by interacting with effector cells to prevent their interaction with DCs [45].

2.1.3 Development/induction and maintenance of CD4⁺CD25⁺Foxp3⁺ Treg occurs either in the thymus (tTreg) or in the periphery (pTreg). For tTreg development, TCR signaling, IL-2, and TGF- β are essential. tTreg emerge in the thymus generally at the CD4 single positive stage [54, 55]. TCRs of Treg, which mostly are non-overlapping with TCR repertoires of naïve T cells, have intermediate affinity to self-antigens [56, 57]. Foxp3⁺ Treg were reported to express higher levels of TCR signal strength compared with non-Treg CD4⁺ T cells [58]. In addition to TCR stimulation, IL-2 signals are critical for tTreg differentiation. IL-2R α deficient mice had approximately half of Foxp3⁺ Treg compared with WT mice [59]. IL-2R γ deficiency completely blocked Treg development [59]. Overexpression of STAT-5, the downstream transcriptional factor of IL-2R, dramatically increased Treg numbers compared with WT mice [60]. TGF- β is another important cytokine for Treg development. Ablation of TGF- β RI resulted in defective tTreg production in neonatal mice. TGF- β receptor deficient tTreg had increased apoptosis due to down-regulated anti-apoptotic gene Bcl-2 and up-regulated pro-apoptotic genes Bim, Bax, and Bak [61]. Moreover, mice deficient in both TGF- β RI and IL-2R α showed a complete absence of tTreg [62].

Naïve CD4⁺ T cells can convert into pTreg in the periphery under certain circumstance. TGF- β and TCR stimulation are required here as well. One of most extensive studied iTreg development model is gut iTreg induced by microbiota. Colonization of germ-free mice with *Clostridium* species increased Treg cell numbers in colon, dependent on TGF- β , as blocking TGF- β with neutralizing antibody abolished Treg induction [63]. Specific TCRs were

required for pTreg generation. Individual TCRs reactive with several colonic bacterial isolates were identified using a GFP-NFAT reporter cell line. Expressing those TCRs could convert naive T cells into Foxp3⁺Treg in the colon lamina propria [64]. Several metabolites in the gut microenvironment also contribute, including vitamin A and its derivative retinoid acid, and short-chain fatty acids such as propionate and butyrate [65–67].

2.2 Treg with TGF- β dependent suppression (Th3 cells and LAP⁺ Treg)

There are also induced Treg that are distinct from the CD4⁺CD25⁺FoxP3⁺ cells. One example is the T helper cells 3 (Th3 cells). They were distinguished from Th1/Th2 cells by the secretion of TGF- β . These cells were class II restricted T cells with identical $\alpha\beta$ TCR as Th1/Th2 cells. They express high levels of TGF- β , low amounts of IL-4 and IL10, but no IL-2 or IFN- γ . They appear to be dependent on IL-4 rather than IL-2 for growth [68]. Secretion of TGF- β is pivotal for their function. Th3 cells are important in oral tolerance and have a reciprocal relationship with Th17 cells, which are highly proinflammatory [68]. A problem with identifying Th3 cells has been the lack of a specific surface marker [69]. In that sense, Treg overexpressing TGF- β and suppressing in a TGF- β dependent fashion are better defined as LAP⁺ Treg, which are discussed below (Fig. 1C).

2.3 Treg with with IL-10 dependent suppression (LAG-3⁺ CD49b⁺ Tr1 cells)

T regulatory cells type 1 (Tr1) were characterized by the production of IL-10 and rely on IL-10 for their function and differentiation [70]. Upon activation, Tr1 cells produce a unique cytokine profile: high levels of IL-10, TGF- β , variable amounts of IL-5 GM-CSF and IFN- γ , low levels of IL-2, and minimal amounts of IL-4 and IL-17 [22, 71]. CD49b and LAG-3 have now been identified as a Tr1 cell-specific combination of markers [72]. Tr1 cells also express CTLA-4, programmed cell death protein 1 (PD-1), inducible costimulatory molecule (ICOS), early response gene 2 (Erg-2), and repressor of GATA-3 (ROG) [70] (Fig.1B). Although transiently up-regulated Foxp3 expression was detected [73], Foxp3 is not constitutively expressed in Tr1 [74]. Tr1 differentiation is independent of Foxp3 [75].

Tr1 cells mainly control immune responses through suppressive cytokines. After activation by their TCR, Tr1 cells secrete high amount of IL-10 and TGF- β , which lead to bystander tolerogenic effects [76]. IL-10 and TGF- β are capable of suppressing T cell responses by decreasing IL-2, IFN- γ , GM-CSF, and T cell proliferation [77, 78]. IL-10 affects APC, by down-regulating MHC class II, co-stimulatory molecules as well as pro-inflammatory cytokines productions, and by up-regulating tolerogenic molecules like immunoglobulin-like transcript (ILT)-3, 4, and the non-classical HLA-G [79–81]. It educates DCs to induce Tregs [82]. IL-10 promotes B cells isotype switching - promotes IgG4 production and suppresses IgE production [83, 84]. Moreover, Tr1 cells inhibit T cell responses by cell-contact dependent mechanisms, metabolic disruption, and directly cytotoxicity. Tr1 cells express inhibitory receptors such as CTLA-4, PD-1, and ICOS, which modulate T cell functions negatively [85, 86]. Tr1 cells express ectoenzymes CD39 and CD73, which were shown to associate with hydrolysis of extracellular ATP and disrupting the metabolic state of effector T cells [87]. Human Tr1 cells can express granzyme B and selectively kill target cells [88, 89] (Fig.1B).

Tr1 cells have poor capacity to proliferate in vitro upon polyclonal TCR or antigen specific activation [86]. IL-10, IL-15 and IL-27 were shown to support Tr1 cell proliferation, expansion, and maintenance [90]. IL-10 drove differentiation of human Tr1 cells dependent on HLA-G and ILT4 molecules [91]. IL-27 decreases DC production of Th17 polarizing cytokines IL-1 β , IL-6 and IL-23, and acts on naïve T cells to drive expression of c-maf, IL-21 and ICOS [90, 92]. IL-27 also increases IL-10 transcription in T cells by activation of STAT1 and 3 [93]. TGF- β may also have a role in priming Tr1 differentiation [94].

3 *In vivo* induction and expansion of Treg using small molecules

One potential approach to control inhibitor formation is to direct the immune response to the coagulation factor antigen toward a Treg-dominated response, resulting in antigen-specific suppression of T and B cells. Recruiting nTreg for this purpose encounters several limitations, as these have poor proliferative capacity, are mostly polyclonal/antigen-nonspecific (less than 0.1% of CD4⁺ T cells are antigen-specific), are available only in low cell numbers (about 5–10% of peripheral CD4⁺ T cells are CD4⁺CD25⁺Foxp3⁺ Treg), and only a minority of CD4⁺CD25⁺ T cells have strong suppressive activity. Thus, iTreg have been a more realistic target [9]. Recent progress in understanding Treg biology facilitates these efforts. Treg develop, differentiate, and function in their specialized local microenvironment, requiring cytokines, metabolites and crosstalk with DCs or other cell types [9, 95]. Interestingly, there are approaches that promote *in vivo* induction and expansion of iTreg by antigen administration combined with drugs that favor Treg over Teff activation.

3.1 Inhibition of mTOR

Mammalian target of rapamycin (mTOR) is an essential serine/threonine protein kinase, which promotes protein synthesis and cell cycle progression upon activation. The mTOR inhibitor rapamycin (sirolimus) is a macrolide antibiotic produced by *Streptomyces hygroscopicus*. It inhibits mTOR via binding to the immunophilin FK506 binding protein-12 (FKBP-12). As a result, it decreases cell cycle progression, suppresses T cell proliferation, and is therefore used as an immunosuppressive drug in allograft transplantation [96]. Interestingly, rapamycin selectively induces and expands Treg cells with time-dependent and dose-dependent manners [96–99] (Fig.2A). Some data suggest that alternate day rapamycin treatment or withdrawal was shown to better promote Treg proliferation compared with continuous administration. Co-administration with other drugs such as tripterygium wilfordii polycoride tablet (TPT), TGF- β , or IL-10 may further increase Treg induction [100–102]. The mechanism of Treg induction by antigen presentation in the presence of rapamycin is not completely clear. However, mTOR is known to interfere with Smad3 signaling, which is a downstream transcriptional factor of TGF- β . Rapamycin enhances TGF- β signaling through mTOR inhibition, thereby facilitating Treg induction [103].

Several studies successfully induced tolerance to clotting factors in hemophilic mice using rapamycin. Combination of oral administration of rapamycin with intravenous FVIII delivery for 1 month effectively prevented inhibitors formation against FVIII upon subsequent intravenous treatment weekly for 3.5 months in hemophilia A mice. Tolerized

mice had increased frequencies of CD4⁺CD25⁺Foxp3⁺ Treg, which suppressed antibody formation upon adoptive transfer [104]. In another study, rapamycin plus IL-10 combined with a FIX-specific peptide was used in treatment in hemophilia B mice 3 times per week for 4 weeks, which significantly suppressed the inhibitors formation against FIX in muscle-directed gene therapy. Gene therapy treated control mice formed inhibitors of 4–19 BU (Bethesda Units), while tolerized mice had <2 BU after gene transfer. Additional studies showed that antigen/rapamycin/IL-10 induced CD4⁺CD25⁺Foxp3⁺ Treg suppressed effector T cells (Teff) in vitro and showed a lower rate of apoptosis in vivo compared to antigen-specific Teff [105]. Combined deletion of Teff and induction of Treg had therefore shifted to a regulated response. Daily oral administration of rapamycin for 1 month combined with frequent low-dose intravenous injection of FIX was effective in not only preventing but also reversing inhibitor formation in this model [106].

The route and dosing schedule of drug administration can affect tolerance and Treg induction. In one study, oral delivery was significantly less effective compared with intravenous, intraperitoneal, or subcutaneous delivery in induction of antigen-specific CD4⁺CD25⁺Foxp3⁺ Treg, so that frequency of oral delivery had to be increased to achieve similar efficacy [106]. In another study, intraperitoneal injection of rapamycin at 2 mg/kg/d for 14 days following by hydrodynamic delivery of naked plasmid DNA expressing FVIII only slightly delayed inhibitor formation in hemophilia A mice, with inhibitor appearing at 3 to 4 weeks post plasmid delivery [107]. Therefore, in some settings such a regimen would have to be further optimized. A recent publication showed biodegradable nanoparticles carrying antigens and rapamycin, but not a free form of rapamycin, substantially inhibited CD4⁺ and CD8⁺ T cell activation, increased Treg numbers, and reduced antibody responses against FVIII in hemophilia A mice, even in previously sensitized animals [108]. These results illustrated the potential for optimization of the formulation of rapamycin for tolerance induction.

3.2 Expansion of dendritic cell (DC) subsets

Treg homeostasis is dependent on DCs. DCs are professional APCs for the capture, processing, and presentation of antigens to T cells. Depending on the type of DC and its activation state, naive T cells may differentiate into Treg or Teff cells [95]. The cytokine FMS-like receptor tyrosine kinase 3 ligand (Flt3L) is critical in the development of DC. Flt3L^{-/-} or Flt3^{-/-} mice have up to 10-fold reduction in different subtypes of DCs in spleen, lymph nodes and thymus [109–111]. STAT3, a downstream transcriptional factor of Flt3L, inhibits the NF-κB pathway, which leads to reduction in the expression of MHC class II and co-stimulatory molecules, and increased transcription of indoleamine-2,3-dioxygenase (IDO) [112–114]. Administration of Flt3L expands DCs and indirectly leads to expansion of Treg in humans and mice [115, 116]. In the NOD (non-obese diabetic) mouse model, Flt3L treatment significantly augmented Foxp3⁺Tregs in the pancreatic lymph node and dramatically suppressed insulinitis [116]. Flt3L facilitates expansion of DC subsets with regulatory properties. In ovalbumin-sensitized mice, Flt3L was able to increase regulatory DCs in the lung and enhance their regulatory capacity [117]. Additionally, Flt3L administration can alter the balance of conventional to plasmacytoid dendritic cells (cDCs to pDCs) - favoring the accumulation of pDCs [118]. In the gut, Flt3L injection increased the

number of CD103⁺DCs, a critical subset of tolerogenic DC, and boosted Foxp3⁺ Treg numbers [119]. Consistent with these observations, Flt3 and Ldlr double knockout mice had reduced Treg and more pro-inflammatory cytokines in the aorta [120].

In order to prevent inhibitor formation in FVIII replacement therapy, one study injected hemophilia A mice (with targeted F8, exon 16 gene deletion) intravenously with a cocktail of Flt3L, rapamycin, and a low dose FVIII (0.3 IU) 3 times per week for 4 weeks, followed by 4 weeks of FVIII protein therapy (1 IU weekly). Mice receiving the cocktail developed substantially lower inhibitor titers compared with mice receiving rapamycin/FVIII, Flt3L/FVIII, or FVIII protein therapy only. Flt3L combined with rapamycin was discovered to synergistically induce Treg *in vivo*. CD4⁺Foxp3⁺ cells induction was much reduced upon antibody-mediated depletion of pDCs. Experiments in BDCA-2-DTR mice (where pDCs are effectively depleted by injection of diphtheria toxin) further supported that Treg induction was dependent on pDCs. Flt3L promotes DC proliferation via signaling through the PI3K-mTOR pathway. However, rapamycin is a strong mTOR inhibitor. Further experiments revealed that pDCs were more resistant to rapamycin and selectively expanded by Flt3L; whereas in cDCs, which have reduced mTOR activity compared to pDCs, rapamycin effectively blocked mTOR1 signaling and its expansion [121] (Fig. 2B).

3.3 Employing antibodies and fusion proteins

Another promising strategy in facilitating immune tolerance induction is targeting costimulatory pathways with antibodies or fusion proteins. Compared with traditional immunosuppressive drugs, antibodies and fusion proteins are more selective, generally well tolerated by recipients with less toxicity, and could induce specific tolerance in the presence of the antigen [5]. Costimulatory pathways such as ICOS and ICOS-L, CD28 and B7 (CD80 and CD86), CD40 and CD40-L, are critical in T cell activation to mount robust immune responses against foreign antigens. Several agents interrupting costimulatory pathways have been reported to induce Treg and promote tolerance in hemophilia. Short-term therapy with an anti-ICOS monoclonal antibody (mAb) transiently blocked the ICOS/ICOS-L pathway in hemophilia A mice receiving FVIII plasmid treatment, resulted in sustained tolerance to FVIII and effective prevention of inhibitors formation. CD4⁺CD25⁺FoxP3⁺ cells were upregulated in peripheral blood, spleen, and lymph nodes in terms of percentages as well as activity. Effector T cells were eliminated in the short term, and T memory cells were suppressed in the long term. CD4⁺ T cells of tolerized mice produced high levels of IL-10 and TGF- β in response to FVIII. Moreover, anti-ICOS treated mice and tolerized to FVIII maintained immune competency since they still generate antibody responses to other antigens [122]. However, blocking ICOS-L with mAb was reported to be ineffective in preventing restimulation of FVIII specific memory B cells, which may be less dependent on this pathway [123].

CTLA4-Ig is a soluble fusion protein containing the extracellular domain of CTLA4 and immunoglobulin heavy-chain. CTLA4-Ig blocks the interaction of B7 and CD28, which resulted in reduced antibody titers to FVIII in hemophilia A mice [124]. Another study using CTLA4-Fc, a fusion protein of murine CTLA4 with Fc part of human IgG1, suppressed the restimulation and differentiation of FVIII specific memory B cells in the presence of FVIII

[123]. Additionally, blocking CD40L with mAb had been reported to temporarily block the inhibitor formation in hemophilia A mice [123, 125, 126]. The combination of CTLA4-Ig and anti-CD40L proved most effective in preventing inhibitor formation against FVIII [107].

Researchers also tried to directly expand Treg using antibodies or fusion proteins. IL-2 mixed with IL-2 mAb (JES6-1) and low dose of FVIII led to a up to 7-fold increase of CD4⁺CD25⁺FoxP3⁺ cells in blood, spleen and lymph nodes for about 4 weeks, which dropped to normal levels within the next 7–14 days [127, 128]. Other lymphocyte populations were not changed. Subsequently, long-term tolerance to FVIII was achieved in hemophilia A mice, which mounted normal immune responses against neoantigens, indicating immune system was not disturbed for long by IL-2/IL-2 mAb treatment [128]. The co-stimulatory molecule GITR is constitutively expressed at high levels in FoxP3⁺ Treg. Injection of Fc-GITR-L into hemophilia B prone mice undergoing liver targeted FIX gene therapy increased the expression of FIX and decreased the immune response to FIX. Additional experiments showed that Fc-GITR-L preferentially drives proliferation of Treg over Teff [129].

Reduced of TCR signaling at the time of initial exposure to antigen may also reduce effector T cells and induce tolerance. A short course of low-dose, non-depleting anti-CD3 significantly increased the proportion of CD4⁺CD25⁺ Treg in the spleen and prevented the inhibitory and non-neutralizing antibodies formation against FVIII in both BALB/c and C57BL/6 hemophilia A mice. Phenotypic characterization of Treg showed a consistently higher number of CD4⁺GITR⁺ and CD4⁺FoxP3⁺ cells in both strains of mice. In addition, *in vitro* cytokine profiling demonstrated that splenocytes derived from tolerized mice were polarized toward a Th1 immune response [130]. In another study, 5 consecutive anti-CD3 treatments concomitant with FVIII plasmid injection successfully prevented inhibitors formation in hemophilia A mice. Here, CD4⁺ and CD8⁺ cells were depleted initially, whereas TGF- β in plasma and the frequency and cell count of CD4⁺CD25⁺FoxP3⁺ Treg were increased [131]. Anti-CD3 treatment transiently depleted other T cells, but selectively expanded FoxP3⁺ Treg. While the underlying mechanism was not fully elucidated, one explanation is heterogeneity in CD3 expression, which is higher in FoxP3⁺ T cells than FoxP3⁺ T cells [132].

4. Adoptive transfer of *ex vivo* induced Treg or genetically modified B cells

Here we discuss the use of cell transfer of *ex vivo* manipulated Treg or B lymphocytes in the *in vivo* induction of antigen-specific Treg.

4.1 Genetically modified B cells induce Treg

Besides their role of producers of antibodies, B cells also have immune regulatory properties. Thus, tolerogenic B cells can be generated from primary B cells (Fig. 3A). Stable retroviral gene transfer of primary B cells of a cDNA encoding the A2 or C2 domains of FVIII fused to the heavy chain of murine IgG led to induction of antigen specific Treg to FVIII and tolerance to the full-length FVIII protein. Tolerogenic B cell adoptive transfer successfully prevented antibody production in naïve hemophilia A mice and also reduced

inhibitor titers in hemophilia A mice with pre-existing inhibitors [133]. This approach also succeeded in a hemophilia B mouse model. Retrovirally transduced primary B cells expressing FIX antigen fused with IgG heavy chain prevented antibody formation to FIX and was also highly effective in desensitizing already primed animals. In either setting, FoxP3⁺ Treg were induced that suppressed anti-FIX formation. These studies were performed in C3H/HeJ mice with targeted F9 gene deletion. This strain develops IgE and fatal anaphylactic after repeated intravenous FIX injections, mimicking the problem of anaphylaxis that is often encountered in humans with FIX inhibitors. Importantly, tolerized mice prevented and reversed anaphylaxis, presumably via Treg induction (transplanted gene modified B cells persist only at very low numbers, with long-term suppression being performed by iTreg). Interestingly, transplant of B cells that received the identical expression cassette via nucleofection of plasmid vector substantially heightened antibody responses that could be blocked by TLR9 inhibition [134]. MHC II presentation of fusion gene product, B7 expression on B cells and FoxP3⁺ Treg are required in the B cell protocol. Tolerogenic antigen presentation by the transduced B cells is dependent on transfer to the endosomes and processing in an IFN- γ -inducible lysosomal thiol reductase-dependent fashion [135]. It is noteworthy that certain epitope domains of IgG heavy chain contain epitopes regulatory properties ("Tregitopes") that recruit and increase nTreg. These may further aid in the antigen-specific tolerance induction through a bystander suppression mechanism [136]. Upregulated expression of IL-10 was detected in gene modified B cells dependent on activation of TLR2 NF- κ B pathways [137]. However, others claimed host but not donor B cells derived IL-10 was necessary for tolerance, as gene modified B cells derived from IL-10 deficient mice had the similar effects as WT B cells in tolerance induction in an experimental autoimmune encephalomyelitis model; while the tolerogenic effects could be abrogated by anti-IL-10 receptor antibody treatment [138].

4.2 Transferring *in vitro* expanded autologous Treg

Autologous Treg can be isolated from peripheral blood, enriched or selected, *ex vivo* expanded, and re-infused back into recipient. Present-day advanced cell isolation and culture systems support efficient safe and reproducible cell processing on a large scale [139]. In fact, a number of clinical studies using adoptive Treg therapy are underway in the transplantation field and already show success in prevention of graft-vs-host disease in bone marrow transplant [140]. Although antigen-specific Treg posse more potent immunosuppressive capacity compared with polyclonal Treg, their isolation is more challenging as they are present at very low frequencies [141]. Interestingly, transplanted *ex vivo* expanded polyclonal Treg can establish a tolerogenic environment that increases induction of endogenous antigen-specific Treg (Fig. 3B). This notion is supported by the fact that adoptive transfer of GFP-marked murine Treg expanded *ex vivo* using anti-CD3/-CD28 beads markedly suppressed inhibitory antibody formation against FVIII and IX in protein and gene therapies in hemophilia A and B mice, even in mice with pre-existing antibodies. The suppression persisted for at least 2 months despite that transplanted Treg were detectable for <1 month 2 weeks [142]. Up regulation of CTLA-4 with subsequent reduction of CD80/86 on DCs might be the main responsible potential molecular mechanism for generating the tolerogenic environment after transplant [142]. Another study using Treg derived from FVIII exposed hemophilia A FoxP3 transgenic mice, produced significantly

reduced antibody titers in Treg recipient hemophilia A mice after initial and second 16 week challenge with FVIII plasmid, although engrafted Treg were undetectable within 8–12 weeks [143].

Other methods not discussed in detail here, such as hematopoietic stem cell gene transfer, and thymic gene transfer or thymic protein treatment, were shown to induce antigen-specific Treg *in vivo* [144–146]. Alternatively, antigen-specific Treg can be generated *in vitro* using methods such as T cell gene modifications with antigen-specific TCR or chimeric antigen receptors, as initially investigated in models of autoimmune disease and now also shown to be a promising strategy to suppress inhibitor formation in hemophilia [15, 147, 148].

5 Treg induction in the liver and the intestine

5.1 Liver-directed gene therapy induces Treg

Treg have been efficiently induced following antigen expression by gene transfer in the liver, which is exposed to a vast number of antigens, which include various foreign antigens as it receives the blood flow from the gut. It has therefore been hypothesized that the liver evolved a mechanism of dampening immune responses, as also evidenced by the fact that liver transplants are well tolerated compared with other organ transplants [149]. Many protocols have now succeeded in Treg and tolerance induction by *in vivo* gene transfer mediated through a viral vector with transgene expression restricted to hepatocytes (Fig. 3C). Among them adeno-associated viral (AAV) vectors have the advantages of strong liver tropism and limited innate immunogenicity [150]. Hepatic AAV gene transfer had been shown to robustly induce transgene specific CD4⁺CD25⁺FoxP3⁺ Treg, which actively suppressed antibody formation and CD8⁺ T cell responses against the transgene products [151–153]. Treg induction correlated with transgene expression levels, and was essential for tolerance maintenance [151–153]. The tolerance mediated by induced Treg extended to other organs and systemically and was therefore effective in animal models of hemophilia A and B and other diseases [154]. A minimal level of transgene expression was required for tolerance induction, for example about 1% of normal FIX levels in hemophilia B mice [155]. TGF- β signaling is required to tip the balance of the response toward FoxP3⁺ Treg and thereby prevent B and CD8⁺ T cell responses to the transgene product [156]. GITR-L, a co-stimulatory molecule, is required for optimal FoxP3⁺Treg induction [157]. Although IL-10 is not required for Treg induction and suppression of antibody formation, this cytokine was found to be necessary for suppression of CD8⁺ T cell responses in the hepatic environment, where it might be produced by Kupffer cells, FoxP3⁺ Treg, or Tr1 cells [156, 158]. Finally, programmed cell death, resulting from Fas-FasL dependent and independent, is required to help tip the balance to Treg by deletion of Teff [155, 159, 160].

Importantly, liver gene transfer with an AAV or lentiviral vector (LV) could reverse pre-existing inhibitors in canine and murine models of hemophilia A and B in a vector-dose dependent fashion, and also protect against anaphylaxis in hemophilia B [153, 161–163]. Once the inhibitor response was reversed, coagulation factor expression from gene therapy provided sustained correction of coagulation. Supplementary intravenous factor injections were also tolerated without relapse of inhibitors or anaphylaxis. Increased levels of FVIII or FIX expression enhanced induction of FoxP3⁺ Treg induction, which were found to be

required for prevention, reversal and long-term suppression of B cell responses/antibody formation [151, 153, 156, 164].

5.2 Induction of Treg by orally administering antigen to the small intestine

It has been estimated that on average every year approximately 30 kg of food proteins reach the gut, and 10^{12} bacteria per g of stool colonize the human intestinal mucosa. Under such a high antigen pressure, the gut immune system has naturally evolved mechanisms to abrogate potentially injurious inflammatory responses and favor a tolerogenic environment [165]. Oral tolerance is defined as the specific suppression of immune responses to an antigen by prior oral administration of the same antigen. Induction of antigen-specific Treg is a key component of the tolerance mechanism. Oral antigens are first sampled by various cells such as M cells, enterocytes, macrophages, or DCs. DCs are educated by local cytokines and crosstalk with different cell types: intestinal epithelial cells releasing TGF- β and retinoic acid, *lamina propria* macrophages producing large amount of IL-10, orally stimulated memory T cells secreting IL-4 and IL-10 to educate DCs and induce naïve T cells to produce similar cytokines. Activated DCs migrate to the mesenteric lymph node (MLN) and induce Treg. Further, the gut immune system contains immune regulatory pDCs and CD103⁺ DCs. CD103⁺ DCs are specialized in inducing Treg via production of retinoic acid, TGF- β , and expression of IDO. TGF- β and IL-10 are high in the gut microenvironment and are essential for Treg function and maintenance. FoxP3⁺ Tregs, Tr1 cells, Th3 cells, and LAP⁺ cells have all been reported to be induced in oral tolerance [166].

Howard Weiner's group first termed Th3 cells when identifying CD4⁺ T cells that established peripheral tolerance in a murine model of experimental autoimmune encephalitis [68, 167]. Th3 cells were initially isolated and cloned from lymphoid tissues of mice tolerized by low-dose antigen feeding and were distinguished from other Th cells by their secretion of TGF- β , which is critical for their suppressor function [68, 168].

TGF- β is a multifunctional and pleiotropic polypeptide. With strong immunosuppressive effects on lymphocytes, TGF- β has emerged as a key regulatory of both innate and adaptive immune responses [169]. Among the three isoforms (TGF- β_1 , TGF- β_2 , and TGF- β_3), TGF- β_1 is the foremost in leukocyte populations [169]. TGF- β produced by T cells is critical for T cell tolerance and autoimmune disease despite the fact that many cell types are capable of secreting TGF- β , as revealed by T-cell specific conditional knockout studies [170]. TGF- β is critical for induction and maintenance of FoxP3⁺ Treg. TGF- β induces FoxP3 expression in activated T cells, which may establish a Treg phenotype [68]. Blocking TGF- β with antibody diminished FoxP3 expression in MLN and spleen in a murine oral tolerance model [171]. TGF- β suppresses T cell proliferation in the absence of exogenous IL-2 and reduces the differentiation of naïve T cells into effector Th1/Th2 cells by downregulation of T-bet and GATA3 [172–174].

Mature TGF- β is a 25kDa dimer protein, derived from two 55 kDa precursors, which are proteolytically processed by furin-like proteases once the precursors dimerize, generating the C-terminal TGF- β and N-terminal end, called LAP [175]. LAP forms a complex with TGF- β via non-covalent association and refrains the function of TGF- β ; hence its name latency-associated peptide [175]. Latent TGF- β can be activated in various ways. For

example, DCs and epithelial cells can activate TGF- β through integrin $\alpha_v\beta_8$ and $\alpha_4\beta_6$ [176, 177]. Mucosal DCs express high levels of $\alpha_v\beta_8$ compared with spleen DCs, release more bioactive TGF- β , and ultimately induce more FoxP3⁺ Treg [176]. Although LAP was found on the surface of some FoxP3⁺ T cells, LAP⁺ cells may or may not express FoxP3 [178]. LAP⁺ Treg may occur in both CD4⁺CD25⁺ and CD4⁺CD25⁻ cell populations. Studies based on feeding of anti-CD3 antibody in animal models of experimental autoimmune encephalitis and autoimmune diabetes showed suppression mediated through LAP⁺CD4⁺CD25⁻ cells [179]. Blocking TGF- β with neutralizing antibody reversed the suppressive effects of LAP⁺ Treg [180].

Taking advantage of this natural route of Treg induction, oral tolerance protocols for hemophilia A and B were developed based on delivery of antigens expressed in transgenic plant leaf cells (Fig. 3D). The plant cell walls provide natural bioencapsulation that protects from stomach acids and enzymes. To achieve optimal delivery to the gut immune system, FVIII and FIX antigens were expressed as fusion to the transmucosal carrier cholera toxin B subunit (CTB). As leaf cells contain high numbers of chloroplasts, each containing multiple genomes, targeting transgene integration and expression to the chloroplast provided high levels of encapsulated antigen [181].

Importantly, oral delivery of plant leaf material expressing FIX effectively suppressed inhibitor formation, modulated FIX specific antibody development (reduced IgG₁ and IgE while modestly increasing IgA responses) and prevented anaphylaxis in FIX replacement therapy in hemophilia B mice of the C3H/HeJ F9^{-/-} strain in a IL-10 dependent manner [182, 183]. Feeding a mixture of transplastomic tobacco plant cells expressing FVIII antigens heavy chain and C2 domain substantially suppressed inhibitor formation against FVIII in mice of 2 different strain backgrounds with hemophilia A [184]. These outcomes correlated with suppression of antigen-specific Th1/Th2 responses and increased IL-10 and TGF- β expression [183, 184]. Tolerance induction also succeeded in mice with pre-existing inhibitors following prolonged oral delivery plant materials [183, 184]. In hemophilia B mice, such a regimen desensitized the animals from anaphylaxis against FIX [183].

Feeding antigen fused with CTB, which exhibits specific and high affinity binding to the oligosaccharide domain of ganglioside GM₁, was initially found in epithelial cells (which high express GM₁), and then taken up by F4/80⁺ cells (mononuclear phagocytes) in the duodenum and DCs (including CD103⁺ DC) throughout the small intestine. Tolerogenic CD103⁺ DCs and pDCs were significantly increased in MLN and Peyer's patches (and for pDCs also in the spleen). Flow cytometry revealed the increased frequencies and cell counts of CD4⁺CD25⁻LAP⁺ cells with upregulated IL-10 and TGF- β expression, while cell counts of FoxP3⁺ Treg, and Tr1 cells remained similar. Nonetheless, adoptive transfer experiments revealed induction of antigen-specific CD4⁺CD25⁺FoxP3⁺ Treg and CD4⁺CD25⁻ LAP⁺ cells, which seem to provide a redundant level of suppression [183, 184]. Data in the FVIII and FIX delivery studies provide first evidence that LAP⁺ Treg can suppress inhibitor formation in the treatment of hemophilia, adding another regulatory cell type to the arsenal against this deleterious immune response. However, further analyses in the oral tolerance model suggested a modest induction of IL-10 expressing LAG-3⁺CD49⁺ Tr1 cells in the lamina propria, which may aid in local IL-10 expression and thereby in induction of other

subsets of Treg in this microenvironment, including LAP⁺ Treg (which depend not only on TGF- β but also on IL-10 for their induction).

Interestingly, these complex changes in APC and Treg were most obvious when oral delivery of antigen was overlapping with intravenous replacement therapy, indicating an interaction between the responses to ingested and systemic antigen that drives maximum Treg induction. Translation of oral tolerance had previously been hampered by the cost prohibitive amounts of antigens that would have to be produced. The plant-based system overcomes this barrier. Moreover, successful generation of an edible crop plant (lettuce) transplastomic for CTB-FIX, along with suppression of inhibitor formation over a wide dose range and scale up using an industrial production system, will move the approach forward toward clinical translation [185].

6. Conclusions

In summary, a host of diverse tolerance strategies and protocols are emerging in pre-clinical studies that are aimed at inducing and harnessing the power of Treg to address the problem of inhibitor formation in hemophilia A and B that currently exists in clinical practice. Some of these may be chosen for clinical studies and perhaps enhance or replace current ITI protocols. Importantly, these advances in basic research increase the potential that a prophylactic/preventive immune tolerance may become a reality in clinical use in the future. This may be of particular relevance for emerging gene therapies. To which extent drugs can be used for this purpose in pediatric patients will in part depend on the extent of global immune suppression and the duration of the regimen. Future pre-clinical studies may focus on methods to further increase the ability to induce and expand Treg and to address whether different subsets of Treg can be used synergistically to tilt the balance of the response toward tolerance.

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Highlights

- Treg play a critical role in immune tolerance in treatment of hemophilia
- Multiple subsets of Treg may suppress antibody formation against factor VIII or IX
- Coagulation factor-specific Treg can be induced *in vivo* by different methods
- Treg inducing gene, cell, oral, and drug therapies have been developed

Fig. 1. Overview of regulatory CD4⁺ T cells, their markers and major mechanisms of suppression

A. Foxp3⁺ Treg express TCR, CD4, CD25, GITR, CTLA-4, and NRP1, among others, on the cell surface, and Foxp3 in the nucleus. Foxp3⁺ Treg secrete suppressive cytokines IL-10, TGF- β , and IL-35 that directly and indirectly inhibit the function of responder T cells. Cell surface molecules may affect effector T cells. For example, Foxp3⁺ Treg express high levels of CD25 (the IL-2 receptor chain), which can compete with effector T cells for IL-2, resulting in effector cells apoptosis. Foxp3⁺ Treg can also function as cytotoxic cells to directly kill effector T cells via releasing granzyme. **B.** Tr1 cells express TCR, CD4, CD49b, LAG-3, CTLA-4, GITR, PD-1, among others, on the cell surface. Tr-1 cells release high amount of IL-10 and also secrete TGF- β . Tr1 cell suppress mainly through IL-10 besides cell contact-mediated, metabolic and cytolytic suppression. **C.** LAP⁺ Treg cells express TCR, CD4, and LAP, among others, on the cell surface. They secrete high amount of TGF- β that is critical for their suppression.

Fig. 2. *In vivo* Treg induction and expansion using small molecule and cytokine drugs such as rapamycin and Flt3L

A. Rapamycin does not block antigen presentation to and initial activation of T cell s.

However, the resulting growth factor signal is blocked through mTOR inhibition, leading to subsequent cell cycle arrest and activation induced cell death. CD4⁺CD25⁺Foxp3⁺ Treg on the other hand are induced and expanded via alternative pathways, and then further suppress effector T cells. These effects can be enhanced by addition of a third component such as certain cytokines. **B.** Flt3L enhances the tolerogenic effects of rapamycin *in vivo*. Flt3L normally expands different types of DCs. However, when combined with rapamycin, Flt3L selectively expands pDCs, because rapamycin suppresses mTOR signaling in cDC (increased basal mTOR activity makes pDCs more resistant to inhibition by rapamycin). Induction of CD4⁺CD25⁺Foxp3⁺ Treg is enhanced by pDCs. Therefore, robust Treg induction and expansion are achieved by the synergetic effects of Flt3L (indirect expansion of Treg by expansion of pDCs) and rapamycin (induction of Treg). Induced Treg then suppress effector T cell and B cell responses.

Fig. 3. Establishment of immune tolerance to FVIII or FIX by different methods of *in vivo* Treg induction

A. Tolerance induction to FVIII or FIX using gene modified B cells. **B.** *Ex vivo* expanded CD4⁺CD25⁺Foxp3⁺ Treg transferred into hemophilia A or B mice followed by treatment with FVIII or FIX enhance induction of antigen-specific Treg. **C.** Viral vector-mediated hepatic gene transfer induces tolerance via induction of CD4⁺CD25⁺Foxp3⁺ Treg and deletion of Teff. **D.** Oral tolerance induction to FVIII or FIX using transgenic plant cells via induction of LAP⁺ Treg, FoxP3⁺ Treg, and Tr1 cells, with LAP⁺ Treg representing the predominantly induced subset.