Progress toward inducing immunologic tolerance to factor VIII

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A major problem in treating hemophilia A patients with therapeutic factor VIII (FVIII) is that 20% to 30% of these patients produce neutralizing anti-FVIII antibodies. These antibodies block (inhibit) the pro-coagulant function of FVIII and thus are termed “inhibitors.” The currently accepted clinical method to attempt to eliminate inhibitors is immune tolerance induction (ITI) via a protocol requiring intensive FVIII treatment until inhibitor titers drop. Although often successful, ITI is extremely costly and is less likely to succeed in patients with high-titer inhibitors. During the past decade, significant progress has been made in clarifying mechanisms of allo- and autoimmune responses to FVIII and in suppression of these responses. Animal model studies are suggesting novel, less costly methods to induce tolerance to FVIII. Complementary studies of anti-FVIII T-cell responses using blood samples from human donors are identifying immunodominant T-cell epitopes in FVIII and possible targets for tolerogenic efforts. Mechanistic experiments using human T-cell clones and lines are providing a clinically relevant counterpoint to the animal model studies. This review highlights recent progress toward the related goals of lowering the incidence of anti-FVIII immune responses and promoting durable, functional immune tolerance to FVIII in patients with an existing inhibitor. (Blood. 2013;121(22):4449-4456)

Introduction: good news, bad news, good news

Hemophilia A is an X-linked bleeding disorder caused by a variety of mutations in the F8 gene encoding factor VIII (FVIII) that interfere with the expression or pro-coagulant function of the translated protein. FVIII is expressed primarily in liver and endothelial vascular beds. Lacking sufficient pro-coagulant activity, hemophilia A patients are prone to bleeding episodes and their sequelae, including increased morbidity and mortality. Fortunately, patients can be treated acutely or prophylactically with either plasma-derived or recombinant FVIII. However, because their immune systems have not been rendered fully tolerant to FVIII, a significant number of patients form neutralizing antibodies, termed “inhibitors,” which block FVIII activity.1 Hemophilic mutations include inversions, deletions, splicing, missense, nonsense, and frameshift mutations.2 Currently the most predictive risk factor for inhibitor formation is the hemophilia-causing mutation: patients with severe hemophilia A are at higher risk, especially those with large gene deletions or early nonsense mutations.3 Patients with mild hemophilia A circulate a dysfunctional FVIII to which they have self-tolerance; thus, their inhibitor incidence is lower.4,6

The accepted method to attempt to eliminate inhibitors is immune tolerance induction (ITI), which consists of intensive high-dose FVIII treatment until the inhibitor titer, measured by a clotting inhibition assay,7,8 subsides.9 ITI in hemophilia A is unique in clinical immunology because the antigen is absolutely known and clinical improvement can be dramatic. ITI does not eliminate all FVIII-reactive T-cell clones,10 and it is often administered in conjunction with other immune-modulating treatments. Nonetheless, animal model studies have shown suppression of FVIII-specific memory B cells following high-dose FVIII administration.11 Some inhibitors resolve (or would have resolved) spontaneously without ITI.12,13 The International Immune Tolerance Induction study, a randomized, prospective study comparing FVIII dosing with outcomes, will provide valuable data to help evaluate the roles of both patient- and treatment-related variables in producing successful outcomes. Although ITI has been used clinically for more than 3 decades14 and is successful in many cases, it is extremely expensive, and clinical management of inhibitor patients remains challenging.15,16 There is a compelling need for more effective and less expensive approaches to induce tolerance to FVIII.

This review highlights recent progress in the field and describes several novel approaches to modulate immunity and induce tolerance to FVIII (Table 1). Some reference will also be made to tolerance protocols for factor IX (FIX) in hemophilia B, because they provide “proof of principle” for novel approaches that could be applied to hemophilia A in the future. Current and upcoming basic and preclinical studies use animal models of hemophilia A, some in conjunction with analysis of blood samples donated by patients. The unifying goals of these studies are to (1) elucidate mechanisms leading to functional immune tolerance, defined as the specific reduction or elimination of inhibitor responses, and (2) translate promising potential therapies to the clinic.

T-cell dependence of inhibitor responses

Recognition of the T-cell dependence of anti-FVIII immune responses was first appreciated in hemophilia A patients infected with HIV.17,18 As these patients’ T-cell counts decreased and HIV progressed, inhibitor titers also decreased. Once effective therapy to increase CD4 counts was implemented, these patients once again produced inhibitors. Experiments in FVIII knockout mice further confirmed the T-cell dependence of inhibitors because blocking
costimulatory B7/CD28 or CD40/CD40L interactions also reduced antibody titers. 19,20

T cells are involved in both initiation and maintenance of inhibitor responses, providing help for immunoglobulin class switching that accompanies the development of high-titer antibodies. Seminal studies of T-cell proliferation following in vitro stimulation of human CD4 T cells with FVIII protein or peptides demonstrated T-cell responses to FVIII A2, A3, and C2 sequences in inhibitor-positive and inhibitor-negative patients. 21,23 More recently, systematic mapping experiments to identify HLA-restricted T-cell epitopes in FVIII have been carried out using major histocompatibility complex class II (HLA-DR) tetramers (i.e., recombinant, fluorescent-labeled proteins that mimic clustered class II molecules on antigen-presenting cells [APCs]). 24–27 When incubated with peptides containing epitopes, tetramers bind to CD4 T cells bearing receptors that recognize specific peptide–MHC complexes. This approach is particularly useful for developing human T-cell clones and lines that can aid in characterizing anti-FVIII immune responses 24 and can also be applied to evaluate patient responses to tolerogenic therapies. MHC II–peptide binding algorithms 28,29 and assays 25,30,31 have also been used to identify potential T-cell epitopes.

Another promising approach to identify epitopes is the generation of hemophilia A mouse models having a human class II (eg, DR1501, which has been associated with inhibitor risk in humans). 31,32 These partially humanized animal models will allow identification of epitopes, preclinical testing of potential tolerogenic therapies, and mechanistic studies of inhibitor responses (with the caveat that murine signaling pathways are not identical to those in humans).

Realization of the T-cell dependence of FVIII responsiveness has led to approaches to reduce FVIII immunogenicity by modifying epitopes. Introducing amino acid substitutions that interfere with class II binding seems particularly promising, because a patient’s class II haplotype is much less diverse than the T-cell repertoire. By targeted mutations of anchoring residues that contact the MHC binding groove, one can “deimmunize” FVIII epitopes so that they cannot be presented and thus do not stimulate T cells. Such a process can lead to FVIII protein (or peptides) that are “ignored” by the immune system, although they are not tolerogenic per se. 28 Deimmunization of an HLA-DRB1*0101–restricted T-cell epitope within FVIII muteins that maintain specific activities similar to that of therapeutic FVIII has recently been achieved. 33 As we learn more about innate immune pathways and stimulation of B cells by FVIII, 34 sequence modification of regions besides T-cell epitopes may also be used to reduce the antigenicity of novel therapeutic FVIII proteins. 35

Substantial progress has been made recently in understanding the uptake, processing, and presentation of FVIII peptides on APCs. 36,37 Specific regions in FVIII are required for efficient uptake. 38 For example, blocking this pathway by monoclonal antibodies during initial FVIII infusions or by modifying the FVIII sequence may have initial FVIII infusions or by modifying the FVIII sequence may have significant overpredict epitopes. As T-cell epitope repertoires become better defined, attention can be focused on deimmunization and promotion of tolerance to the most immunostimulatory regions of FVIII.

### Immunosuppression

Immunosuppressive drugs are used to modulate undesirable immune responses such as transplant rejection, graft-versus-host disease, and autoimmune diseases. Generally nonspecific, this class of agents includes cyclophosphamide, Tacrolimus, mycophenolate mofetil (MMF), rapamycin (RAP), corticosteroids, and intravenous immunoglobulin. Used alone and over extended periods, they can run the risk of susceptibility to viral and bacterial infections, and even cancer. 41 Nonetheless, when used transiently in combination with a specific antigen, immunosuppressive drugs can induce immune tolerance to FVIII in experimental models. For example, cyclophosphamide has been used to suppress the immune response following F8 gene transfer. 42–44 Short-term cyclophosphamide treatment of hemophilia B dogs prevented inhibitors following adenov-associated virus (AAV)-mediated gene delivery to skeletal muscle. 45

In a non-human primate gene-therapy trial, coupling of transient immune suppression with MMF and RAP 46 or MMF and Tacrolimus 47 with AAV-mediated gene transfer of FIX improved the effectiveness of the gene therapy. Repeated FIX dosing combined with RAP and interleukin (IL)-10 prevented antibody formation and induced FIX-specific tolerance in hemophilia B mice following AAV-mediated gene therapy. 48 The same protocol can reverse inhibitor formation. 49 Furthermore, treatment of hemophilia A mice with orally delivered RAP and repeated injections of low-dose FVIII prevented inhibitor responses. 50 This regimen induced effector T-cell responses and concomitant substantial increases in regulatory T cells (Tregs). Nevertheless, in FVIII plasmid gene therapy–treated hemophilia A mice, application of either single-agent or combined MMF, cyclosporin A, and RAP therapy delayed but did not prevent immune responses because inhibitors appeared quickly upon withdrawal of the drug(s). 42

### Blockade of costimulatory pathways

Regimens using monoclonal antibodies (mAbs) targeting a variety of immunological pathways have been investigated extensively in FVIII knockout mice. 6,11,20,42,51–56 MAbs have emerged as a new class of immunosuppressive agents that appear to be both more effective and more selective in facilitating ITI, and they are generally well tolerated by recipients. That these agents target specific pathways makes them less toxic than traditional immunosuppressive agents. When administered together with antigen, they can block responsiveness and may promote antigen-specific tolerance via T-cell apoptosis or anergy. Multiple T-cell costimulatory pathways, including CD28 and B7 (CD80, CD86), ICOS and ICOSL, CD40L and CD40, PD-1/PD-L1, OX40 (CD134) and OX40-L, ensure robust T-cell activation to mount immune responses against foreign antigens, and each of these molecules is a potential target for tolerogenic mAb therapy.

Specific agents that interrupt costimulation have been used to induce tolerance to FVIII. For example, CTLA4-immunoglobulin (CTLA4-Ig) blocks the B7/CD28 interaction and also prevents inhibitor formation in hemophilia A mice. 20 It acts partly by inducing indoleamine 2,3-dioxygenase, a tryptophan-degrading enzyme. Co-delivery of FVIII and indoleamine 2,3-dioxygenase genes in
### Table 1. Protocols for immune tolerance induction to factor VIII

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ATS, anti-thymocyte serum; cFVIII, canine factor VIII; CSA, cyclosporin A; HemA, hemophilia A; HemB, hemophilia B; HSC, hematopoietic stem cell; ICOS, inducible costimulatory molecule; IDO, indoleamine 2,3-dioxygenase.
a transposon system yielded long-term therapeutic FVIII expression and significantly reduced anti-FVIII antibody titers. Similar to CTLA4-Fc, anti-CD40L blocked restimulation and differentiation of FVIII-specific memory B cells in the presence of FVIII antigen. Dual blockade of CD40/CD40L and B7/CD28 pathways using combined anti-CD40L and CTLA4-Ig demonstrated that these agents act synergistically to prevent antigen-specific immune responses and that this therapy induced long-term tolerance to FVIII in F8-plasmid treated hemophilia A mice.

A mAb against the inducible co-stimulatory molecule (ICOS) blocks the interaction between ICOS and ICOS-ligand (ICOS-L). Anti-ICOS treatment prevented inhibitory antibody formation following nonviral F8 gene transfer. Sequential changes included transient depletion of CD4 T cells, followed by a reduction of T-effector cells and upregulation of CD4+CD25+Foxp3+ Tregs and regulatory cytokines. These results indicated the involvement of antigen-specific Tregs in tolerance induction.

### T-cell depletion therapy

T-cell depletion can significantly reduce the number of effector T cells capable of mounting an immune response following initial antigen exposure. Five consecutive anti-CD3 treatments concomitant with F8-plasmid injection prevented inhibitory antibodies and achieved persistent, therapeutic FVIII expression levels in hemophilia A mice. Furthermore, these tolerated mice received repeated plasmid F8-gene transfers that did not elicit an inhibitor. The mechanism involved increased transforming growth factor-β levels and generation of adaptive FVIII-specific CD4+Foxp3+ Tregs. Anti-CD3 treatment also induced tolerance to FVIII in hemophilia A mice that received repeated injections of FVIII protein. This tolerance was also characterized by a heightened Treg-dependent response. The dosages and schedules were comparable with those used in human trials.

### B-cell depletion

The effect of B-cell depletion on tolerance induction to FVIII has also been investigated. In FVIII-primed mice, a single dose of IgG1 anti-CD20 pretreatment prevented increased inhibitor formation in the majority of mice receiving high-dose replacement therapy. This antibody can selectively deplete follicular B cells while sparing marginal zone B cells as potential tolerogenic APCs. Transient B-cell depletion by anti-CD20 IgG2a prevented inhibitor formation in mice receiving protein therapy but failed to induce long-term tolerance. In FVIII plasmid-treated hemophilia A mice, administration of anti-murine CD20 IgG2a significantly reduced CD19+ B cells in blood, spleen, and lymph nodes as well as inhibitor titers. Anti-CD20 (Rituximab) therapy represents another immunomodulation strategy to regulate antigen-specific immune responses following protein replacement or gene therapy.

### Oral tolerance

Exposure of the immune system to antigens via the mucosal route has been known for decades to promote hypo- or unresponsiveness. This “oral tolerance” involves delivery of antigens via oral gavage or even in drinking water. FIX knockout mice that imbibed transgenic milk containing FIX were unresponsive to FIX, and they even showed corrected partial thromboplastin times (O. Alpan and P. Matzinger, NIH, personal communication). Delivery of FVIII peptides or FVIII-C2 domain has been shown to reduce FVIII-C2–specific antibody titers in hemophilia A mice, but not to FVIII per se. Oral delivery of full-length FVIII would require large amounts of protein and would not currently be feasible due to the high cost of therapeutic FVIII.

Another novel approach to induce oral tolerance involves engineering of plants to express FVIII or FIX. FIX knockout mice fed extracts of such plants had reduced anti-FIX titers, whereas FIX knockout mice did not mount an anti-FIX immune response. Importantly, engineering plants to express protein antigen allows for scaled up production with low cost and thus may lead to effective oral tolerance to therapeutic proteins.

### Gene and cellular therapy for tolerogenic FVIII delivery

Initial clinical trials with retroviral gene therapy received a setback because of severe consequences of insertional mutagenesis in scid patients. The issue of insertional mutagenesis may be moot because potential hemophilia A recipients are immunologically competent. Efforts to correct FVIII deficiency by gene therapy have evolved tremendously over the past decade. However, 2 major problems persist: immune responses to viral vectors and lack of tolerance to the F8-transgene. Current approaches fall into 2 broad categories: (1) direct in vivo injection of expression constructs in retroviral, lentiviral, adeno-associated, or nonviral vectors, and (2) ex vivo FVIII expression in various cell types. The goal is to express functional FVIII (or its major immunodominant epitopes/domains) in cells that can present these proteins in a tolerogenic fashion, thus avoiding neutralization of therapeutic FVIII by antibodies.

### Bone marrow gene therapy

Expression of FVIII or its domains in hematopoietic stem cells presumably leads to presentation of FVIII epitopes in the regenerating immune system to achieve tolerance. For example, retroviral delivery of FVIII in bone marrow hematopoietic cells following pretreatment with either antithymocyte serum or CTLA4-Ig + anti-CD40L resulted in sustained FVIII expression without eliciting a significant immune response. Busulfan treatment and bone marrow transduction have been used to lower irradiation risks to achieve myeloablation and induce tolerance to immunodominant FVIII domains, but this also led to hyporesponsiveness to subsequent FVIII challenge.

Mesenchymal stem cells (MSC) have been used as cellular delivery vehicles to transfer the F8 gene in utero or postnatally in hemophilia A sheep. Transplantation of “normal” MSCs in utero produced widespread cell engraftment. However, FVIII expression was too low for therapeutic efficacy. Transfer of gene-corrected MSCs in utero may elevate FVIII expression and facilitate tolerance because MSCs are normally regarded as hypoinmunogenic. Treating hemophilia sheep postnatally with porcine FVIII-encoding lentiviral vector-transduced paternal MSCs in the absence of preconditioning resolved all existent hemarthroses, and spontaneous bleeds ceased. Unfortunately, high-titer inhibitors then appeared, indicating that durable tolerance had not been achieved.

### Tolerogenic APCs: dendritic cells, macrophages, and B cells

A central immunologic dogma is that antigen presentation via nonprofessional APCs should be tolerogenic. This is presumably
because this presentation mode is not accompanied by significant costimulation known as “signal 2,” or “danger.” This concept, although of fundamental importance in understanding many immune responses, may be a somewhat oversimplified description of immune responses to infused antigens such as FVIII. A good example of tolerogenic presentation is the use of immature dendritic cells (iDC) as professional APCs.77,78 For example, a foamy virus vector transgene was used to express human FVIII in murine iDC expanded from lineage-negative bone marrow cells. Recipients of these transduced iDC, which expressed FVIII and IL-10, had reduced inhibitor titers and lower T-cell responses.77 Adoptive transfer of antigen-pulsed dendritic cells treated with IL-10 and transforming growth factor-β(1) also inhibited anti-FVIII antibody responses in hemophilia A mice.79 Such iDC isolated from murine bone marrow and pulsed with canine FVIII have been used to achieve hyporesponsiveness. As noted previously, the mechanism appeared to involve increased Treg production.78

B-cell expression of FVIII domains

B cells have been used as tolerogenic APCs expressing FVIII-C2 and A2 domains (which contain immunodominant T-cell and B-cell epitopes) on an IgG heavy chain scaffold to exploit the tolerogenic carrier properties of IgG.80-82 B-cell presentation of antigen may promote tolerance in part because of a lack of costimulation; the full mechanism is under investigation. Significant suppression of T-cell responses and inhibitor titers was achieved in both naive and FVIII-primed recipients. Although assembly and secretion of tolerogenic fusion proteins was hypothesized, it appeared that presentation by class II–expressing B cells was required. This process also required Tregs. Interestingly, tolerance was induced more effectively with constructs having the IgG scaffold.83,84

Platelet expression of FVIII

A novel approach for effective gene therapy is to express clotting factors in platelets, which home to injury sites and thus release FVIII when needed without exposing it to the immune system ahead of time. Delivery and expression of FVIII via platelets in animal models has been achieved with increasing efficiency.85-87 Importantly, this FVIII delivery method was effective in recipient mice with preexisting inhibitors,86 possibly because of antigen masking (resulting in immunologic ignorance) or tolerance (eg, if platelets constitute an immune privileged site). These interesting possibilities have not yet been formally tested. Moreover, to avoid specific challenges posed by ex vivo gene delivery, intravenous delivery of lentiviral vectors expressing FVIII in platelets leads to long-term correction of bleeding in both unprimed and FVIII-primed hemophilia A mice.88

Improving gene therapy for tolerance

In addition to strategies involving costimulatory blockade and short-term immunosuppression to overcome inhibitor responses following hydrodynamically delivered naked DNA constructs,35,42-44 several other strategies are being developed to create a milieu for inducing tolerance to FVIII. Injection of viral expression vectors (or naked DNA) can lead to rapid, but often evanescent, production of functional clotting factors. Major problems are immunogenicity of the vectors and transgene, and stimulation of innate immunity. Lentiviral expression of transgenes such as FIX, FVIII, or GFP led to short-term expression, which was curtailed by a strong CD8 immune response to the transgene.89-91 MicroRNA (mir-142-3p) prevented this response by suppressing expression in hematopoietic lineages while permitting expression in nonhematopoietic cells, which led to Treg-mediated tolerance.

AAV vectors containing Treg epitopes discovered in IgG92 were recently shown to mediate CD8 tolerance to AAV capsid epitopes, in a process involving Tregs (F. Mingozzi et al, personal communication). Testing this approach with FVIII domains in these novel vectors will be important proof of principle for their applicability to promote CD4 tolerance to FVIII.

In vivo genome editing via delivery to liver of a gene-targeting vector using zinc finger nucleases has been shown to stimulate gene repair and concomitant targeted gene insertion at the zinc finger nuclease–specified locus,93,94 leading to long-term expression of the corrected gene and presumably tolerance, although direct challenge has not yet been reported. Hepatocytes, in particular when using AAV vectors, are a tolerogenic site for transgene expression.95,96 Improved gene expression in hepatocytes using a codon-optimized F8-cDNA following AAV-mediated gene transfer enhanced tolerance via an increased Treg response.97 Notably, intrahepatic delivery can be considered a tolerogenic route.

Novel tolerogenic fusion proteins and Tregitopes

The construction of Fc-fusion proteins has been used to increase the half-life of many biologics such as cytokines, and most recently FVIII and FIX. The mechanism of this extended half-life depends in part on binding of the therapeutic protein to the neonatal Fc receptor, FcRn.97-100 IgG-Fc have been found to contain peptide epitopes, termed “Tregitopes,” which appear to activate and/or recruit FoxP3 Tregs.92 This may partly explain the success of B-cell gene therapy using FVIII-domain-Fc fusions,81 and it may provide a mechanistic explanation for recent reports of lowered immunogenicity of FVIII-Fc fusions designed to have longer half-lives.101,102

Additional tolerogenic approaches

Crosslinking of antigens to peripheral blood or spleen cells (using ethylenediamine carbodiimide) has been exploited as a method to induce tolerance.103 These studies have focused primarily on the treatment of autoimmune diseases and have progressed to a clinical trial (Martin et al., in press). Recent data suggest that coupled cells actually become apoptotic and are processed by splenic marginal zone macrophages.104 This has also been applied successfully in a mouse hemophilia A model.105 Coupling of antigen to biodegradable nanoparticles was efficacious in an animal model of multiple sclerosis,106 and applications to FVIII tolerance may be on the horizon.

Central role and utilization of Tregs

A dominant tolerance can be induced when activated T cells are suppressed by Tregs. T-cell homeostasis is achieved by balancing the CD4+CD25+ Tregs and effector T cells, and tolerance induction can thus be accomplished by inducing a balance shift between Treg and T-effector cells. Recently, Tregs have been induced and/or expanded...
and shown to suppress autoimmune and alloimmune responses.\textsuperscript{107-110} Many successful protocols to modulate FVIII-specific immune responses involve increases in the percentages and/or total numbers of CD4+Foxp3+ Tregs in either protein replacement\textsuperscript{111} or gene therapy\textsuperscript{112} settings. Importantly, these induced Tregs must be activated to exert their regulatory function. Immuno modulation strategies with such capacity could prevent inhibitor induction and also induce long-term tolerance to FVIII, even in patients with a measurable preexisting inhibitor titer. The successful results reported for animal model studies indicate that a shift from an immune activating to a regulatory environment by induction of activated Tregs is important both in blocking antibody responses and in facilitating the induction and maintenance of antigen-specific tolerance.

Adoptive Treg cell therapy

In an adoptive transfer experiment, Foxp3-positive Tregs from FVIII-exposed hemophilia A mice expressing the Foxp3-GFP transgene reduced antibody titers in Treg-recipient hemophilia A mice compared with untreated control mice.\textsuperscript{52} This suggested that the transferred Tregs activated endogenous Tregs in the recipient mice via an infectious tolerance mechanism, leading to long-term tolerance and limited recall responses following a second challenge. This therapy has potential advantages over conventional treatments, including antigen-specific protection without general immunosuppression and the possibility of long-lasting regulation in vivo with limited or no significant side effects. However, precautions should be taken in designing potential clinical trials, considering the plasticity of Tregs in vivo.\textsuperscript{113}

In vivo expansion of Tregs

Recently, an in vivo approach for inducing selective expansion of Treg cells by injecting hemophilia A mice with IL-2 plus a particular IL-2 mAb (JES6-1)\textsuperscript{114} was used to modulate FVIII-specific immune responses. Mice treated with IL2/IL2mAb complex did not generate inhibitory anti-FVIII antibodies and therapeutic-level FVIII gene expression was achieved in FVIII plasmid\textsuperscript{115}–treated or protein\textsuperscript{116}–treated mice. The treatment led to a marked increase in Treg cells in peripheral blood on the peak day (day 6 following the last IL2-IL2mAb complex treatment); these levels gradually returned to normal in patients with mild and moderate hemophilia A: systematic review and meta-analysis. Blood. 2012;119(12): 2922-2934.

Perspectives for the future

Multiple facets of the mechanisms by which hemophilic inhibitors develop and of how they might be prevented or suppressed are coming into focus, and our increasing understanding of tolerogenic mechanisms provides excellent opportunities for the development of novel treatment strategies. Clinical testing of promising regimens identified in animal model studies is highly anticipated, although safety will always be the first and most important consideration in deciding whether and how to test any new strategies in patients. Oral tolerance seems to be an option that would be favored among many clinicians if costs can be reduced and effectiveness improved, because it appears to be relatively safer and most closely resembles existing ITI protocols. Prophylactic tolerance induction protocols with or without a short immunosuppressive regimen having minimum side effects and toxicity are also promising strategies for patients at high risk of inhibitor formation, especially because early intervention may favor tolerance.\textsuperscript{117} Use of new FVIII variants/ formulations or manipulation of antigen presentation by gene-cell therapy could also prevent or reduce the inhibitor incidences. Some therapeutic agents could be combined with transient immunosuppressive protocols targeting B and/or T cells to improve success rates. It is noteworthy that many of the successful immunomodulation protocols for regulating FVIII inhibitors in animal models involve increasing Treg levels. Novel strategies to activate/expand/recruit Tregs could facilitate induction of tolerance to FVIII. These evolving new strategies show tremendous potential to not only reduce costs, but also to shorten treatment times and increase success rates in achieving durable immune tolerance to FVIII.

Authorship

Contribution: D.W.S., K.P.P., and C.H.M. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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