

B-Cell and T-Cell Epitopes in Anti-factor VIII Immune Responses

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Abstract Adequate hemostasis is achieved for many hemophilia A patients by infusion of plasma-derived or recombinant factor VIII (FVIII), but unfortunately, a significant subset of patients develop an immune response in which anti-FVIII antibodies, referred to clinically as “inhibitors,” interfere with its procoagulant activity. Inhibitors are the subset of anti-FVIII antibodies that bind to surfaces on FVIII (B-cell epitopes) that are important for its proper functioning in coagulation. Less antigenic FVIII molecules may be designed by identifying and then modifying the amino acid sequences of inhibitor B-cell epitopes. Conversely, characterization of these epitopes can yield important information regarding functionally important surfaces on FVIII. The production of inhibitor antibodies is driven by T cells. T cells recognize FVIII as foreign when FVIII-derived peptides bind to major histocompatibility complex (MHC) class II molecules on the surface of antigen-presenting cells. The class II–peptide complexes must then be recognized by T-cell receptors (TCRs). T-cell stimulation requires sustained association of antigen-presenting cells and T cells through formation of a class II–peptide–TCR complex, and peptide sequences that mediate this association are termed “T-cell epitopes.” MHC class II tetramers that bind FVIII-derived peptides and recognize antigen-specific TCRs are proving useful in the

characterization of human leukocyte antigen-restricted T-cell responses to FVIII.

Keywords Hemophilia A · Inhibitory antibody · B-cell epitopes · T-cell immune responses

Introduction

Factor VIII (FVIII) serves as a cofactor in blood coagulation; after being proteolytically activated to FVIIIa, it associates with the serine protease FIXa at activated membrane surfaces in the presence of calcium, forming a catalytic complex that converts another serine protease, FX, to its active form FXa [1]. This “intrinsic tenase” complex increases the Vmax for FX activation by four orders of magnitude [2]. FXa then forms an analogous complex with cofactor FVa, accelerating the proteolytic conversion of prothrombin to thrombin. The human *F8* gene consists of 26 exons; it encodes a FVIII precursor protein that is 2,332 amino acid residues in length [3–5]. This precursor is modified post-translationally by attachment of carbohydrates, sulfation of tyrosine residues, proteolytic processing, etc., as it passes through the endoplasmic reticulum and Golgi apparatus [6, 7]. It is then secreted into the plasma, where it circulates in a complex with von Willebrand factor (VWF), another plasma protein that serves as its molecular chaperone, protecting FVIII from proteolytic degradation [8], extending its half-life in the circulation, and delivering it to sites of injury.

Hemophilia A (HA) is an X-linked genetic disease in which a mutation in the *F8* gene results in either a lack of circulating functional factor VIII (FVIII) protein or production of a defective FVIII with impaired activity. HA is treated by infusing patients with FVIII. Approximately 25%

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of infused HA patients develop an immune response in which anti-FVIII antibodies, referred to clinically as “inhibitors,” block the procoagulant effect of FVIII [9–13]. Hemophilic amino acid substitutions that occur at sites of FVIII interaction with other proteins or membranes can highlight the importance of specific residues for proper functioning of FVIII as a procoagulant cofactor. Figure 1 shows a ribbon depiction of the three-dimensional structure of human FVIII, as recently determined by X-ray crystallography [14, 15]. Antibodies binding to epitopes in these regions would be expected to interfere with FVIII function, leading to their classification as “inhibitors.”

FVIII mutations resulting in HA and their association with inhibitor risk

Mutations resulting in HA [16, 17] include common DNA inversions, large deletions or rearrangements, small deletions or insertions (usually leading to frameshifts), and single point transitions or transversions. Most of the less severe hemophilic phenotypes are due to missense mutations that can lead to production of an impaired but still

somewhat functional FVIII and/or a protein that retains function but has impaired secretion. The majority of nonsense mutations, in which a premature termination codon is introduced into the FVIII mRNA open reading frames, result in transcripts that are degraded by the cell [18], so truncated nonfunctional FVIII proteins would not be expressed in these cases. The risk of developing an inhibitor is elevated in patients with nonsense mutations [19]. Exon skipping, in which a FVIII transcript missing the exon with the nonsense mutation is generated, has been documented [20], although it is not prevalent, so the available evidence does not support in-frame exon skipping leading to expression of variant FVIII proteins as an explanation for the observed tolerance of most severe HA patients for infused FVIII. The most common cause of HA is a DNA inversion between a repeat sequence in intron 22 with one of two copies of this sequence that are telomeric to *F8*, resulting in a severe HA phenotype [21]. Although intron-22 inversions are responsible for about half of all severe HA cases, the risk of developing an inhibitor for these patients is only about 20% [22]. The reason for the discrepancy in inhibitor risk for severe HA patients with an inversion mutation versus risks associated with other FVIII mutations is not understood. One can speculate that trace production and secretion of proteins from partial transcripts could promote tolerance to infused FVIII; this possibility deserves further investigation.

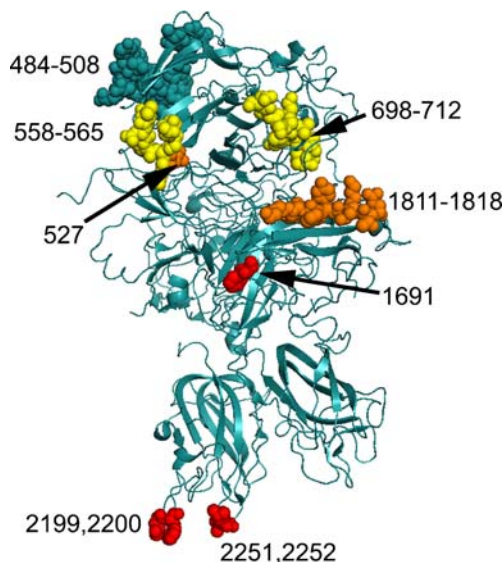


Fig. 1 Ribbon diagram representation of the FVIII crystal structure determined by Ngo et al. [14]. Amino acids that interact with factor IXa are shown as *space-filling spheres colored yellow* (558–565 and 698–712) and *orange* (R527 and 1811–1818). Amino acids that contribute to FVIII-Von Willebrand factor affinity are shown as *space-filling red spheres*, specifically the “hydrophobic feet” residues M2199, F2200, L2251, and L252, as well as residue 1691, which indicates the location of the acidic peptide 1649–1689 that constitutes a critical part of a VWF-binding interface. This region was not visible in the crystal structures, but the location of residue 1691 provides a pointer to a VWF-binding interface. Residues 484–508, which comprise a dominant A2 domain epitope, are shown as blue space-filling spheres

Inhibitory antibodies and B-cell epitopes

The most prevalent complication encountered when HA patients receive infusions of recombinant or plasma-derived FVIII is the development of inhibitor antibodies that neutralize the procoagulant function of FVIII. Although more common in severe and moderately severe patients [9–13], they can also occur in individuals with mild or moderate HA [23–31], as well as in nonhemophilic individuals who develop immunity to their own FVIII [32]. Antibodies that develop in HA patients tend to involve a larger number of epitopes than auto-immune inhibitors, which develop very rarely in nonhemophiliacs [33]. Both allo- and auto-immune inhibitors can be life threatening, and they are difficult and extremely expensive to treat [34–36]. There is a compelling need for improved therapies to reduce the incidence of FVIII inhibitors and to provide effective alternatives to FVIII infusions when they do occur.

Polyclonal IgG from hemophilic and nonhemophilic inhibitor plasmas have been shown to bind primarily to the FVIII A2 and C2 domains, and antibodies directed to the $\alpha 3$ -A3-C1 region are also fairly common [37–41]. Non-inhibitory anti-FVIII antibodies also occur in infused HA patients [42–44], and they are not isotypically restricted [42]. Although many of these antibodies do not result in

clinical bleeding disorders, some may affect FVIII clearance and half-life, but this effect will not be evident in standard *in vitro* assays for inhibitors. Non-inhibitory antibodies can be detected by enzyme-linked immunosorbent assays (ELISA) but not by Bethesda assay or chromogenic assays of FVIII costimulation. Inhibitor antibodies are generally identified by measuring the Bethesda titer, which is the reciprocal of the dilution of inhibitor plasma resulting in 50% inhibition of FVIII activity [45, 46].

Some inhibitor antibodies are associated with limited catalytic activity, neutralizing FVIIIa procoagulant activity by proteolysis of FVIII at multiple sites, implying there is an additional set of clinically relevant B-cell epitopes that may or may not affect interactions of FVIII with membranes or other proteins [47]. Most of the cleavages occur after Arg or Lys residues, implying that the epitopes include substrates for a serine protease-like catalytic IgG [48]. Sequential cleavages would of course affect the FVIII structure and could expose sites that are inaccessible in the intact, properly folded FVIII. Catalytic anti-FVIII antibodies have been found in IgG from subjects with severe HA [48, 49], with auto-immune inhibitors [50], and also in healthy control subjects [51]. The epitopes recognized by these IgGs are still not well characterized, but they are certainly of interest. Proteolysis of FVIII by endogenous proteases and/or by catalytic IgG results in the presentation of “naturally-processed” T-cell epitopes on major histocompatibility complex (MHC) class II molecules. Variations in this process due to the inflammatory state of the affected individual or to the specific site(s) at which the anti-FVIII response develops may also account for different susceptibilities of individuals to inhibitor formation [52]. Binding of antibodies to FVIII may induce conformational changes that make it more susceptible to cleavage by endogenous proteases; the prevalence of this potential mechanism of FVIII inactivation is unknown. Another variable that may affect the presentation of FVIII to the hemophilic immune system is the source of the FVIII protein. The question has been raised whether recombinant FVIII may undergo proteolytic processing that differs somewhat from that of plasma-derived FVIII or from preparations containing both von Willebrand factor and FVIII. In principle, such differences, if they exist, could affect binding of FVIII degradation products to MHC class II molecules on antigen-presenting cells, as affinity for the class II is affected by peptide length as well as by the amino acid sequence of the epitope. Both recombinant and plasma-derived FVIII can provoke immune responses. Although the relative immunogenicity of various FVIII preparations is an important concern [53], new breakthroughs in immune system modulation, e.g., immune tolerance induction protocols, as well as rational design of less immunogenic FVIII proteins are likely to have a

greater eventual impact on the clinical problem of FVIII inhibitors.

Anti-FVIII A2 domain inhibitors block the activation of FX by the intrinsic tenase complex [54]. They have been mapped by the Lollar group using recombinant human/porcine FVIII proteins; these studies exploited the limited cross-reactivity of the human and porcine FVIII [55], which indeed has proven useful for the clinical management of some high-titer inhibitor patients with antibodies against the human FVIII protein [56]. The predominant A2 epitope was first localized by noting the hybrid FVIII with the porcine sequence substituted for the human at residues 484–508 was much less antigenic than the other hybrids [57]. Scanning alanine mutagenesis was then carried out in this region, followed by Bethesda assays carried out with allo- and auto-immune plasmas to measure the antigenicity of the mutants. Several of the substitutions significantly reduced the antigenicity, indicating a significant fraction of the anti-A2 inhibitory IgG bound to epitopes in this loop [58]. A mildly inhibitory monoclonal antibody specific for the FVIII A2 domain was used to immunoprecipitate peptides from a protease-treated FVIII A2 protein. Mass spectrometry identified peptides 497–510 and 584–593 as comprising part of this B-cell epitope, which likely overlaps the known inhibitor epitope at residues 484–508 [59]. A human inhibitory alloantibody directed against residues 351–365 has also been characterized [60], but inhibitor binding to this region is not common.

Anti-FVIII C2 domain inhibitors bind to multiple epitopes in this domain [37, 61, 62]. Experiments with human–porcine FVIII hybrids identified residues 2181–2243 as containing important antigenic sites [63], and immunoblotting experiments utilizing recombinant C2 fragments have localized epitopes for both murine monoclonal and patient-derived inhibitor antibodies to additional distinct regions on the C2 domain [64]. Human and murine antibodies have been shown to block FVIII binding to phospholipid membranes [65–67] and to VWF [65, 68, 69]; some but not all inhibitors prevent binding to both [64, 70], indicating a partial overlap of these binding sites on the C2 domain. Overlapping of phospholipid- and VWF-binding sites has also been established by assays not involving antibodies [71, 72]. The mild hemophilic in-frame deletion of A2201 affected binding of the mutant FVIII to an inhibitory monoclonal antibody, indicating that the hairpin turn at residues 2198–2202 contributes to a B-cell epitope [73]. A missense substitution associated with mild hemophilia A also occurs at this same site, A2201P; one individual with this mutation developed inhibitory antibodies that had only limited cross-reactivity against his endogenous hemophilic FVIII [74].

It is interesting to note that although the membrane- and VWF-binding regions on the C2 domain are essential for

FVIII procoagulant function, hemophilic substitutions occur here only rarely. This likely follows from the redundant design of this region, in that four hydrophobic side chains (M2199, F2200, L2251 and L2252) are proposed to insert into the membrane interior, while arginine and lysine side chains form favorable ionic interactions with the negatively charged surfaces of membranes that expose phosphatidylserine [75]. Thus, a single missense substitution at this interface region would generally remove only some of the interactions conferring affinity, and the mutant FVIII could still function as an effective cofactor for FIXa. Experiments testing the binding of FVIII/FV C2 domain chimeras to human polyclonal inhibitory IgG were consistent with the proposed membrane-binding mode of the FVIII C2 domain [75], and they also indicated that inhibitor epitopes were clustered at this surface [76].

A distinct C2 domain epitope is recognized by a murine monoclonal antibody (ESH8) and also by a patient-derived polyclonal inhibitor; these antibodies were shown to stabilize the FVIII–VWF complex but have no effect on phospholipid binding [77]. Their neutralization of FVIII activity thus results from the delayed transfer of FVIII from VWF to the membrane surface upon platelet activation. Monoclonal antibodies recognizing a similar epitope were generated recently [78], and the ESH8 epitope has been localized by peptide array analysis to a C2 loop distal from the membrane-binding region, including residues 2271–2275 [79]. Inhibitor alloantibodies have been neutralized by peptides corresponding to the C-terminus of the C2 domain, specifically residues 2315–2330, suggesting that residues in this region contribute to inhibitory epitopes [80].

It has been proposed that conformational changes in the FVIII C2 domain can affect its antigenicity; in support of this, removal of the acidic region from the FVIII light chain lowered its affinity for the monoclonal anti-C2 antibody NMC-VIII/5 [81]. Conformational changes in the C2 domain have also been proposed to explain the enhanced immunogenicity of a few batches of plasma-derived FVIII concentrates that were subjected to virus inactivation by solvent-detergent plus pasteurization or other methods involving heat inactivation [82, 83]; an increased incidence of C2-specific inhibitors in patients infused with these proteins suggested that some variation in the virucidal treatments may have led to increased solvent exposure of the membrane-binding surface of FVIII. This idea was supported by enhanced phospholipid binding and altered thrombin activation of FVIII treated in this manner [82]. The epitopes were not characterized further, but these incidents and the follow-up studies have established that conformational heterogeneity can result in enhanced immunogenicity and antigenicity of FVIII products.

The FVIII C2 domain binds to both thrombin [84] and FXa [85, 86]. The most comprehensive characterization of

C2 domain epitopes to date utilized competition ELISA assays carried out for a panel of 29 murine monoclonal antibodies and one patient-derived human monoclonal antibody against the human FVIII C2 domain [78]. The results indicated the antibodies could be divided into five groups, two of which bound to overlapping sites recognized by two other antibody groups. A significant fraction of these monoclonal anti-C2 antibodies interfered with the proteolytic activation of FVIII by thrombin or FXa [78]. The relevance of these epitopes to inhibitory antibodies in humans was recently established by ELISA assays in which the murine monoclonal antibodies competed effectively with several samples from a panel of plasmas donated by 26 allo- or auto-immune inhibitor subjects for binding to FVIII [87].

The epitope for the human-derived anti-FVIII C2 domain monoclonal antibody BO2C11 has been characterized both biochemically [88] and by determining the three-dimensional structure of its corresponding Fab fragment bound to the recombinant FVIII C2 domain at 2.0 Å resolution [89]. Thus, the B-cell epitope has been visualized at atomic resolution (Fig. 2). BO2C11 binds to the C2 domain of FVIII, interfering with FVIII binding to both activated phospholipid membranes and to VWF [88]. The high-resolution BO2C11–C2 crystal structure allowed precise identification of interactions between amino acids at the FVIII–antibody interface. A recent investigation characterized an anti-idiotypic antibody that bound to and neutralized BO2C11; a three-dimensional homology-based model of this anti-idiotypic Fab region was constructed, and its properties were quite similar to those of the FVIII

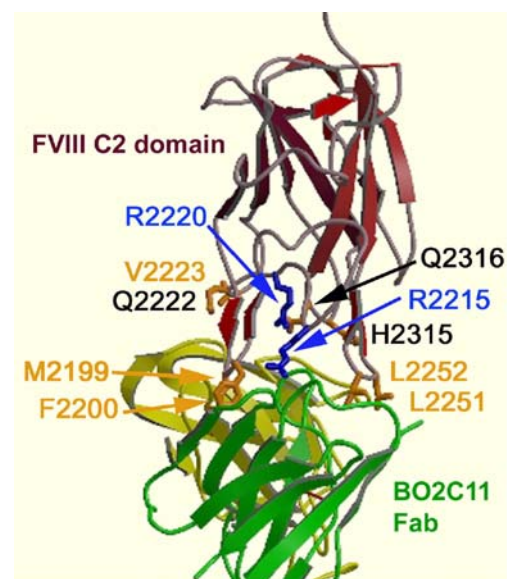


Fig. 2 Three-dimensional crystal structure of the FVIII C2 complex with the inhibitory human monoclonal antibody BO2C11 Fab fragment. Several of the residues at the antigen–antibody interface are labeled and shown explicitly

C2 domain, with which it competed for binding to BO2C11 [90].

Inhibitors that bind to epitopes in other FVIII domains are less prevalent, but they can be very significant clinically for some individuals. Some polyclonal inhibitory antibodies are neutralized more effectively by the FVIII light chain than by a recombinant C2 domain [33] or by a combination of A2 and C2 proteins [40], indicating the presence of epitopes in the A3 or C1 domains. Inhibitors have been shown to bind a recombinant A3-C1 fragment [26, 91] and to block the association of FIXa and the FVIII light chain [91–93]. Human monoclonal single-chain variable domain antibody fragments (ScFv) that inhibit FVIII activity have identified an epitope within A3 residues 1778–1840 [94]; furthermore, a synthetic peptide corresponding to A3 residues 1804–1819, which overlaps a known FIXa binding site at residues 1811–1818 [92], competed with inhibitor IgGs for binding to FVIII [91]. Inhibitor antibodies that block FVIII binding to VWF were shown to be specific for the 41-residue a3 sequence; anti-a3 antibodies have also been identified by cross-reactivity of inhibitor plasmas against a series of hybrid porcine–human FVIII proteins [95]. Shima et al. generated anti-peptide antibodies by immunizing rabbits with peptides corresponding to this region; an epitope within residues 1663–1669 was recognized by antibodies that neutralized FVIII procoagulant activity, and neutralization of this inhibitory activity by sequence-modified peptides suggested that residues D1663 and Y1664 are particularly important to FVIII function [96]. Inhibitors binding to the heavy chain acidic regions a1 and a2 have also been identified [65, 97, 98]. A patient-derived IgG4κ monoclonal antibody directed against a C1 domain epitope, which blocks FVIII binding to VWF, has also been characterized [99]. A subset of polyclonal IgG from a mild HA inhibitor subject with missense genotype R2150H recognized a B-cell epitope present only on FVIII–VWF complexes [100]; this inhibitor did not compete with the anti-C2 domain monoclonal antibody ESH8 [101]. Inhibitory antibodies directed to the A3 and/or C1 domains may affect membrane binding as well, as indicated by experiments in which phospholipid vesicles blocked binding of FVIII to ScFVs derived from inhibitor patients [102]. Antibodies from one severe HA inhibitor subject were shown to bind primarily to the C1 domain, and they inhibited the tenase activity by blocking binding of FX to FVIII [103].

Synthetic peptides with amino acid sequences corresponding to some of the B-cell epitopes described above have been tested for their ability to bind to and block the inhibitory effect of anti-FVIII antibodies or ScFVs [104–107]. Although some of the inhibitors were indeed blocked and could be removed from subjects' plasma, the effectiveness of this approach was quite variable, as the epitope

profiles varied among different patients and even during their course of treatment. Synthetic peptides with FVIII-derived sequences have been used to affinity-purify anti-FVIII IgG from pooled plasma samples donated by nonhemophilic individuals [108]. Another interesting approach to block inhibitor IgG has utilized FVIII or recombinant FVIII fragments containing electrophilic compounds at or near the relevant epitopes. These modified proteins have been shown to covalently bind and inactivate FVIII inhibitors [109]. The use of smaller synthetic peptides with electrophilic substituents would be an attractive alternative to using electrophilic-modified FVIII because of the high concentrations of FVIII protein that would otherwise be required to neutralize an antibody response. However, synthetic peptides were much less effective, reflecting the fact that many FVIII inhibitors recognize conformational rather than linear epitopes. This approach is worthy of further investigation.

Modification of FVIII antigenicity

In attempting to evaluate the feasibility of modifying specific residues in FVIII to evade inhibitory antibodies in an established immune response, it would be helpful to know (a) how many amino acids in FVIII contribute significantly to antigen–antibody complex formation, (b) the relative contributions of specific residues to the total binding energy and (c) whether or not conservative substitutions at or near these residues can be tolerated without drastically affecting activity or pharmacokinetics of FVIII. Our laboratory is addressing these questions by analyzing a physiologically relevant antigen–antibody pair, specifically the monoclonal antibody BO2C11 (described above) and the recombinant FVIII C2 domain. Surface plasmon resonance experiments indicated that this inhibitory IgG bound to FVIII with an association rate of $k_{\text{on}} \sim 7.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and dissociation $k_{\text{off}} \leq 1 \times 10^{-5} \text{ s}^{-1}$, giving an apparent $K_d = k_{\text{off}}/k_{\text{on}} = 1.4 \times 10^{-11} \text{ mol/L}^{-1} \text{ s}^{-1}$ [88]. The extended buried interface between this IgG4κ Fab and the C2 domain includes extensive hydrophobic interactions as well as a network of specific hydrogen and ionic bonds [89]. However, only a limited subset of these interactions contributes significantly to the strong binding affinity (Lin, JC personal communication). Determination of binding affinities using surface plasmon resonance or other methods can help to identify amino acid substitutions as candidates to reduce the antigenicity of FVIII. Next-generation “designer” FVIII proteins incorporating such substitutions may eventually provide therapeutic benefit for some inhibitor patients, for example high-titer inhibitor patients who fail immune tolerance induction or patients who develop high-titer inhibitor antibodies that cross-react with porcine FVIII.

In fact, the use of FVIII proteins with variant sequences to evade inhibitors is more than promising; it has clinical precedence, especially in patients with autoimmune responses to FVIII. Plasma-derived porcine FVIII has been used to treat allo-immune inhibitor patients who fail tolerance induction [110], and a recombinant porcine FVIII has been produced and is being characterized [111, 112]. The Lollar lab has shown that in many immune responses to factor VIII there is only limited cross-reactivity between anti-human FVIII inhibitory antibodies and porcine FVIII [113], and this lack of antigenic cross-reactivity has been exploited to map B-cell epitopes within particular domains or regions of FVIII using porcine–human hybrid molecules [95, 114]. The effects of the substitutions were evaluated by ELISA and/or immunoblot assays, and IgG binding and Bethesda assays established whether the substitutions affected interactions with the subset of antibodies that inhibited FVIII function. Taking this approach further, individual amino acids within an A2 domain epitope at residues 484–508 were substituted systematically to alanine, and the effects of the substitutions were evaluated in a similar manner. The result was that a major inhibitory epitope was removed by substitution of just a few residues [58]. B- and T-cell epitopes in other domains, particularly the C2 domain, are more complex (or numerous); nevertheless, the approach of site-specific sequence modification is promising, as reduction of inhibitor titers by modifying immunodominant epitopes could be clinically significant for at least some patients.

Although porcine FVIII has been used successfully for initial treatment of patients with high-titer inhibitors, antibodies that inhibit the porcine protein can develop after repeated infusions [115]. A concern in modifying the sequence of any protein therapeutic is the possibility that the modification will introduce new T-cell epitopes that could stimulate production of neutralizing antibodies [116]. It is highly unlikely that a completely non-immunogenic or non-antigenic version of a protein the size of FVIII could be developed, due to the number of potential T-cell and B-cell epitopes. However, the development of FVIII proteins that are *less* antigenic (likely to bind epitopes) or immunogenic (likely to stimulate T cells) than the human, porcine, or porcine–human hybrids is well within reach.

A testable premise in attempts to reduce FVIII antigenicity is that a limited number of amino acid substitutions (which must not substantially interfere with the procoagulant FVIII cofactor activity) may be sufficient to allow the modified FVIII to avoid neutralization by a substantial fraction of the anti-FVIII antibodies that are causing the inhibitor response. If this were indeed the case, then these versions of FVIII could be utilized therapeutically when patients develop a high-titer inhibitor or when they fail immune tolerance induction therapy. It is possible that use

of a less antigenic protein to treat high-titer inhibitor patients could extend the therapeutic window for achieving hemostasis by infusions with the modified FVIII. Use of a modified FVIII would not preclude other therapeutic interventions, e.g., methods to induce tolerance via high-dose or lower-dose administration of FVIII, bypass therapies, or immunoadsorption [117–119]. Tolerance to a less *immunogenic* FVIII would, by definition, be achieved more readily than tolerance to wild-type human FVIII due to a reduced number of T-cell epitopes. Would a protein designed to be less *antigenic* also be less immunogenic? T-cell and B-cell epitopes do not necessarily overlap, although in several cases, the overlap of a human leukocyte antigen (HLA)-restricted T-cell epitope with the corresponding B-cell epitope in FVIII has been documented in human subjects with mild HA who developed an inhibitor [27, 74, 120, 121]. A similar overlap of B- and T-cell epitopes was also indicated in a study utilizing a hemophilic mouse model [122]. Analysis of inhibitor responses in a greater number of subjects, including individuals with severe HA, is needed to address this question properly. Finally, very little is known about the roles of particular B- and T-cell epitopes in immune tolerance induction (ITI). During ITI, the patient is infused with superphysiological amounts of FVIII, and in successful cases, the antibody titer decreases in the presence of high levels of antigen. The molecular interactions leading to these clinical successes are not well understood. It would be interesting to know, for example, how ITI affects the interactions of FVIII epitopes with dendritic cells, memory B cells, regulatory T cells, and other components of the immune system.

T-cell responses to FVIII and HLA restriction

An early indication of the role of CD4⁺ T cells in hemophilic patients with a FVIII inhibitor came from clinical observations of inhibitor patients infected with HIV whose inhibitor titers decreased as their CD4⁺ cell counts decreased [123]. Additional evidence of CD4⁺ T-cell help comes from the fact that inhibitory antibodies undergo somatic hypermutation [88, 124, 125]. There is a preponderance of anti-FVIII inhibitory antibodies that are IgG1 and IgG4 [42, 126], and this observed class switching indicates the involvement of T cells in the anti-FVIII immune response. Despite the compelling evidence for T-cell involvement in anti-FVIII antibody production, the HLA dependence of inhibitor responses has not been readily apparent [127], and this reflects the complexity of these immune responses. Low-resolution HLA typing does not adequately reflect the heterogeneity of MHC class II molecules, for example, and variations in T-cell receptor

(TCR) V-alpha and V-beta regions that interact with peptide–class II complexes will also affect avidities of binding and subsequent signaling events. However, the possibility of manipulating initial MHC class II and TCR binding events leading to T-cell activation is compelling because effective intervention at this stage could prevent inhibitor formation.

In order for antibody maturation to occur, the protein antigen must be processed proteolytically into short peptide fragments by professional antigen presenting cells (APCs), including dendritic cells and B cells, in a subcellular “MHC class II compartment.” If a class II protein encoded by one of an individual’s MHC class II alleles binds to a FVIII peptide with sufficient affinity, then the resulting peptide–bound class II molecule will be transported to the cell surface and “presented” to the intercellular environment. In order for T-cell signaling to occur, the peptide–class II complex must be recognized by a T-cell receptor on an antigen-specific T cell. Sustained binding of the peptide–class II complex to its cognate TCR results in intermolecular signaling and cytokine release. The types of intermolecular signals generated and the specific cytokines released depend on the type of T cell bearing the receptor, the TCR subclass, and the number and type of costimulatory and other molecules on the cell surface. Proliferation of CD4⁺ effector T cells (also referred to as “helper” T cells) can stimulate the proliferation and class switching of B cells, which then undergo affinity maturation to become antibody-producing plasma cells or APCs. Conversely, stimulation of regulatory T cells, e.g., CD4⁺ CD25⁺ FoxP3⁺ cells, Tr1 cells that secrete interleukin (IL)-10, or T cells that secrete TGF- β family cytokines, can lead to down-regulation of immune responses to foreign antigens such as FVIII. Several distinct peptide-binding motifs may be recognized by a given MHC class II molecule, and effective class II–peptide–TCR complex formation and subsequent signaling can occur in response to peptides having different amino acid sequences. A result of this “polyspecificity” (recognition of several distinct amino acid sequences) is that T cells and APCs may recognize several related amino acid sequences [128]. Conversely, “promiscuous” T-cell epitopes may bind to multiple class II molecules or TCRs.

Effector T cells may be assigned to three broad categories or phenotypes based on the cytokines they secrete and the presence of characteristic transcription factors (Fig. 3) [129, 130]. Th1 cells secrete interferon gamma (IFN- γ), IL-2, and tumor-necrosis factor- α ; utilize the transcription factor T-bet; and are important in “cellular” immunity; Th2 cells secrete IL-4, IL-5, IL-6, and IL-13, utilize the transcription factor GATA-3, and are important in “humoral” immunity. The importance of a third distinct class of effector CD4⁺ T cells, known as Th17 cells, has recently been recognized [131–133]; these cells

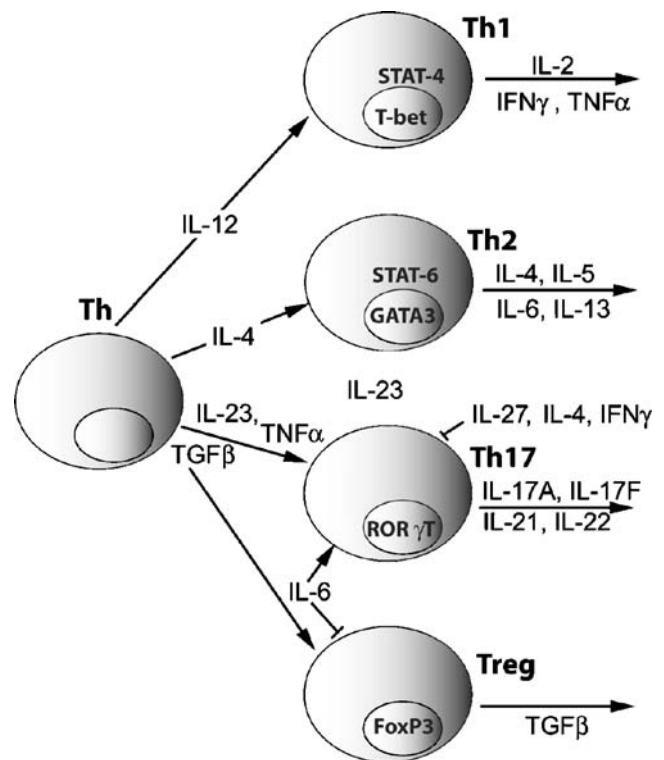


Fig. 3 Simplified scheme showing differentiation of uncommitted helper T cells into Th1, Th2, Th17 or Tregs. Arrows indicate cytokines that promote a particular differentiation pathway, while lines without arrowheads indicate cytokines that inhibit a given pathway. Characteristic transcription factors for each T-cell type are indicated at or near the nuclei. Cells with mixed phenotypes are not shown explicitly, nor are additional classes of Tregs, e.g., Tr1 and Th3

secrete IL-17A, IL-17F, IL-21, and IL-22, utilize the transcription factor ROR- γ t, and are important in “inflammatory” immunity; Th17 cells also become up-regulated in various auto-immune disorders [134, 135]. The possible roles or significance of Th17 cells in either allo- or auto-immune responses to FVIII are unknown at present, whereas the involvement of Th1 and Th2 cells in anti-FVIII immune responses is well established [136, 137]. Both Th1 and Th2 cells secrete cytokines promoting B-cell differentiation and antibody synthesis [138–141]. Th1 cells promote the development of IgG1 and IgG2 subclasses in humans, which bind complement, while Th2 cytokines promote development of IgG4, which does not bind complement. Thus, by analyzing the subclasses of anti-FVIII IgG, one can determine which T-cell subsets contributed to the immune response. This analysis was carried out for a series of inhibitor subjects with hemophilia A or with an auto-immune response to FVIII [137], and higher inhibitor titers correlated with IgG4 levels, indicating that Th2-driven antibody synthesis characterizes strong anti-FVIII immune responses.

Polarization toward a Th1, Th2, or Th17 cytokine response characterizes many immune processes and clinical

disorders. For example, Th0 (Th1+Th2) cytokine patterns are often seen in early stages of an immune response [142]. Cytokine production by CD4⁺ T cells has been analyzed after stimulating peripheral blood mononuclear cell (PBMC) from HA subjects with and without an inhibitor, as well as from nonhemophilic controls [143]. IFN- γ was produced by FVIII-stimulated CD4⁺ T blasts from HA subjects with and without an inhibitor and also by controls whose PBMC proliferated in response to FVIII, but only inhibitor subjects had a Th2 response characterized by IL-4 production. TGF- β 1 production increased when cells from controls or from HA subjects without an inhibitor were stimulated with FVIII, but this was not seen for inhibitor subjects; these results indicated that Th3 cells may help down-regulate T-cell and humoral antibody responses to FVIII.

Costimulatory molecules and immunosuppression strategies

Costimulatory molecules on both T cells and APCs play essential roles in the development of FVIII inhibitors. Their myriad functions in various immune and auto-immune responses have been demonstrated by experiments in animal models as well as in human clinical studies. B7/CD28 and CD40/CD40L are well established as important regulatory signaling pairs involved in costimulation pathways. CD28 is a glycoprotein on the T-cell surface that interacts with B7-1 (CD80) and B7-2 (CD86) on APCs, initiating signals that are essential for antigen-specific T-cell responses [144]. Cytotoxic T lymphocyte-associated antigen-4 (CTLA4) negatively signals T cells to down-regulate pro-inflammatory responses [145, 146], and it is a competitive inhibitor of B7/CD28 binding [147]. The reagent CTLA4-IgG binds to B7-1 and B7-2, preventing B7 interactions with both CD28 and CTLA4 [148]. Blockade of CD40/CD40L has been shown to block inhibitor development in hemophilic mice [149–152] and blockade of CD40/CD40L interactions has been investigated in some inhibitor patients [153]. Blockade of B7/CD28 by murine CTLA4-Ig prevented inhibitor formation in hemophilic mice, and hemophilic mice that were also deficient in B7-2 did not develop inhibitory antibodies after repeated infusions of FVIII, establishing the importance of this pathway in FVIII inhibitors in this animal model. Furthermore, administration of CTLA4-Ig to hemophilic mice with an established inhibitor modulated the inhibitor titers relative to titers in hemophilic mice when both groups were subsequently injected with FVIII [149]. Blocking the interaction of CD28 with B7-1 and B7-2 using CTLA4-Fc or using anti-B7-1 and anti-B7-2 monoclonal antibodies, respectively, as well as blockade of

CD40/CD40L binding using an anti-CD40L monoclonal antibody has been shown to specifically prevent re-stimulation and differentiation of memory B cells *in vitro* and *in vivo* in a mouse HA model. However, blocking the inducible costimulator (ICOS)/inducible costimulator ligand (ICOSL) interaction did not down-regulate memory B cell stimulation by FVIII [154]. Short-term administration of an anti-ICOS monoclonal antibody to block the ICOS/ICOSL signaling pathway led to depletion of CD4⁺ ICOS⁺ cells. It also led to sustained tolerance in hemophilic mice that expressed FVIII following gene transfer using a plasmid system [155], indicating a role for this pathway in T-cell responses to FVIII. Other immunosuppressive agents used to treat patients with acquired hemophilia due to development of anti-FVIII auto-antibodies include cyclosporine A [156] and rituximab [157, 158], while hemophilic patients with inhibitors have been treated with cyclophosphamide, intravenous immunoglobulin therapy, or various protocols to induce tolerance [159] and with combinations of the above therapies [160–162]. These results from animal and clinical studies suggest that a strategy of administering less antigenic or immunogenic versions of FVIII *together with agents affecting costimulation and/or with immunosuppressive drugs* may produce a synergistic effect in reducing antibody titers in inhibitor patients or in preventing inhibitor development following FVIII infusions.

T-cell epitopes in FVIII

T cells isolated from peripheral blood of HA subjects with and without inhibitors [163], and even from some non-hemophilic individuals [164], proliferate when cultured with FVIII. Longitudinal studies have shown that these responses are often transient, and the magnitude of the proliferative responses tended to vary over time [165]. Attempts to characterize the epitopes eliciting these T-cell responses have involved stimulation of polyclonal CD4⁺ T cells by culturing them with and without synthetic peptides having amino acid sequences corresponding to regions in FVIII, then measuring their proliferation, e.g., by ³H-thymidine incorporation. The ratios of proliferation in the presence and absence of FVIII peptides (reported as significance index, or SI values) are then calculated and used to determine the statistical significance of peptide-stimulated proliferation. Several studies have involved stimulation of human CD4⁺ T cells with pools of peptides spanning the FVIII A2 [163], A3 [166], and C2 [122, 167] domains, and T-cell proliferation was measured by incorporation of ³H-thymidine. Mouse models of HA have also been utilized to investigate T-cell responses to FVIII protein and peptides [122, 136, 149, 168, 169]. The murine

HA mice offer several advantages for carrying out studies of T-cell responses to FVIII, as a number of unavoidable variables inherent in human studies can be well controlled in animal studies. The mice are genetically identical, they can be given the same treatment regimes, e.g., immunization with adjuvant or infusion of FVIII at therapeutic doses, and samples can be obtained at regular time intervals. Continued investigation of both human and animal T-cell responses will be essential to furthering understanding of anti-FVIII immune responses.

A recent study utilized peptides spanning the entire FVIII sequence to stimulate CD4⁺ T cells from several HA subjects [170]. This study identified a promiscuous T-cell epitope in the FVIII C1 domain that bound to several common DRB1 receptors. One challenge in attempting to map T-cell epitopes in FVIII by this method is that many peptides are needed to span the sequence of the large FVIII protein, even if peptides corresponding to the B domain are omitted, while the amount of blood available from individual donors is a limiting factor. In order to carry out triplicate or quadruplicate assays of T-cell responses to particular peptides, either large volumes of blood are required, or else the T cells must be expanded substantially in culture before proliferation assays are conducted. Phenotypic changes may occur during expansion, and various subpopulations of T cells may expand at different rates; both of these phenomena can affect the measured proliferation of the expanded population in response to particular peptides. Therefore, physiologically relevant epitopes that stimulate effector T cells, leading to stimulation of B cells and production of plasma cells, may not be detected in assays measuring proliferation of polyclonal T cells because the responding antigen-specific T cells may constitute only a minor fraction of the cell population. In such a case, the proliferation and cytokine release of these critical early-responding T cells is masked by responses of the bulk polyclonal cells, some of which may proliferate nonspecifically under the culture conditions. The C1 domain epitope identified by this study was undoubtedly significant, but alternative approaches will be needed to circumvent the problems noted above and to identify additional clinically relevant epitopes.

Another approach to identifying T-cell epitopes and characterizing T-cell responses to FVIII is to isolate antigen-specific T-cell clones. For example, Jacquemin et al. isolated and characterized FVIII-specific T-cell clones from a mild HA subject with missense substitution R2150H [27]. All of the clones obtained from this subject responded to synthetic peptides containing R2150, and their HLA restriction was partially characterized by using lymphoblastoid cell lines of known MHC class II HLA-DR types to present the peptides. Peptide FVIII-2144-2161 also bound to multiple recombinant DR molecules, suggesting

that this region contains a promiscuous epitope. Interestingly, the corresponding peptide with the hemophilic sequence bound to the DR molecules with a similar affinity, suggesting that the distinction between self and nonself, at least with respect to this epitope, occurred at the level of T-cell recognition of the peptide-DRB1 complex. In other words, the affinities of the hemophilic and nonhemophilic peptides for the different class II receptors were similar, but the hemophilic histidine residue at position 2150 likely interfered with the binding or subsequent signaling through the T-cell receptors of the responding T cells. In contrast, the wild-type peptide with the wild-type arginine at position 2150 was presented to the same TCRs, but this peptide led to more effective engagement and activation of the T cells, leading to an anti-FVIII immune response.

MHC class II tetramers

Understanding the process of epitope recognition by class II molecules and their cognate TCRs is essential to efforts to modulate immune responses at the level of T-cell signaling. Our laboratory has utilized fluorescent MHC class II tetramers [171] as tools to investigate HLA-restricted T-cell responses in both hemophilic and nonhemophilic subjects. T-cell activation in response to a protein antigen, e.g. FVIII, involves proteolytic antigen processing, binding of a resulting peptide to an MHC class II protein on the surface of an APC, and sustained interaction of the MHC-peptide complex with a TCR on the surface of a T cell [172]. Class II-peptide-TCR signaling involves receptor clustering *in vivo*. MHC class II tetramers mimic receptor clustering, as the extracellular region of the class II-DR molecules is produced in a recombinant system, the purified DR proteins are biotinylated at a single site and incubated with peptides, and they are converted to fluorescent tetramers by the addition of PE-labeled streptavidin. Tetramers thus “loaded” with either single peptides or with pools of three to eight peptides are added to CD4⁺ cells that have been cultured for ~2 weeks with adherent APCs in the presence of FVIII peptides. T cells bearing TCRs that recognize the peptide-loaded tetramers become labeled by the fluorescent tetramers. Because the number of available CD4⁺ T cells is a limiting factor, initial screening is often carried out using pooled peptides, and then positive responses to tetramers loaded with pooled peptides are “decoded” using tetramers loaded with individual peptides that comprised the positive pool(s). This process of identifying epitopes within peptides is termed “tetramer-guided epitope mapping,” or TGEM. T cells labeled by particular tetramers may then be counted (generally counterstaining for CD4 and/or CD25) and/or single-cell sorted and expanded as antigen-specific T-cell clones.

Our first application of the TGEM methodology was to investigate FVIII-specific T-cell responses in a mild hemophilia A subject with missense substitution A2201P, who developed a high-titer inhibitor following FVIII infusions given as support during and immediately after tonsillectomy [74]. Tetramers corresponding to his DRB1*0101 phenotype were loaded with pooled peptides spanning the FVIII C2 domain sequence (tetramers corresponding to his second allele, DRB1*1503, were not available). The peptides were 20-mers that overlapped by 12 residues; the overlap was included in the design to present potential T-cell epitopes, which are generally ten to 14 residues in length in more than one binding register. The subject's T cells were stained by tetramers loaded with peptides containing the wild-type alanine at position 2201, indicating that the T-cell epitope overlapped with the missense site. His T-cell responses were interrogated using these class II tetramers periodically for over a year following initial detection of his immune response. Interestingly, tetramers loaded with a peptide containing the hemophilic proline at position 2201 stained only T cells obtained at the first time point, 3 days following detection of his inhibitor, indicating a transient auto-immune response to his endogenous hemophilic FVIII. His clotting activity (FVIII:C) at this time point was well below his usual level of 8–10% normal, consistent with a transient antibody response against both the infused wild-type FVIII and his endogenous FVIII-A2201P. Antigen-specific T cells recognizing the same peptide, FVIII-2194–2213, were single-cell sorted from samples obtained at various time points, and T-cell clones were established. The cloned lines demonstrated dose-dependent proliferation when stimulated with peptide FVIII-2194–2213, with SI values ranging from 10 to several hundred, in other words well above nonspecific background proliferation (Fig. 4). Further analysis of these types of FVIII-specific clones will yield additional insights into T-cell responses to particular epitopes. T-cell clones with strong proliferative responses will also prove useful in

experiments testing various methods to produce T-cell tolerance to FVIII.

The possible roles of regulatory T cells in modulating anti-FVIII immune responses are of strong interest, as stimulation of a regulatory T-cells is one of several possible approaches to promote tolerance to infused FVIII [173]. A major category of regulatory T cells is CD4⁺ CD25⁺ cells that utilize the transcription factor FoxP3 [174]. We wanted to know whether these types of Tregs were up-regulated during or after the initial immune response to FVIII. Accordingly, TGEM was carried out for T cells isolated from serial blood samples, assaying both the total CD4⁺ fraction and a CD25-depleted CD4⁺ fraction. Removal of the CD25-high cells depletes the culture of activated CD25⁺ cells, including CD4⁺ CD25⁺ Tregs, and this should allow antigen-specific CD4⁺ cells to proliferate in the absence of signaling from Tregs. Enhanced tetramer staining of the CD4⁺ CD25[−] fraction, compared to the total CD4⁺ fraction, was indeed seen for samples obtained 11 and 19 weeks following the initial immune response. T cells from these time points also showed increased FoxP3 expression, which was consistent with a Treg response but was not conclusive evidence for Treg involvement, as both CD25⁺ and transient FoxP3 expression are characteristic of activated T cells as well as Tregs. However, these results were provocative, and further investigation of Tregs in anti-FVIII immune responses is warranted. It is interesting to note that CD4⁺ CD25⁺ Tregs have recently been shown to suppress proliferation of T cells from healthy nonhemophilic subjects in response to FVIII [175].

The study of T-cell responses to FVIII C2 domain sequences was recently extended to include family members of the original subject, including his hemophilic brother, cousin, and grandfather, as well as two mothers who were obligate carriers of the FVIII-A2201P missense genotype [176]. The hemophilic brother had a DRB1*0101-restricted T-cell response to the same peptide as did the original subject, although he did not have a clinically detectable

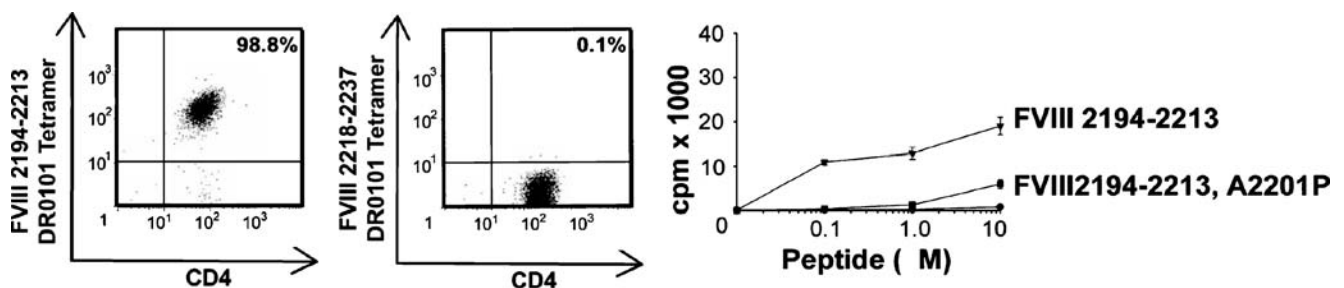


Fig. 4 *Left panel* Expanded FVIII-specific T-cell clones stain positive for CD4⁺ and for the relevant MHC class II tetramers loaded with the correct FVIII-derived peptide. *Center panel* The same DR0101 tetramer loaded with an irrelevant peptide as a control is not recognized by the same subject's T cells. *Right panel* The clone

proliferates strongly in response to the wild-type peptide (corresponding to infused FVIII) but not in response to a peptide with the hemophilic sequence (corresponding to the subject's endogenous FVIII protein)

inhibitor, while T cells from an HLA-matched control subject were not stained by tetramers carrying any of the FVIII C2-derived peptides. The carrier mothers also showed no tetramer-positive responses. T-cell responses to epitopes in the FVIII A2 domain are also under investigation [177].

Summary

The identification of specific amino acids that are essential for formation of FVIII–IgG complexes (B-cell epitopes) and for promoting high-avidity MHC class II–peptide–TCR complexes (T-cell epitopes) will help us to better understand the molecular mechanisms underlying these clinically important immune reactions. Modification of FVIII immunogenicity and/or antigenicity can be attempted as the epitope repertoires become better characterized. Development of less immunogenic versions of FVIII through epitope modification is feasible; this approach can potentially decrease but not eliminate the incidence of FVIII inhibitors. FVIII-specific CD4⁺ T cells and B cells are critical to the development of inhibitors [173]. Future experiments to better characterize their responses to specific epitopes, especially in the context of inflammatory states and immune modulators, and the presence or absence of additional “danger signals” will help us better understand and improve existing methods for immune tolerance induction to FVIII.

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