DISTRIBUTION AGREEMENT

In presenting this dissertation as a partial fulfillment of the requirement for an advanced degree from Emory University, I herby grant to Emory University and its agents the non- exclusive license to archive, make accessible, and display my dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this dissertation. I retain all ownership rights to the copyright of the dissertation. I also retain the right to use in future works (such as articles or books) all or part of this dissertation.

_____ _____

Rebecca C. Markovitz

The Complexity of the Humoral Immune Response to Factor VIII in Hemophilia A

By: Rebecca C. Markovitz

Doctor of Philosophy

Immunology and Molecular Pathogenesis

_____

John S. (Pete) Lollar, M.D.
Advisor

_____

Max Cooper, M.D.,
Committee Member

_____

Richard Cummings, Ph.D.
Committee Member

_____

Christopher Doering, Ph.D.
Committee Member

_____

Timothy Denning, Ph.D.
Committee Member

_____

Joshy Jacob, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____Date

The Complexity of the Humoral Immune Response to Factor VIII in Hemophilia A

By

Rebecca C. Markovitz

B.A., Yale University, 2007

Advisor: John S. (Pete) Lollar, III, M.D.

An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Immunology and Molecular Pathogenesis

2014

Abstract

The Complexity of the Humoral Immune Response to Factor VIII in Hemophilia A

By: Rebecca C. Markovitz

The humoral immune response is the most serious complication in the treatment of hemophilia A patients. Approximately thirty percent of patients with severe hemophilia A develop anti-factor VIII (fVIII) antibodies (Abs), termed inhibitors, with associated increases in both morbidity and mortality. The Abs most commonly target the A2 and C2 domains of fVIII. Consequently, we used a murine immunogenicity model to study the humoral immune response to the A2 domain of fVIII. This study confirmed the importance of the immunodominant epitope between 484-508. In addition, by identifying novel inhibitory mechanisms, we gained a better understanding of the pathogenicity of fVIII inhibitors.

We identified anti-A2 MAbs that inhibited cleavage at Arg372 and Arg1689, thus inhibiting thrombin's activation of fVIII cofactor function and fVIII's dissociation from von Willebrand Factor (VWF) respectively. *In vivo* studies with anti-A2 MAbs then demonstrated that the epitopes and mechanisms of inhibition of the MAbs in combination with inhibitory titer may better correlate with *in vivo* pathogenicity than inhibitory titer alone. Our collaborators then used the MAbs to compare the antigenicity of different fVIII molecules. They found that ovine fVIII (ofVIII) could be a good alternative treatment for some patients with inhibitors to both porcine and human fVIII.

In addition to reducing antigenicity, we were interested in reducing the immunogenicity of fVIII and in inducing tolerance in murine models of fVIII inhibitor formation. We hypothesized that by targeting fVIII-specific naïve and memory B cells, we could decrease or inhibit the formation of anti-fVIII Abs. We chose the toxin saporin, a type I ribosome inactivating protein (RIP), and conjugated it to fVIII using N-succinmidyl-3-(2-pyridyldithio)propionate (SPDP) a heterobifunctional cross linker. We have used both an adoptive transfer and naïve mouse model to test our hypothesis with variable results. This may be due to experimental error or due to a flaw in our hypothesis. If ultimately successful this strategy could be a novel mechanism of inhibiting anti-fVIII Ab formation as well as inducing tolerance to fVIII after inhibitors have formed. In addition, it could increase knowledge regarding mechanisms of tolerance to fVIII.

The Complexity of the Humoral Immune Response to Factor VIII in Hemophilia A

By

Rebecca C. Markovitz

B.A., Yale University, 2007

Advisor: John S. (Pete) Lollar, III, M.D.

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Immunology and Molecular Pathogenesis

2014

Acknowledgements

This work would not have been possible without the mentorship, immense efforts and friendship of numerous generous individuals.

First and foremost, my utmost gratitude to my laboratory group: my mentor, Dr. John S. (Pete) Lollar, and my co-workers, John F. Healey and Ernest T. Parker. I will be forever indebted to Dr. Pete Lollar for his dedication to my education and for the inspiring example he makes with his personal commitment to independent learning. In addition, I am deeply grateful to John Healey and Ernest Parker for their guidance, intelligence, patience and friendship. Thank you for being the best mentors imaginable.

Next, I would like to deeply thank Mr. Hanselman, my high school biology teacher, who first made me realize the true beauty of a cell.

Dr. Renhao Li for his innovative ideas, insights and patience at crucial moments during my thesis work.

Dr. Shannon Meeks for her valuable guidance and insights throughout my training.

Hunter Baldwin and Courtney Cox for their friendship, guidance and assistance throughout my thesis work.

Dr. Periasamy Selvaraj for his wisdom, innovative suggestions and kindness throughout my thesis work.

My thesis committee: Dr. Max Cooper, Dr. Richard Cummings, Dr. Chris Doering, Dr. Timothy Denning, and Dr. Joshy Jacob for collectively serving as an exceptional thesis committee. I am immensely grateful to them all for their insights, patience, and generosity of time.

The MD/PhD program at Emory University, especially Mary Horton and Dr. Kerry Ressler. They have been unfailing in their support and I am deeply grateful for their kindness and insights.

Heartfelt thanks go out to my dear friends Oloruntosin Adeyanju and Naima Farrell for their remarkable support and kindness throughout this process.

Thank you to my brother, Adam, and my sisters, Lara and Allison, for their support and love. I would like to extend special thank yous to my mother, Ruth Markovitz, for everything she has done to support me throughout my life and especially her invaluable assistance during my thesis work and to my father, David Markovitz, who gave me my first pipette and introduced me to the joy of science, a bond we still share today.

Table of Contents

Figures and Tables

Introduction

Approximately 40 years ago, a little boy, around 18 months old, was admitted to the hospital with a very high titer of anti-factor VIII (fVIII) Abs of over 500 Bethesda Units (BU/milliliter.) The toddler was bleeding into his right shoulder, arm and chest. In order to stop this patient's severe bleeding episode, Dr. Hans-Hermann Brackmann treated him with high doses of fVIII in combination with prothrombin concentrate. Dr. Brackmann's goal was to control the bleeding but, as he later said, "astonishingly" the boy's titer dropped to about 40 BU/mL during his three-week hospital course and eventually disappeared. In addition, the half-life of his fVIII normalized[1]. Today, we still use a modified version of this protocol, called immune tolerance induction (ITI), which uses multiple doses of fVIII to eliminate inhibitory anti-fVIII Abs. Although other regimens are used to eliminate anti-fVIII Abs, ITI is the only regimen whose efficacy has been proven in clinical trials[2]. Despite the clinical importance of this regimen, its mechanism of action remains unknown.

Despite the improvement in care, treatment with high-dose fVIII ITI protocols fail in about 30% of cases[2]. Moreover, this regimen is very expensive, often exceeding one million USD per patient, and onerous for patients, because it takes approximately 9 to 36 months to complete[3]. Therefore, numerous pre-clinical studies have focused on inducing tolerance to fVIII. We will discuss these pre-clinical studies and ITI therapy. Our approach to decreasing or eliminating inhibitors, using a targeted toxin that eliminates fVIII-specific naïve and memory B cells, will be introduced at the end of the review. We will discuss proposed

mechanisms of ITI. In addition, we will discuss the mechanisms of tolerance induction to fVIII used in pre-clinical studies. When successful, these studies further our understanding of the pathogenesis of inhibitors. This knowledge is critical because inhibitors are the most serious complication in the treatment of hemophilia A patients, and yet the mechanisms for their development remain unknown. In addition, we still do not know why the immune system targets fVIII more often than other clotting factors nor can we predict the patients that will develop inhibitors. Unraveling the immune response to fVIII could contribute to improved care for hemophilia A patients and also provide insights into other immune-mediated disorders.

Both responders and non-responders to ITI treatment experience significant time periods during which their disease must be managed in the presence of inhibitory Abs. Clinically, patients are mainly defined by their inhibitory titer. High titer patients are treated with bypassing agents such as recombinant factor VIIa (rfVIIa) while lower titer patients are treated with fVIII. However, some patients do not have a good hemostatic response to bypassing agents. Recent experimental results, including results described in this thesis, suggest that better characterizing anti-fVIII Abs by studying their epitopes could allow for more individually tailored therapy and better outcomes.

We discuss studies characterizing the humoral immune response to the two most immunogenic regions of fVIII, the A2 and C2 domains. We will introduce a study of the diversity of the humoral immune response to the A2 domain (Chapter I.) This study determining the epitopes, anticoagulant properties, and inhibitory

mechanisms of murine anti-human anti-A2 monoclonal antibodies (MAbs) followed a previous study of anti-C2 domain MAbs[4]. Using our library of MAbs, we were able to investigate the hypothesis that the specific epitope in combination with inhibitory titer is more important for determining pathogenicity *in vivo* than inhibitory titer alone (Chapter II.) Finally, there is considerable interest in developing new treatments for inhibitor patients with reduced antigenicity due to the considerable complications in their treatment. In chapter III, the anti-A2 MAbs were used as a tool to characterize a potential novel treatment for inhibitor patients, ovine fVIII (ofVIII.)

Diagnosis of Neutralizing Anti-FVIII Antibodies in Hemophlia A Patients

Hemophilia A is caused by a deficiency or a defect in the coagulation protein fVIII. FVIII is a ~300-kDa glycoprotein composed of the domain sequences A1-A2-B-*ap*-A3-C1 and C2. It is cleaved intracellularly at R1648 between the B and ap domains to produce an A1-A2-B/*ap*-A3-C1-C2 heterodimer. FVIII circulates in plasma bound to von Willebrand factor (VWF) and is activated proteolytically by thrombin or factor Xa (fXa). Proteolytic cleavages catalyzed by thrombin and fXa occur at R372 between the A1 and A2 domains, at R740, between the A2 and B domains, and at R1689, between the ap and A3 domains. Activated fVIII (fVIIIa) is an A1/A2/A3-C1-C2 heterotrimer that acts as a cofactor for factor IXa (fIXa) in the activation of factor X (fX) on phospholipid membrane surfaces in the intrinsic

pathway of blood coagulation. Cleavage at R372 activates the cofactor function of fVIIIa and cleavage at R1689 releases fVIIIa from VWF.

The gold standard for treatment is replacement of the missing clotting factor with recombinant or plasma-derived fVIII. The most common presentation of anti-fVIII antibodies (Abs) is a poor clinical response to fVIII infusions or a lower than expected levels of fVIII after infusions. Individuals who do not have congenital hemophilia A can also develop anti-fVIII Abs, thus breaking tolerance to their own fVIII protein and causing an autoimmune bleeding disorder called acquired hemophilia A. The most common presentation of acquired hemophilia A is spontaneous, often severe, bleeds.

Treatment of inhibitors consists of controlling bleeding, diagnosing the presence of the inhibitor and, if an inhibitor is found to be present, eradicating the inhibitor once the patient is stable. Inhibitor tests should be done after a washout period so that fVIII levels can return to baseline. FVIII *in vivo* recovery and half-life studies are the most critical and sensitive tests for inhibitors[3]. In addition, the modified Bethesda assay is used to identify and quantify inhibitors. This assay uses the activated partial thromboplastin time (APTT) to determine fVIII activity level. If an inhibitor is present in patient plasma, mixing the plasma with normal plasma prolongs the APTT and reduces fVIII activity level. A Bethesda titer is defined as the dilution of patient plasma that gives 50% residual activity of fVIII in normal plasma and is expressed in Bethesda units per milliliter (BU/mL)[5]. The Nijmegen modification of the Bethesda assay is commonly used, because during the two hour

incubation changes in pH and possible loss of fVIII due to adsorption had led to unacceptable variation in the original Bethesda assay[6].Additionally, in low titer plasmas, fVIII may neutralize the low concentrations of Ab. In these plasmas, the Nijmegen assay can be modified by heating the patient plasma for 30 minutes at 56°C in order to denature and thus inactive any residual fVIII left in the sample[7].

Clinically, patients are classified as having low or high titers and are treated accordingly. Bleeds in patients with low titer inhibitors (≤ 5 BU/mL) are usually responsive to high doses of fVIII, while bleeds in high titer patients (≥5 BU/mL) can be treated with agents that bypass the intrinsic pathway of coagulation, namely (activated prothrombin complex concentrate (aPCC) or recombinant Factor VIIa (rFVIIa). For some severe bleeds in patients with high titer inhibitors, high doses of fVIII are used in addition to bypassing agents[8]. Alternatively, recombinant porcine fVIII has been approved for use in patients in acquired hemophilia A that have anti-fVIII Abs that cross-react poorly with porcine fVIII.

Risk of Inhibitor Development

Out of all the coagulation factors, FVIII is the most commonly targeted in autoimmunity. Additionally, in the congenital hemophilias, 25-30% of severe and moderate hemophilia A patients develop inhibitors, in contrast to 1-3% of hemophilia B patients (which is due to factor IX deficiency) in response to infusion of the missing clotting factor[9]. Some suggest that this may be due to the higher prevalence of high risk, non-missense mutations in hemophilia A as opposed to hemophilia B patients. However, even when taking into account the prevalence of

high-risk mutations in hemophilia A vs. B patients, the immune response against fVIII appears to be more severe, leading to a higher rate of inhibitor formation. The type of mutation, along with severity of disease, race, clinical factors such as number of exposure days to treatment product, intensity of exposure, age of first exposure, and circumstance of exposure e.g., prophylaxis vs. surgery, all have been identified as possible risk factors for developing inhibitors[10, 11]. The type of causative mutation in fVIII has been identified as an important risk factor with large deletions, nonsense mutations, and inversions leading to the highest rate of inhibitor formation. These mutations are predicted, and in some cases have been shown, to completely stop the synthesis of the fVIII protein[12]. In contrast, missense or small deletions can lead to the synthesis of non-functional fVIII. The hypothesis is that in the first case central tolerance cannot occur while in the second it can. In the thymus, autoreactive T cells are deleted if they react with self-proteins. Thus, if fVIII is not expressed, central tolerance cannot occur. In contrast, if non-functional fVIII is produced with missense or small deletions, fVIII-specific autoreactive T cells can still be deleted while in the thymus as long as fVIII is present.

Recent studies suggest that, in addition to causative mutations, other genetic factors may be risk factors for the development of inhibitors. Black and Hispanic patients are approximately twice as likely to develop inhibitors as White patients[13]. Some have proposed that these groups are more likely to develop inhibitors due to a higher prevalence of high-risk mutations. The most recent survey of hemophilia patients in the United States does not support this hypothesis, because Blacks and Hispanics with the same mutations as Whites had significantly

higher rates of inhibitors[14]. This is consistent with a previous study that found the spectrum of fVIII mutations to be similar between ethnic groups[10, 15]. This suggests that Blacks and Hispanics have a genetic predisposition to inhibitor formation. These findings suggest a possible role of immune modulators in the risk of developing inhibitors.

Astermerk et al. used the Malmo International Brother Study (MIBS) to analyze genetic polymorphisms of interleukin (IL)-1β, IL-4 and IL-10 genes and *F8* gene mutations in 78 unrelated families. These three cytokines have been identified as playing a role in antibody production in autoimmunity. Of the three, only a polymorphism of IL10 in the promoter region of the gene showed a strong association with inhibitor development[11, 14, 16-19]. Also analyzing the MIBS study, a protective effect was found between a SNP in the promoter region of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and inhibitor production. Polymorphisms in CTLA-4, IL-10, tumor necrosis factor α (TNF-α) and the role of human leukocyte antigen (HLA) class II molecules in inhibitor formation were also examined in a case-controlled cohort study using mutation-matched patients. The results confirmed the previous studies that polymorphisms in IL-1O and TNF-α increase risk of inhibitor formation[20]. Additionally, this study found a higher frequency of DRB1*15 and DQB1*0602 alleles in patients who develop inhibitors, suggesting a role, although weakly predictive, for HLA in inhibitor formation. These studies suggest that inhibitor formation is a polygenic process influenced by immune mediators such as IL-10, TNF-α, CTLA-4.

Inhibitor development is a $CD4^+$ T cell dependent process involving B cells, T cells, and antigen presenting cells (APCs)[21-23]. The MHC II molecules present fVIII to the T cells, and with a co-stimulatory signal the T cells can then activate B cells. CTLA-4 is a receptor on T cells that competes and down regulates the binding CD28 on T cells with their binding to CD80/86 on APCs and in the process down regulates activation of T cells. There are numerous steps to anti-fVIII Ab formation and many of these steps may be associated with inhibitor development. Despite incremental progress in the field, we cannot yet predict the patients who will develop anti-fVIII Abs[24].

How the Immune System Sees fVIII

Why 25-30% of severe congenital hemophilia A patients develop inhibitors is unclear. In addition, when hemophilia A mice are injected intravenously with adjuvant –free human fVIII at amounts that mimic the dosing for patients they produce greater than 350 μg/ml after four injections[21]. Because the formation of these anti-fVIII Abs is a $Cd4^+$ T cell dependent process, co-stimulatory signals are necessary to generate an immune response[21]. Therefore, what is producing the co-stimulatory signal for these mice? The immune response to fVIII can be temporarily inhibited when co-stimulatory signals between B7-1 and B7-2 and CD28 are blocked using CTLA4-Ig[23] or using a MAb against CD40L to antagonize the interaction of CD40 with CD40L[22, 25, 26]. Hemophilia A mice used for experiments are healthy and do not have chronic joint disease or evidence of occult bleeding post-mortem that would be an activating signal to an APC. Giving fVIII

intravenously to congenital hemophilia A patients introduces a foreign protein to their immune systems. In patients with several hemophilia A, the frequency of fVIII treatments is on average 3 to 14 days throughout their lifetime. It is thought that one job of the immune system is to distinguish between self and non-self, with non-self proteins evoking an immune response if introduced with an adjuvant[27-29]. However, no clear adjuvant has been established. Thus, the question remains unanswered: what mechanism lies behind the immune response to fVIII?

Three potential characteristics of fVIII have been tested as potential drivers of co-stimulatory signaling. Our group and others have addressed whether the (1) procoagulant activity or (2) intrinsic structural elements in fVIII or (3) VWF, fVIII's carrier protein, could be the cause of its immunogenicity. The role of VWF has been addressed in pre-clinical experiments. Meeks et al. compared the immune response to fVIII in $fVIII^{-/-}$/ $VWF^{-/-}$ and $fVIII^{-/-}$ mice[30]. The data suggested that VWF increased the immunogenicity of fVIII, because higher doses of fVIII were needed to generate the same level of anti-fVIII Abs in the $fVIII^{-/-}$/ $VWF^{-/-}$ mice. One caveat to these experiments is that fVIII is cleared more rapidly in the absence of VWF. Therefore the immune systems of the $fVIII^{-/-}$/ $VWF^{-/-}$ mice may have had less time after each injection to generate a response to the fVIII making it an uneven comparison. In the same paper, an inactive mutant fVIII molecule that cannot be released from VWF, R372A/R1689A, was found to be less immunogenic than wild-type fVIII, suggesting a protective role for VWF, although the difference only trended towards statistical significance. Meeks et al. concluded that the immune response to

fVIII is both positively and negatively affected by its association with VWF[30]. Dasgupta et al. reported a mechanism for the protective role of VWF, demonstrating VWF's ability to inhibit the endocytosis of fVIII by human peripheral blood-derived dendritic cells (DCs) (19). This study had potential flaws, such as the supraphysiological concentrations of fVIII and VWF used in the work. More studies are needed to determine the role of VWF in the immunogenicity of fVIII.

Rather than focusing on VWF, Skupsky et al. tested the hypothesis that fVIII's unusual immunogenicity stems from its involvement in the coagulation cascade. They and others have hypothesized that, during coagulation, pro-inflammatory factors could be released and act as an adjuvant for inhibitor production. They propose that the bidirectional relationship between coagulation and inflammation[31] creates many possible adjuvants for inhibitor formation. Specifically, they hypothesized that the immunogenicity of fVIII is linked to thrombin production during the coagulation cascade. They compared the immunogenicity of OVA, fVIII, and a heat inactivated fVIII in order to test whether fVIII's immunogenic properties were linked to its structure or its procoagulant function. They found that the heat-inactivated fVIII had a reduced immunogenicity. This, along with their findings that warfarin, an anti-coagulant, and hirudin, a direct thrombin inhibitor, decreased the immune response to fVIII, led them to conclude that the immune response to fVIII is linked to its procoagulant function. Nonetheless, they demonstrated destruction of numerous B cell epitopes in their heat-inactivated fVIII, thus affecting structure as well as function. This would

warrant further studies to parse out structure vs. function in the immunogenicity of fVIII[32].

Meeks et al. addressed both this question and also the possible role of VWF in the immunogenicity of fVIII using two conformationally intact, inactive mutant fVIII proteins. The first mutant, V634M fVIII, has a single point mutation in the A2 domain that leads to a loss of procoagulant function. The second mutant, R372A/R1689A, also lacks procoagulant function due to a loss of the necessary recognition sites for thrombin and fXa cleavage. In contrast to V634M fVIII, it cannot be released by its carrier protein VWF, because it cannot be cleaved by thrombin. Thus, R372A/R1689A cannot localize to procoagulant sites while V634M fVIII should be able to localize but not participate in the clot. In contrast to Skupsky et al., Meeks et al. found that despite the difference in their procoagulant function, wild type fVIII and V634M fVIII, which has less than 1% the specific procoagulant activity of wild type fVIII, were equally immunogenic. Meeks et al. concluded that the immunogenicity of fVIII was independent from its procoagulant role[30].

Meeks et al. pointed out that the B cell epitope loss that Skupsky demonstrated could be affecting the overall structure and immunogenicity of fVIII. Unlike T cells that recognize and bind short peptides, B cells recognize intact native antigens. Thus, changes in the conformation of fVIII due to heating could easily cause changes to both antigenicity and immunogenicity due to changes in B cell receptor binding, and downstream signaling. In addition, the specific epitopes that were lost with heating have been previously implicated in antigen presentation[32].

Based on these studies, it appears that the immunogenicity of fVIII is not due to its procoagulant function.

In a recent study Gangadharan et al. similarly concluded that the immunogenicity of fVIII is not linked to thrombin generation nor, more generally, to its procoagulant function. As did Meeks et al., they compared the immunogenicity of V634M fVIII to a wild-type fVIII. In addition, they looked at the possible role of tissue factor (TF) as an initiator of the immune response to fVIII. They found no change in the immunogenicity of fVIII after inhibiting coagulation by neutralizing TF. This addressed the concern that the presence of TF in hemophilia A mice could lead to coagulation from the extrinsic pathway in the mice given V634M fVIII in previous experiments.

In addition, Gangadharan et al. inhibited coagulation using warfarin in an experiment similar to one performed by Skupsky, et al. The two groups had opposite results. Gangadharan found no significant reduction in titers after the treatment while Skupsky et al. did find a significant reduction in titers. Skupsky et al. used an enzyme-linked immunosorbent assay (ELISA) that measured all anti-fVIII Abs while Gangadharan used a chromogenic assay that quantified inhibitory titers[33]. Overall, Gangadharan's results further strengthen the argument that fVIII's immunogenicity is not derived from its procoagulant function. These studies do not address the possible effect of a pro-inflammatory milieu, independent from coagulation, on the immunogenicity of fVIII. This should be addressed in studies

designed to compare the immunogenicity of fVIII when given in pro-inflammatory or anti-inflammatory environments.

The Diversity of the Immune Response to FVIII

The majority of severe hemophilia A patients who develop inhibitors have Abs directed against the A2 and/or C2 domains of fVIII[34-38]. Congenital hemophilia A inhibitor patients are more likely to develop Abs against both the A2 and C2 domains of fVIII, whereas autoimmune hemophilia A patients are more likely to have either an A2 or a C2 Ab[39]. Interestingly, non-neutralizing anti-fVIII Abs have been found in the plasma of healthy individuals. The prevalence of non-neutralizing anti-fVIII Abs can be as high as 19% in this healthy population and approximately 34% in non-inhibitor hemophilia A patients[40]. The majority of anti-fVIII Abs studies have focused on inhibitory Abs, but the possibility has not been eliminated that other MAbs could be pathogenic by increasing the clearance rate of fVIII or the formation of immune complexes and activation of inflammatory pathways.

Our group undertook a study of the polyclonal humoral immune response to human fVIII in hemophilia A mice. Anti- fVIII Abs produced by hybridomas from the spleens of hemophilia A mice exposed to human fVIII were characterized, with approximately 300 hybridomas produced per spleen. Consistent with results seen in patients, the majority of these MAbs were directed against the A2 and C2 regions of fVIII. Although previous studies had characterized a few individual anti-fVIII MAbs, this study presented a comprehensive anaylsis[41].

From these hybridomas, a panel of 56 anti-C2 MAbs were created and characterized. The MAbs had 18 distinct epitopes and were split into five groups based on their overlap patterns as determined by ELISA. The anticoagulant properties of each MAb were determined, as well as each one's ability to block fVIII binding to VWF and/or phospholipid. The activation of fVIII by thrombin results in the formation of the heterotrimer, A1/A2/A3-C1-C2. This is caused by the release of the B domain and light chain peptide, and cleavage between the A1 and A2 domains[42]. Following its release from VWF, fVIIIa associates with phospholipid membranes. The C2 domain is known to play a role in fVIII binding to VWF and phospholipid, making this a potential inhibitory mechanism for anti-C2 MAbs.

Previous studies identified multiple anti-C2 MAbs that inhibit fVIII's binding to phospholipid membranes[43] and VWF[44, 45] and one Ab was found that appears to slow cleaved fVIII's dissociation from VWF[46]. Using the large panel of anti-C2 MAbs obtained from the hybridomas, multiple additional MAbs were identified that block fVIII's ability to bind phospholipid and/or VWF. In addition to these "classical" anti-C2 MAbs, a novel group of non-classical MAbs was identified that appear to inhibit activation of fVIII by thrombin or fXa. After defining MAb groups by epitope, it became apparent that a relationship exists between the epitopes of the MAbs and their inhibitory mechanism, a structure-function relationship. Validating this immunogenicity model, Meeks et al. found that the majority of patients with anti-C2 inhibitors have both classical and non-classical inhibitors [4].

Patients are most likely to develop a humoral response against the A2 and/or C2 domains of fVIII[39]; this is consistent with the results from our group's study of the humoral immune response of fVIII. Therefore, we undertook to study the diversity of the immune response to the A2 domain of fVIII (Chapter I). The A2 domain has been implicated in binding fX to the intrinsic pathway fX activation complex (intrinsic fXase complex). A murine anti-A2 MAb, MAb 413, has been investigated by our group and others, and was shown to compete with human plasmas for binding to fVIII. MAb 413 binds to an immunodominant A2 epitope bounded by R484 and I508 (4). It interferes with fVIIIa binding to fX and is a noncompetitive inhibitor of the intrinsic fXase complex (6). Two other anti-A2 MAbs, CLB-CAg 9 and GMA-012, were characterized by other groups, and were included in our panel of MAbs. In Chapter I we examine the humoral immune response to the A2 domain of fVIII. We created and characterized 29 monoclonal anti-human fVIII Abs. We first identified their epitopes using competition ELISA and homolog scanning mutagenesis. We then studied their inhibitory properties with the Bethesda bioassay. Finally, we studied their inhibitory mechanisms. We found novel inhibitory mechanisms and identified an important role for VWF in the inhibitory mechanism of anti-A2 MAbs. We identified eight MAbs overlapping the epitope of the noncompetitive inhibitor of the Xase complex, MAb 413, with all but one having a high specific inhibitory activity. Also, we found anti-A2 MAbs that inhibit proteolytic cleavage at R372 and a separate group of MAbs that inhibit cleavage at R1689, representing two novel mechanisms of inhibition. Finally, we determined that there is a relationship between the mechanisms of inhibition,

epitopes, and kinetics of inhibition of anti-A2 MAbs.

Based on this work and earlier work by our group[47], we hypothesized that the *in vivo* pathogenicity would more directly correlate with epitope and the anticoagulant properties of the anti-A2 MAbs than with inhibitory titer alone. In Chapter II we used a murine tail snip model to examine the pathogenicity of anti-A2 MAbs. The result of this study suggests that inhibitory titers are not sufficient information with which to characterize anti-A2 MAbs.

In Chapter III, the characterization of the antigenicity of the A2 domain of recombinant ovine fVIII (rofVIII) is described. Similar to recombinant porcine fVIII, Zakas et al. hypothesized that ofVIII could be used therapeutically in fVIII inhibitor patients because it retains its procoagulant function in human plasma while displaying a reduction in antigenicity due to differences in the amino acid sequences of human and ovine fVIII[48].

Immune Tolerance Therapy

The current standard of care calls for inducing tolerance to fVIII, thus decreasing the inhibitor level to undetectable levels. ITI is the only proven regimen for achieving antigen-specific tolerance[2, 49], but numerous other therapies such as steroids and cytoxan[50], rituximab[51, 52], and IVIG[53] are also used clinically. ITI consists of giving high doses of fVIII until normalization of fVIII pharmacokinetics. Several different regimens of ITI are used. The three most commonly used are the so-called Bonn, the van Creveld, and the Malmö protocols. Although the protocols have varying dosing and scheduling, they have similar

success rates[54]. The International ITI study, compared results from treating "good-risk" severe high-titer hemophilia A patients with a high dose of fVIII (200 IU/kg/d) versus giving "good-risk" patients a low dose (50 IU/kg 3 times/week.) The overall success rate was 69.7%, consistent with previous studies[55].

ITI is a very expensive and lengthy process with quite varied duration. In the International ITI Study, patients were included whose therapy lasted from nine to 33 months, demonstrating the broad range of treatment schedules for this regimen[2]. ITI costs approximately one million dollars per patient[3], but this figure can vary significantly depending upon the length of treatment. Cost can be a barrier to treatment in the United States and often proves prohibitive in less affluent countries.

In addition, despite considerable investigation, the mechanism behind ITI is not known. ITI developed as a result of Dr. Brackmann's fortuitous observation that giving high doses of fVIII could reduce inhibitor levels, but true understanding of this treatment has remained elusive. Considering its long duration, failure rate of 30%, and expense, improvements are necessary.

Numerous studies have looked at potential predictors of successful outcomes for ITI therapy. The recent International ITI Study has been particularly influential due to its study design as a randomized, multicenter prospective study, in contrast to earlier small retrospective cohort studies. Despite ending the study early due to bleeding concerns and feasibility the study revealed that titer while on ITI and historical peak titer were inversely correlated with time to normal recovery of fVIII and a negative titer[55]. Previous studies have identified pre-ITI titer as being

an important indicator of success[55, 56]. The predictive value of pre-ITI titer has been recognized as so critical that ITI is not initiated until a patient's titer has fallen below 10 BU/mL. Therefore, it is important to quickly diagnose inhibitors before the titer rises above 10 BU/mL.

Debate continues over whether the type of fVIII product used for ITI affects its outcome. This idea that VWF might have a protective effect became popular when Kreuz et al. reported a large decline in the success of ITI using the Bonn protocol with plasma when a recombinant VWF-free fVIII product was substituted for a VWF-containing plasma-derived fVIII product [57]. A retrospective study comparing recombinant fVIII products with plasma-derived fVIII products (containing VWF) found that the adjusted relative risk for inhibitor development with recombinant fVIII vs. plasma-derived fVIII was 2.4[58]. This difference is surprising because recombinant fVIII quickly associates with VWF once it is infused. Thus, it appears likely that the immune system sees fVIII-VWF complexes whether recombinant fVIII or pfVIII is used. In contrast, two studies found that the type of product used had no effect on outcome of ITI[55, 59]. One possible explanation for a variable effect of VWF- containing product is that the epitopes of the Abs contained in the patient plasma are influencing outcome. Potentially, VWF and an Ab with overlapping binding sites for fVIII could compete for binding to fVIII, thus influencing the outcome of ITI[57]. Finally, another negative predictor for ITI is infection of the central venous access device [60].

Possible Mechanisms of ITI and Alternative Mechanisms of Tolerance

Central venous catheter infections are likely to activate inflammatory pathways downstream from Toll-like receptors (TLRs) that could interfere with the onset of tolerance. There is debate regarding the cell populations targeted by ITI therapy. It has been proposed that memory B cells are targeted and deleted during ITI therapy. Allacher et al. used an *in vitro* assay to study the effect of fVIII stimulation on memory B cells[61]. They found that depending upon the concentration, fVIII could either stimulate or inhibit memory B cell differentiation into plasma cells. This mimics the clinical setting, in which fVIII is immunogenic at low concentration and tolerogenic at the higher concentrations used in ITI. The authors used a range of concentrations of fVIII and a range of TLR agonists. They found that TLR 7 and 9 agonists were most effective at stimulating differentiation of memory B cells into plasma cells. TLR7 recognizes single stranded RNA, a common feature of viral genomes, while TLR9 recognizes unmethylated CpG sequences in DNA commonly found in bacteria. Both agonists amplified restimulation of memory B cells at low concentrations of fVIII and countered the inhibition caused by high concentrations of fVIII. [54, 61].

Because the humoral immune response to fVIII is a $CD4^+$ T cell dependent response, APCs, $CD4^+$ helper T cells, and B cells could all potentially be involved in changing an activating signal to an inhibitory or anergic signal[21-23]. The B cell receptor allows memory B cells to bind fVIII with high affinity. Because antibody-secreting plasma cells do not express B cell receptors, antigen-based

immunotherapy targeting memory B cells may be the most direct way to decrease the production of anti-fVIII plasma cells.

Moreover, as the immune response progresses, memory B cells become crucial APCs due to their high affinity for antigen[62]. Therefore, targeting memory B cells could decrease APCs, decreasing primed $CD4^+$ T cells and ultimately feeding back to decrease fVIII-specific memory B cells. Another potential target of ITI is the $CD4^+$ T cell. Depending on the environment in which antigen is presented T cells can become effector cells or T regulatory cells (Tregs.)

Tregs developed in the thymus or periphery are called natural and adaptive Tregs, respectively[63]. Tregs in the thymus are thought to develop as an alternative pathway to T cell deletion when T cells. *In vitro* work shows that adaptive Tregs develop after cross-linking their TCRs while exposing them to transforming growth factor β (TGF-β), while *in vivo* adaptive Tregs develop after infusing low doses of soluble antigen[64].

It has been suggested that Tregs play a crucial role in the mechanism underlying ITI, pointing out that Tregs develop when low doses of soluble antigen are infused, a regimen with similarities to ITI. These experimental systems do not closely mimic ITI because they are done using naïve mice, where fewer "danger" signals are present[54, 65, 66]. In contrast, ITI therapy occurs in an already primed immune system in which T cells and B cells have already differentiated into effector cells. Therefore, examining the role of Tregs in a primed fVIII specific system would be most useful for elucidating the mechanism of ITI.

.

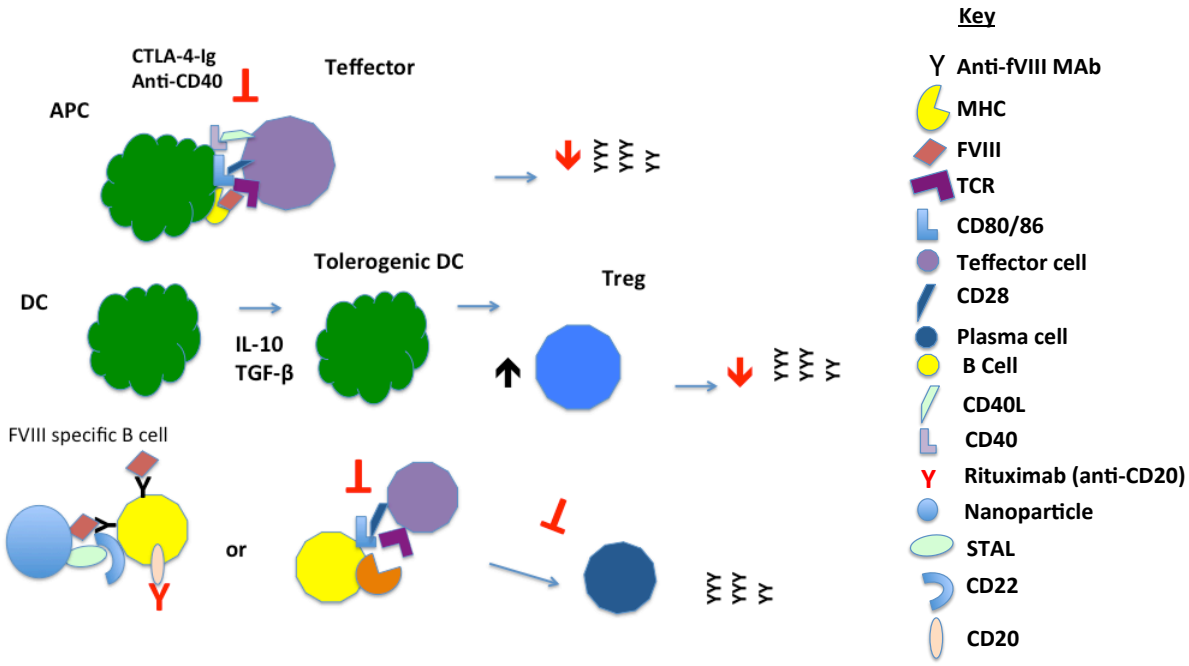


Figure 1. Approaches used for tolerance induction to fVIII

Groups have focused on decreasing antigen presentation of fVIII using CTLA4-Ig, anti-CD40 or tolerogenic DCs[67-69]. Others have focused on decreasing fVIII-specific B cells due to their role as APCs and as precursors to fVIII-specific plasma cells. They have used rituximab or a nanoparticle containing both fVIII and the ligand for CD22, an inhibitory receptor[70].

Studies in mice have examined the role of Tregs in the immune response to fVIII. Oral tolerance studies have shown that oral delivery of fVIII can inhibit the formation of anti-fVIII Abs. In a recent paper, Sherman et al. created tobacco transplastomic lines expressing the heavy and C2 domains of fVIII and fed the heavy

chain/C2 mixture to hemophilia A mice[71]. They found that this mixture inhibited the formation of anti-fVIII Abs and decreased the inhibitor titer in preimmune mice by 3 to 7 fold after 2 to 3 months of feeding. Adoptive transfer studies demonstrated that the inhibition of anti-fVIII Ab formation induced by the oral tolerance regimen transferred most effectively with $CD4^+CD25^+LAP^+$ Treg cells[71]. These Tregs express high levels of TGF-β and less is known about them than the more thoroughly characterized $CD4^+CD25^+Foxp3^+$ Tregs.

Research from before the work of Sherman et al. suggested that oral tolerance could offer a feasible approach to reducing inhibitor titers. Rawle et al. administered the C2 domain of fVIII orally and intranasally and they were able to decrease or inhibit the anti-fVIII antibody formation with those two protocols, respectively[72]. After intranasal injection, adoptive transfer experiments were performed using isolated $CD4^+$ T cells that suggested Tregs were involved in inducing tolerance through mucosal factor C2-fVIII administration. Hemophilia A recipient mice had significantly reduced inhibitory titers after receiving the $CD4^+$ T cells from hemophilia A treated mice compared to $CD4^+$ T cells from hemophilia A control mice. Nonetheless, after tolerance was induced using the intranasal protocol, the mice were challenged with one dose of fVIII and tolerance was broken. This highlights the question of how to define tolerance. For example, Sherman et al. showed very promising results, but they continued their tolerance protocol by feeding the mice fVIII throughout their experiments. In future studies, challenging their mice after ceasing to feed them fVIII could determine whether lasting tolerance had been achieved.

Some groups have focused more directly on generating Tregs either by *in vivo* Treg expansion therapy or in donors for adoptive transfer experiments[73]. In one study, $Foxp3^+$ Tregs were adoptively transferred into naïve hemophilia A mice and, similar to the studies described above, the transferred Tregs reduced inhibitor levels in recipient mice. Another group expanded Tregs in hemophilia A mice by creating immune complexes with IL-2 and a specific anti-IL-2 MAb, JES-6-1A12, a combination that had been previously shown to expand $CD4^+CD25^+$ Tregs[74]. This treatment led to a short-lived expansion of Tregs and inhibition of the formation of anti-fVIII Abs. One caveat to this approach is that T cells are phenotypically labile *in vivo*[75]. Therefore, determining a regimen that will induce expansion of Tregs and maintain them must be determined.

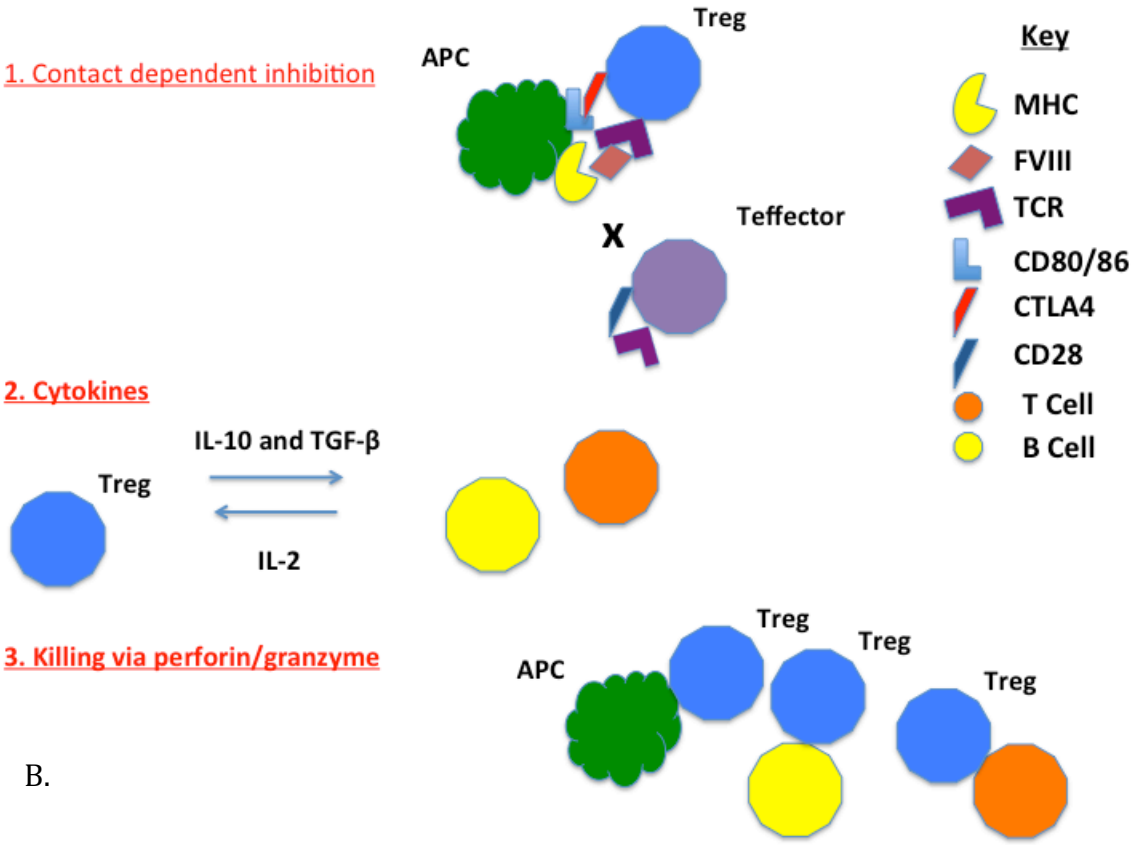
Rapamycin, a drug that blocks IL-2 signaling in T cells, has been used to induce tolerance to fVIII. Rapamycin's ability to decrease inhibitor formation is part of an evolving story about the role of IL-2 in inflammation and autoimmunity. IL-2's involvement in the differentiation of $CD4^+$ and $CD8^+$ T cells was previously the main focus of investigations, but recent studies suggest that it also plays a major role in the development and expansion of Tregs[76].

In a study in hemophilia A mice, intravenous administration of fVIII was combined with oral delivery of rapamycin. This regimen inhibited the formation of anti-fVIII Abs during subsequent weekly intravenous injections of fVIII [77]. This combination of fVIII and rapamycin led to the formation of $CD4^+CD25^+Foxp3^+$ Treg cells which, when adoptively transferred, suppressed antibody formation. However, in another study with hemophilia A mice, when fVIII was co-administered into mice

along with rapamycin and another immunosuppressive agent, mycophenylate mofetil (MMF), neutralizing anti-fVIII antibody production was delayed but not inhibited[78]. The authors tried other combinations of immunosuppressive drugs, the most effective being an anti-CD40 ligand MAb, MR1, with CTLA-4-Ig. Both of these drugs inhibit co-stimulatory pathways between APCs and effector T cells. Treating mice with this combination inhibited the production of anti-fVIII Abs. In addition, the response appeared to be specific to fVIII, because the mice had normal primary and secondary responses to another antigen, the bacteriophage Φx174[78].

Using transposon-based gene deliver fVIII has also been co-expressed with indoleamine 2,3 dioxygenase, a tryptophan degrading enzyme downstream of CTLA-4-Ig. This has led to long-term expression of fVIII and a decrease in inhibitor formation consistent with the previous finding that one of CTLA-4-Ig's mechanisms of action is induction of indoleamine 2,3 dioxygenase expression[79]. Co-stimulatory signals were also targeted in a study using a MAb against inducible co-stimulatory molecule (ICOS) that inhibits its binding to ICOS-ligand (ICOS-L.) Neutralizing anti-fVIII Ab formation were inhibited while $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs were upregulated[80]. These studies induced tolerance by inhibiting co-stimulatory signals thus modifying the "second signal" that T cells receive from APCs to differentiate into effector T cells. A similar approach is to create tolerogenic APCs and use these cells to send a modified "second signal."

A.



B.

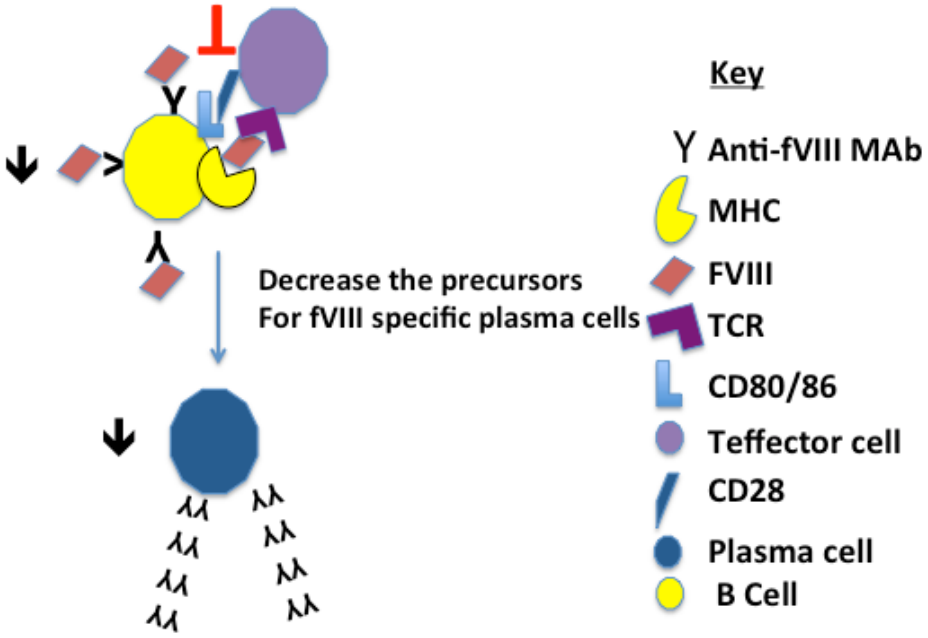


Figure 2. Possible Mechanisms of ITI

A) ITI therapy may be creating $CD4^+$ Tregs that down regulate the inflammatory response to fVIII. The key mechanisms of inhibition of Tregs are thought to be cytokine-mediated by both release of inhibitory cytokines such as IL-10 and TGF-β and soaking up IL-2. This is mediated by a high affinity receptor thus leading to apoptosis of near-by effector T cells. In addition, Tregs act by cytolysis mediated by both granzyme A and B and perforin dependent killing and by targeting DCs e.g., CTLA4 and CD80/86 interactions leading to immunosuppression[64].
B) FVIII-specific naïve and memory B cells are another potential target of ITI therapy. By down-regulating these cells, ITI would decrease both the precursors of fVIII-specific plasma cells and crucial APCs

In one of these studies tolerogenic DCs, tDCs, expressing low levels of co-stimulatory molecules and anti-inflammatory cytokines, were modified to express human fVIII. Hemophilia A mice pre-treated with the tDCs showed a decreased humoral immune response to fVIII and an increased Treg population. Consistent with other Treg tolerance studies, when $CD4^+$ T cells from hemophilia A mice treated with tolerogenic DCs were adoptively transferred, they inhibited the formation of anti-fVIII titers for up to four fVIII injections[67]. In addition, when DCs were pulsed with antigen and treated with IL-10 and TGF-β they inhibited the formation of anti-fVIII Abs in recipient hemophilia A mice[68, 69].

Reducing fVIII's presentation to APCs has also been approached through

T cell epitope modification. One recent paper, building on numerous previous studies from the same group, used a method called deFT, “de-immunization for Functional Therapeutics”, employing bioinformatics to select and de-immunize immunodominant epitopes in the C2 domain. They hypothesized that with a decrease in T cell epitope binding to MHCII molecules, there would be a decrease in the immunogenicity of fVIII. Their method led to a decrease in both the antigenicity and immunogenicity of fVIII[81].

Our group has modified B cell epitopes in order to create a less immunogenic fVIII molecule that retained its procoagulant function. Alanine modifications were made in a singe immunodominant loop in the A2 domain that led to a significant decrease in the immunogenicity of fVIII[82]. As more B cell epitopes are characterized more candidate amino acids for mutagenesis may become evident.

In addition to work on decreasing B cell epitopes, work is also focusing on targeting B cells, particularly memory B cells. The ability of these cells to differentiate into plasma cells makes them targets for tolerance therapy. Studies with isotype-specific anti-CD20 MAbs targeting precursor, naïve, and memory B cells have had variable success. An IgG1 specific MAb stopped an increase in the inhibitor titer in pre-immune hemophilia A mice, while the IgG2 specific MAb succeeded in temporarily halting inhibitor formation, but eventually inhibitor levels did rise[83, 84]. Rituximab, a monoclonal MAb composed of human IgG1 and κ constant regions linked to a murine anti-human CD20 variable region, has been used with patients with inhibitors refractory to ITI. Individual cases have reported

success with this regimen[51, 52, 68] but there have also been failures[52]. To date it has not been tested in a large prospective study[85]. As previously mentioned patients are usually treated with rituximab when they have failed ITI. One hypothesis regarding the failure of ITI is an inability to target long-lived plasma cells. If fVIII-specific long-lived plasma cells are responsible for a subset of patients failing ITI rituximab would likely not help them as its target is not on plasma cells. Based on the current practice of inhibitor treatment it is hard to assess what rate of success rituximab would have in the general population of hemophilia A patients with inhibitors.

Another research group that targeted B cells did successfully induce tolerance, employing a novel method involving liposomal nanoparticles. The nanoparticles expressed fVIII and glycan ligands for the inhibitory co-receptor CD22, and induced apoptosis of mouse and human B cells, leading to tolerance. Referred to as STALs (SIGLEC-engaging tolerance inducing antigenic liposomes), they induced antigen-specific tolerance that was maintained even after challenge with fVIII[70].

In our work described in the Appendix, we targeted fVIII-specific naïve and memory B cells using fVIII conjugated to a toxin as a novel tolerance regimen. We conjugated fVIII to saporin using Sulfo-LC-SPDP, a heterobifunctional crosslinker that links amines to sulfhydryls, producing a disulfide bond. Targeted toxins are routinely used for cancer therapies[86], but they have not been exploited for immune and autoimmune diseases. Although numerous alternatives to ITI are being developed, this B cell targeting drug could be an important addition. We predict,

consistent with results published in 2013 by Macauley et al., that targeting B cells will lead to tolerance [70]. Using a targeted toxin for B cell depletion has potential practical merit, because immuntoxins have been widely used in experimental cancer treatments in both preclinical and clinical trials over the past thirty years.

The concept of an immunotoxin was first described by Paul Ehrlich as part of his "magic bullet theory." His groundbreaking theories began to solidify in 1897 and he won his noble prize in 1908 for work that has been the foundation for cancer therapy today[87]. In 1970, Moolten and Cooperband demonstrated that diphtheria toxin could be conjugated to an antibody and could deplete cells in an antigen-specific manner[88]. A phase I study published in 1996 by E.S. Vitetta's group, documented treating patients with B cell lymphomas using the infusion of a deglycosylated ricin A chain immunotoxin linked to CD19 by a disulfide bond[89].

For our toxin, we chose saporin, a type I RIP. Saporin is a very stable protein, being resistant to high temperatures, denaturation by urea and guanidine, or attack by proteolytic enzymes. Saporin, like other type I RIPs, lacks a lectin B chain and has been presumed to enter cells with low efficiency unless given an independent mode of entry[90]. Nonetheless, it has been proposed that there is a receptor for saporin itself, based on the finding that some cell types and organs are more sensitive than others to saporin intoxication. Recent work suggests that the low-density lipoprotein receptor-related protein (LRP) could be the receptor for saporin[91]. Our results (shown in the Appendix) suggest that saporin may be entering cells non-specifically or through a receptor. More studies are needed to determine whether or

not saporin can enter cells without an independent mode of entry and if this may be influencing our experiments.

Saporin causes cell death by depurinating the 28S RNA of the large 60S subunit of the ribosome. This leads to the inability of the ribosome to bind elongation factor 2 (ef2), thus inhibiting protein synthesis. Saporin may also cause apoptosis by depurinating DNA and other nucleic acids. Signs of apoptosis have been observed, e.g., chromatin fragmentation, apoptotic bodies and hypodiploid cells, in lymphocytes and cancer cell lines after treatment with saporin[37,38]. In mice the LD_{50} of saporin is calculated to be 4 to 8 mg/kg suggesting a wide therapeutic window[92].

Saporin has been used in numerous pre-clinical and clinical studies with very limited side effects. More success has been found at targeting hematological rather than solid tumors[92]. Hematological malignancies are often more accessible to immunotoxins *in vivo*, because surface molecules on the hematological target cells are better characterized and, once the immunotoxin has bound to their surface molecules, it is often endocytosed and kills the target cell. Saporin immunotoxins have been used to target T cell lymphomas, T cell leukemias, B cell leukemias and B cell lymphomas in a mix of *in vitro* and *in vivo* studies. Overall, these studies demonstrate saporin immunotoxins' ability to eliminate target cancer cells while sparing host cells[92].

Saporin has also been tested in a number of clinical trials. Saporin immunotoxins targeting CD22 have been used to treat B cell lymphoma patients and

non-Hodgkin's lymphoma patients. In these trials, the immuntoxins rapidly decreased the size of the target tumors. In addition, only grade I toxicity effects were reported e.g., mild fevers, myalgias, and weakness all directly after treatment. A few of the patients did develop an anti-mouse Ab response, a key challenge to immunotoxin therapy[92].

A saporin immunotoxin targeting CD30 was used to treat Hodgkin's disease (HD), leading to partial remission in approximately half of the patients and full remission in two of 12 patients. These patients had advanced disease. The immunotoxin had originally been tested in refractory advanced HD with worse results, suggesting that the burden of disease is an important factor in outcome. Thus, these studies suggest that saporin has good potential as a targeted toxin in a system where the burden of target cells is potentially less than in advanced cancers. In addition, the toxicity has been low in these pre-clinical and clinical trials[92]. Finally, the development of anti-mouse Abs has been addressed for immunotoxins made with alternative toxins to saporin by modifying proteins to creating less immunogenic immunotoxins. For example, an immunotoxin was made with *Pseudomonas* exotoxin (PE) that originally induced an Ab response. Next, seven mutations were made that reduced its immunogenicity by 90% but did not affect its killing ability[93]. We could follow similar strategies if anti-drug Abs were to develop during fVIII-saporin therapy.

In the work presented below, we further characterized the humoral immune response to fVIII. We studied the diversity of anti-A2 Abs, their epitopes, and their

mechanisms of inhibition. When looking at the various approaches to decreasing the immunogenicity of fVIII, one showing promise involves modifying B cell epitopes, specifically in the A2 domain. This approach could be more fully realized once we have gathered more information about B cell epitopes, such as those presented below. Additionally, we tried to decrease the immune response to fVIII more directly by using a targeted toxin. By linking fVIII to a type one RIP, we aimed to combine the specificity and targeting potential of fVIII with the potent killing ability of saporin. We aimed to decrease antigen presentation and to reduce the precursors to plasma cells by targeting fVIII-specific naïve and memory B cells with our targeted toxin.

References

1. Brackmann HH, Oldenburg J, Schwaab R. Immune tolerance for the treatment of factor VIII inhibitors--twenty years' 'bonn protocol'. *Vox Sang* 1996,**70 Suppl 1**:30-35.
2. Hay CR, DiMichele DM, International Immune Tolerance S. The principal results of the International Immune Tolerance Study: a randomized dose comparison. *Blood* 2012,**119**:1335-1344.
3. Colowick AB, Bohn RL, Avorn J, Ewenstein BM. Immune tolerance induction in hemophilia patients with inhibitors: costly can be cheaper. *Blood* 2000,**96**:1698-1702.
4. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Antihuman factor VIII C2 domain antibodies in hemophilia A mice recognize a functionally complex continuous spectrum of epitopes dominated by inhibitors of factor VIII activation. *Blood* 2007,**110**:4234-4242.
5. Kasper CK, Aledort L, Aronson D, Counts R, Edson JR, van Eys J*, et al.* Proceedings: A more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975,**34**:612.
6. Giles AR, Verbruggen B, Rivard GE, Teitel J, Walker I. A detailed comparison of the performance of the standard versus the Nijmegen modification of the Bethesda assay in detecting factor VIII:C inhibitors in the haemophilia A population of Canada. Association of Hemophilia Centre Directors of Canada. Factor VIII/IX Subcommittee of Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis. *Thromb Haemost* 1998,**79**:872-875.
7. Miller CH, Platt SJ, Rice AS, Kelly F, Soucie JM, Hemophilia Inhibitor Research Study I. Validation of Nijmegen-Bethesda assay modifications to allow inhibitor measurement during replacement therapy and facilitate inhibitor surveillance. *J Thromb Haemost* 2012,**10**:1055-1061.
8. Lanzkowsky P. *Manual of pediatric hematology and oncology*. 2nd ed. New York: Churchill Livingstone; 1995.
9. Lusher JM. Inhibitor antibodies to factor VIII and factor IX: management. *Semin Thromb Hemost* 2000,**26**:179-188.
10. Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factors VIII and IX. *Haemophilia* 2006,**12 Suppl 6**:15-22.
11. Ragni MV, Ojeifo O, Feng J, Yan J, Hill KA, Sommer SS*, et al.* Risk factors for inhibitor formation in haemophilia: a prevalent case-control study. *Haemophilia* 2009,**15**:1074-1082.
12. Schwaab R, Brackmann HH, Meyer C, Seehafer J, Kirchgesser M, Haack A*, et al.* Haemophilia A: mutation type determines risk of inhibitor formation. *Thromb Haemost* 1995,**74**:1402-1406.

13. Astermark J, Berntorp E, White GC, Kroner BL, Group MS. The Malmo International Brother Study (MIBS): further support for genetic predisposition to inhibitor development in hemophilia patients. *Haemophilia* 2001,**7**:267-272.
14. Miller CH, Benson J, Ellingsen D, Driggers J, Payne A, Kelly FM*, et al.* F8 and F9 mutations in US haemophilia patients: correlation with history of inhibitor and race/ethnicity. *Haemophilia* 2012,**18**:375-382.
15. de Alencar JB, Macedo LC, de Barros MF, Rodrigues C, Cadide RC, Sell AM*, et al.* Importance of immune response genes in hemophilia A. *Rev Bras Hematol Hemoter* 2013,**35**:280-286.
16. Astermark J, Oldenburg J, Pavlova A, Berntorp E, Lefvert AK, Group MS. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood* 2006,**107**:3167-3172.
17. Santagostino E, Mancuso ME, Rocino A, Mancuso G, Mazzucconi MG, Tagliaferri A*, et al.* Environmental risk factors for inhibitor development in children with haemophilia A: a case-control study. *Br J Haematol* 2005,**130**:422-427.
18. Gouw SC, van der Bom JG, Marijke van den Berg H. Treatment-related risk factors of inhibitor development in previously untreated patients with hemophilia A: the CANAL cohort study. *Blood* 2007,**109**:4648-4654.
19. d'Oiron R, Volot F, Reynaud J, Peerlinck K, Goudemand J, Guerois C*, et al.* Impact of choice of treatment for bleeding episodes on inhibitor outcome in patients with mild/moderate hemophilia a and inhibitors. *Semin Hematol* 2006,**43**:S3-9.
20. Pavlova A, Delev D, Lacroix-Desmazes S, Schwaab R, Mende M, Fimmers R*, et al.* Impact of polymorphisms of the major histocompatibility complex class II, interleukin-10, tumor necrosis factor-alpha and cytotoxic T-lymphocyte antigen-4 genes on inhibitor development in severe hemophilia A. *J Thromb Haemost* 2009,**7**:2006-2015.
21. Qian J, Borovok M, Bi L, Kazazian HH, Jr., Hoyer LW. Inhibitor antibody development and T cell response to human factor VIII in murine hemophilia A. *Thromb Haemost* 1999,**81**:240-244.
22. Reipert BM, Sasgary M, Ahmad RU, Auer W, Turecek PL, Schwarz HP. Blockade of CD40/CD40 ligand interactions prevents induction of factor VIII inhibitors in hemophilic mice but does not induce lasting immune tolerance. *Thromb Haemost* 2001,**86**:1345-1352.
23. Qian J, Collins M, Sharpe AH, Hoyer LW. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. *Blood* 2000,**95**:1324-1329.
24. Astermark J, Wang X, Oldenburg J, Berntorp E, Lefvert AK, Group MS. Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. *J Thromb Haemost* 2007,**5**:263-265.
25. Rossi G, Sarkar J, Scandella D. Long-term induction of immune tolerance after blockade of CD40-CD40L interaction in a mouse model of hemophilia A. *Blood* 2001,**97**:2750-2757.

26. Qian J, Burkly LC, Smith EP, Ferrant JL, Hoyer LW, Scott DW*, et al.* Role of CD154 in the secondary immune response: the reduction of pre-existing splenic germinal centers and anti-factor VIII inhibitor titer. *Eur J Immunol* 2000,**30**:2548-2554.
27. Burnet FM. A modification of Jerne's theory of antibody production using the concept of clonal selection. *CA Cancer J Clin* 1976,**26**:119-121.
28. Bretscher P, Cohn M. A theory of self-nonself discrimination. *Science* 1970,**169**:1042-1049.
29. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994,**12**:991-1045.
30. Meeks SL, Cox CL, Healey JF, Parker ET, Doshi BS, Gangadharan B*, et al.* A major determinant of the immunogenicity of factor VIII in a murine model is independent of its procoagulant function. *Blood* 2012,**120**:2512-2520.
31. Levi M, van der Poll T, Buller HR. Bidirectional relation between inflammation and coagulation. *Circulation* 2004,**109**:2698-2704.
32. Skupsky J, Zhang AH, Su Y, Scott DW. A role for thrombin in the initiation of the immune response to therapeutic factor VIII. *Blood* 2009,**114**:4741-4748.
33. Gangadharan B, Delignat S, Ollivier V, Gupta N, Mackman N, Kaveri SV*, et al.* Role of coagulation-associated processes on FVIII immunogenicity in a mouse model of severe hemophilia A. *J Thromb Haemost* 2014.
34. Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T*, et al.* Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet* 1992,**339**:594-598.
35. Barrow RT, Healey JF, Gailani D, Scandella D, Lollar P. Reduction of the antigenicity of factor VIII toward complex inhibitory antibody plasmas using multiply-substituted hybrid human/porcine factor VIII molecules. *Blood* 2000,**95**:564-568.
36. Barrow RT, Healey JF, Jacquemin MG, Saint-Remy JM, Lollar P. Antigenicity of putative phospholipid membrane-binding residues in factor VIII. *Blood* 2001,**97**:169-174.
37. Healey JF, Barrow RT, Tamim HM, Lubin IM, Shima M, Scandella D*, et al.* Residues Glu2181-Val2243 contain a major determinant of the inhibitory epitope in the C2 domain of human factor VIII. *Blood* 1998,**92**:3701-3709.
38. Healey JF, Lubin IM, Nakai H, Saenko EL, Hoyer LW, Scandella D*, et al.* Residues 484-508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. *J Biol Chem* 1995,**270**:14505-14509.
39. Prescott R, Nakai H, Saenko EL, Scharrer I, Nilsson IM, Humphries JE*, et al.* The inhibitor antibody response is more complex in hemophilia A patients than in most nonhemophiliacs with factor VIII autoantibodies. Recombinate and Kogenate Study Groups. *Blood* 1997,**89**:3663-3671.
40. Whelan SF, Hofbauer CJ, Horling FM, Allacher P, Wolfsegger MJ, Oldenburg J*, et al.* Distinct characteristics of antibody responses against factor VIII in healthy individuals and in different cohorts of hemophilia A patients. *Blood* 2013,**121**:1039-1048.

41. Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. The humoral response to human factor VIII in hemophilia A mice. *J Thromb Haemost* 2007,**5**:512-519.
42. Lollar P, Parker CG. Subunit structure of thrombin-activated porcine factor VIII. *Biochemistry* 1989,**28**:666-674.
43. Arai M, Scandella D, Hoyer LW. Molecular basis of factor VIII inhibition by human antibodies. Antibodies that bind to the factor VIII light chain prevent the interaction of factor VIII with phospholipid. *J Clin Invest* 1989,**83**:1978-1984.
44. Shima M, Scandella D, Yoshioka A, Nakai H, Tanaka I, Kamisue S*, et al.* A factor VIII neutralizing monoclonal antibody and a human inhibitor alloantibody recognizing epitopes in the C2 domain inhibit factor VIII binding to von Willebrand factor and to phosphatidylserine. *Thromb Haemost* 1993,**69**:240-246.
45. Shima M, Nakai H, Scandella D, Tanaka I, Sawamoto Y, Kamisue S*, et al.* Common inhibitory effects of human anti-C2 domain inhibitor alloantibodies on factor VIII binding to von Willebrand factor. *Br J Haematol* 1995,**91**:714-721.
46. Saenko EL, Shima M, Gilbert GE, Scandella D. Slowed release of thrombin-cleaved factor VIII from von Willebrand factor by a monoclonal and a human antibody is a novel mechanism for factor VIII inhibition. *J Biol Chem* 1996,**271**:27424-27431.
47. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Non-classical anti-factor VIII C2 domain antibodies are pathogenic in a murine in vivo bleeding model. *J Thromb Haemost* 2009,**7**:658-664.
48. Zakas PM, Gangadharan B, Almeida-Porada G, Porada CD, Spencer HT, Doering CB. Development and characterization of recombinant ovine coagulation factor VIII. *PLoS One* 2012,**7**:e49481.
49. Di Michele DM. Immune tolerance induction in haemophilia: evidence and the way forward. *J Thromb Haemost* 2011,**9 Suppl 1**:216-225.
50. Zeitler H, Ulrich-Merzenich G, Hess L, Konsek E, Unkrig C, Walger P*, et al.* Treatment of acquired hemophilia by the Bonn-Malmo Protocol: documentation of an in vivo immunomodulating concept. *Blood* 2005,**105**:2287-2293.
51. Stasi R, Brunetti M, Stipa E, Amadori S. Selective B-cell depletion with rituximab for the treatment of patients with acquired hemophilia. *Blood* 2004,**103**:4424-4428.
52. Fox RA, Neufeld EJ, Bennett CM. Rituximab for adolescents with haemophilia and high titre inhibitors. *Haemophilia* 2006,**12**:218-222.
53. Kubisz P, Plamenova I, Holly P, Stasko J. Successful immune tolerance induction consisting of high-dose factor VIII rich in von Willebrand factor and pulsed intravenous immunoglobulin: a case report. *J Med Case Rep* 2012,**6**:350.
54. Reipert BM, van Helden PM, Schwarz HP, Hausl C. Mechanisms of action of immune tolerance induction against factor VIII in patients with congenital haemophilia A and factor VIII inhibitors. *Br J Haematol* 2007,**136**:12-25.

55. DiMichele DM, Kroner BL, North American Immune Tolerance Study G. The North American Immune Tolerance Registry: practices, outcomes, outcome predictors. *Thromb Haemost* 2002,**87**:52-57.
56. Lenk H, Group ITTS. The German Registry of immune tolerance treatment in hemophilia--1999 update. *Haematologica* 2000,**85**:45-47.
57. Di Minno G, Coppola A. A role for von Willebrand factor in immune tolerance induction in patients with haemophilia A and inhibitors? *Blood Transfus* 2011,**9 Suppl 2**:s14-20.
58. Goudemand J, Rothschild C, Demiguel V, Vinciguerrat C, Lambert T, Chambost H*, et al.* Influence of the type of factor VIII concentrate on the incidence of factor VIII inhibitors in previously untreated patients with severe hemophilia A. *Blood* 2006,**107**:46-51.
59. Coppola A, Margaglione M, Santagostino E, Rocino A, Grandone E, Mannucci PM*, et al.* Factor VIII gene (F8) mutations as predictors of outcome in immune tolerance induction of hemophilia A patients with high-responding inhibitors. *J Thromb Haemost* 2009,**7**:1809-1815.
60. Oldenburg J, Schwaab R, Brackmann HH. Induction of immune tolerance in haemophilia A inhibitor patients by the 'Bonn Protocol': predictive parameter for therapy duration and outcome. *Vox Sang* 1999,**77 Suppl 1**:49-54.
61. Allacher P, Baumgartner CK, Pordes AG, Ahmad RU, Schwarz HP, Reipert BM. Stimulation and inhibition of FVIII-specific memory B-cell responses by CpG-B (ODN 1826), a ligand for Toll-like receptor 9. *Blood* 2011,**117**:259-267.
62. Rodriguez-Pinto D. B cells as antigen presenting cells. *Cell Immunol* 2005,**238**:67-75.
63. Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol Rev* 2006,**212**:60-73.
64. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008,**8**:523-532.
65. Apostolou I, von Boehmer H. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 2004,**199**:1401-1408.
66. Chen TC, Cobbold SP, Fairchild PJ, Waldmann H. Generation of anergic and regulatory T cells following prolonged exposure to a harmless antigen. *J Immunol* 2004,**172**:5900-5907.
67. Su RJ, Epp A, Feng J, Roy J, Latchman Y, Wu X*, et al.* Suppression of the immune response to FVIII in hemophilia A mice by transgene modified tolerogenic dendritic cells. *Mol Ther* 2011,**19**:1896-1904.
68. Qadura M, Othman M, Waters B, Chegeni R, Walker K, Labelle A*, et al.* Reduction of the immune response to factor VIII mediated through tolerogenic factor VIII presentation by immature dendritic cells. *J Thromb Haemost* 2008,**6**:2095-2104.
69. Sule G, Suzuki M, Guse K, Cela R, Rodgers JR, Lee B. Cytokine-conditioned dendritic cells induce humoral tolerance to protein therapy in mice. *Hum Gene Ther* 2012,**23**:769-780.

70. Macauley MS, Pfrengle F, Rademacher C, Nycholat CM, Gale AJ, von Drygalski A*, et al.* Antigenic liposomes displaying CD22 ligands induce antigen-specific B cell apoptosis. *J Clin Invest* 2013,**123**:3074-3083.
71. Sherman A, Su J, Lin S, Wang X, Herzog RW, Daniell H. Suppression of inhibitor formation against FVIII in a murine model of hemophilia A by oral delivery of antigens bioencapsulated in plant cells. *Blood* 2014,**124**:1659-1668.
72. Rawle FE, Pratt KP, Labelle A, Weiner HL, Hough C, Lillicrap D. Induction of partial immune tolerance to factor VIII through prior mucosal exposure to the factor VIII C2 domain. *J Thromb Haemost* 2006,**4**:2172-2179.
73. Miao CH, Harmeling BR, Ziegler SF, Yen BC, Torgerson T, Chen L*, et al.* CD4+FOXP3+ regulatory T cells confer long-term regulation of factor VIII-specific immune responses in plasmid-mediated gene therapy-treated hemophilia mice. *Blood* 2009,**114**:4034-4044.
74. Liu CL, Ye P, Yen BC, Miao CH. In vivo expansion of regulatory T cells with IL-2/IL-2 mAb complexes prevents anti-factor VIII immune responses in hemophilia A mice treated with factor VIII plasmid-mediated gene therapy. *Mol Ther* 2011,**19**:1511-1520.
75. Sharma MD, Huang L, Choi JH, Lee EJ, Wilson JM, Lemos H*, et al.* An inherently bifunctional subset of Foxp3+ T helper cells is controlled by the transcription factor eos. *Immunity* 2013,**38**:998-1012.
76. Nelson BH. IL-2, regulatory T cells, and tolerance. *J Immunol* 2004,**172**:3983-3988.
77. Moghimi B, Sack BK, Nayak S, Markusic DM, Mah CS, Herzog RW. Induction of tolerance to factor VIII by transient co-administration with rapamycin. *J Thromb Haemost* 2011,**9**:1524-1533.
78. Miao CH, Ye P, Thompson AR, Rawlings DJ, Ochs HD. Immunomodulation of transgene responses following naked DNA transfer of human factor VIII into hemophilia A mice. *Blood* 2006,**108**:19-27.
79. Liu L, Liu H, Mah C, Fletcher BS. Indoleamine 2,3-dioxygenase attenuates inhibitor development in gene-therapy-treated hemophilia A mice. *Gene Ther* 2009,**16**:724-733.
80. Peng B, Ye P, Blazar BR, Freeman GJ, Rawlings DJ, Ochs HD*, et al.* Transient blockade of the inducible costimulator pathway generates long-term tolerance to factor VIII after nonviral gene transfer into hemophilia A mice. *Blood* 2008,**112**:1662-1672.
81. Moise L, Song C, Martin WD, Tassone R, De Groot AS, Scott DW. Effect of HLA DR epitope de-immunization of Factor VIII in vitro and in vivo. *Clin Immunol* 2012,**142**:320-331.
82. Parker ET, Healey JF, Barrow RT, Craddock HN, Lollar P. Reduction of the inhibitory antibody response to human factor VIII in hemophilia A mice by mutagenesis of the A2 domain B-cell epitope. *Blood* 2004,**104**:704-710.
83. Zhang AH, Skupsky J, Scott DW. Effect of B-cell depletion using anti-CD20 therapy on inhibitory antibody formation to human FVIII in hemophilia A mice. *Blood* 2011,**117**:2223-2226.

84. Sack BK, Merchant S, Markusic DM, Nathwani AC, Davidoff AM, Byrne BJ*, et al.* Transient B cell depletion or improved transgene expression by codon optimization promote tolerance to factor VIII in gene therapy. *PLoS One* 2012,**7**:e37671.
85. DeFrates SR, McDonagh KT, Adams VR. The reversal of inhibitors in congenital hemophilia. *Pharmacotherapy* 2013,**33**:157-164.
86. Pastan I, Hassan R, Fitzgerald DJ, Kreitman RJ. Immunotoxin therapy of cancer. *Nat Rev Cancer* 2006,**6**:559-565.
87. Strebhardt K, Ullrich A. Paul Ehrlich's magic bullet concept: 100 years of progress. *Nat Rev Cancer* 2008,**8**:473-480.
88. Moolten FL, Cooperband SR. Selective destruction of target cells by diphtheria toxin conjugated to antibody directed against antigens on the cells. *Science* 1970,**169**:68-70.
89. Tsai LK, Pop LM, Liu X, Vitetta ES. A comparison of the anti-tumor effects of a chimeric versus murine anti-CD19 immunotoxins on human B cell lymphoma and Pre-B acute lymphoblastic leukemia cell lines. *Toxins (Basel)* 2011,**3**:409-419.
90. Bergamaschi G, Perfetti V, Tonon L, Novella A, Lucotti C, Danova M*, et al.* Saporin, a ribosome-inactivating protein used to prepare immunotoxins, induces cell death via apoptosis. *Br J Haematol* 1996,**93**:789-794.
91. Fabbrini MS, Rappocciolo E, Carpani D, Solinas M, Valsasina B, Breme U*, et al.* Characterization of a saporin isoform with lower ribosome-inhibiting activity. *Biochem J* 1997,**322 (Pt 3)**:719-727.
92. Polito L, Bortolotti M, Pedrazzi M, Bolognesi A. Immunotoxins and other conjugates containing saporin-s6 for cancer therapy. *Toxins (Basel)* 2011,**3**:697-720.
93. Oh S, Stish BJ, Sachdev D, Chen H, Dudek AZ, Vallera DA. A novel reduced immunogenicity bispecific targeted toxin simultaneously recognizing human epidermal growth factor and interleukin-4 receptors in a mouse model of metastatic breast carcinoma. *Clin Cancer Res* 2009,**15**:6137-6147.
94. Lollar P, Parker ET, Curtis JE, Helgerson SL, Hoyer LW, Scott ME*, et al.* Inhibition of human factor VIIIa by anti-A2 subunit antibodies. *J Clin Invest* 1994,**93**:2497-2504.

The Diversity Of The Immune Response To The A2 Domain Of Human Factor VIII

Short title: The immune response to the A2 domain of factor VIII

Rebecca C. Markovitz[1], John F. Healey[1], Ernest T. Parker[1], Shannon L. Meeks[1], and Pete Lollar[1]

[1]Aflac Cancer and Blood Disorders Center, Department of Pediatrics, Emory University, Atlanta, GA.

Corresponding author: Pete Lollar, M.D., Emory Children's Center, Room 426D, 2015 Uppergate Drive, Atlanta, GA 30322; e-mail: jlollar@emory.edu

Abstract

Approximately 30% of patients with severe hemophilia A develop inhibitory anti-factor VIII (fVIII) antibodies. We characterized 29 anti-human A2 monoclonal antibodies (MAbs) produced in a murine hemophilia A model. A basis set of non-overlapping MAbs was defined by competition ELISA, producing 5 major groups. The overlapping epitopes covered nearly the entire A2 surface when mapped by homolog scanning mutagenesis. Most Group A MAbs recognized a previously described epitope bounded by Arg484-Ile508 in the N-terminal A2 subdomain, resulting in binding to activated fVIII and noncompetitive inhibition of the intrinsic fXase complex. Group B and C MAbs displayed little or no inhibitory activity. Group D and E MAbs recognized epitopes in the C-terminal A2 subdomain. A subset of Group D MAbs inhibited the activation of fVIII by interfering with thrombin-catalyzed cleavage at Arg372 at the A1-A2 domain junction. Other Group D MAbs displayed indeterminate or no inhibitory activity despite inhibiting cleavage at Arg740 at the A2-B domain junction. Group E MAbs inhibited fVIII light chain cleavage at Arg1689. Inhibition of cleavages at Arg372 and Arg1689 represent novel mechanisms of inhibitor function and along with the extensive epitope spectrum identified in this study reveal hitherto unrecognized complexity in the immune response to fVIII.

Introduction

The standard of care for patients with congenital hemophilia A is the infusion of recombinant or plasma-derived factor VIII (fVIII). Approximately 30% of patients with severe hemophilla A develop detectable inhibitory anti-fVIII antibodies (inhibitors) in response to these infusions[1-3], which is the most serious complication in the treatment of hemophilia A. In addition, non-hemophiliacs can break tolerance to their native fVIII protein. This results in acquired hemophilia A, which can produce life- or limb-threatening bleeding.

FVIII is a glycoprotein that contains an A1-A2-B-*ap*-A3-C1-C2 domain sequence. The A domains are homologous to corresponding triplicated domains in factor V and ceruloplasmin. Each A domain contains two ~20 kDa cupredoxin-like subdomains.[4] Intracellular cleavages within the B domain of fVIII or at the A2-B junction prior to its secretion produce A1-A2-B or A1-A2 heavy chain species and an *ap*-A3-C1-C2 light chain. FVIII circulates predominantly as an A1-A2-B/*ap*-A3-C1-C2 heterodimer tightly bound to von Willebrand Factor (VWF). FVIII is activated by thrombin-catalyzed heavy chain cleavages at Arg372 at the A1-A2 junction and Arg740 at the A2-B junction and by light chain cleavage at Arg1689 at the *ap*-A3 junction, which results in dissociation of fVIIIa from VWF.[5] The product is a 160-kDa A1/A2/A3-C1-C2 fVIIIa heterotrimer. FVIIIa is a cofactor for factor IXa in the activation of factor X in the intrinsic pathway of blood coagulation. The A2 subunit participates in the

binding of factor X to the intrinsic fXase complex.[6] Additionally, it has been reported that fIXa interacts with the A2 subunit.{Fay, 1994 1908 /id} Following thrombin activation, the A2 domain spontaneously dissociates resulting in loss of cofactor activity.[8]

FVIII inhibitors in congenital and acquired hemophilia A are polyclonal IgG populations that typically recognize both the A2 and C2 domains.[9] Scandella et al. identified an immunodominant A2 epitope using a murine monoclonal antibody (MAb), designated 413.[10] 413 competes with human inhibitor plasmas for binding to fVIII, indicating that the Arg484-Ile508 epitope is clinically relevant. The 413 epitope was mapped to a linear segment bounded by residues Arg484-Ile508.[11]413 interferes with factor X binding and is a noncompetitive inhibitor of the intrinsic fXase complex.[6] We have produced a large panel of murine anti-human fVIII hybridomas by immunizing mice with human fVIII under conditions that mimic therapeutic use of fVIII. [12] Anti-A2 MAbs were identified that that do not overlap the 413 epitope. In this study, 29 murine anti-human A2 MAbs were characterized to develop a comprehensive epitope map and to identify additional inhibitory properties of A2 inhibitors.

Materials and Methods

Materials

MAb 413 was a gift from the American Red Cross. CLB-CAg 9 was a gift from Dr. Jan Voorberg, Sanquin-AMC Landsteiner Laboratory, Amsterdam, the Netherlands. GMA-012 (originally described as R8B12[13]) was purchased from Green Mountain Antibodies. Enzyme-linked immunosorbent assay (ELISA) plates were purchased from Thermo Fisher Scientific. Pooled citrated normal plasma and fVIII-deficient plasma were purchased from George King Biomedical. Phosphatidylcholine/phosphatidylserine (PCPS) (75/25, w/w) vesicles were prepared as described previously.[14] Human B domain-deleted (BDD) fVIII and BDD human/porcine hybrid constructs were prepared as described previously.[11, 15] Recombinant full-length human fVIII (Helixate) was a gift from Hemophilia of Georgia. Human VWF was purified and characterized as described previously.[5] Molar VWF concentrations are reported in terms of the 265-kDa monomeric subunit.

Anti-human fVIII A2 domain MAbs

Anti-human fVIII A2 domain IgG MAbs were purified from supernatants of splenic B cell hybridomas that were produced following intravenous immunization of mice with adjuvant-free human fVIII as described previously.[12] IgG concentrations were calculated

using an extinction coefficient at 280 nm of 1.37 $(mg/mL)^{-1}\,cm^{-1}$. IgG isotypes and subclasses were determined by ELISA using alkaline phosphatase isotype and sub-class specific Abs from SouthernBiotech as previously described.[12]

Identification of overlapping and non-overlapping A2 epitopes by competition ELISA

A competition sandwich ELISA was performed using immobilized anti-fVIII A2 domain primary MAb, human fVIII, biotinylated anti-A2 secondary MAb, and streptavidin-alkaline phosphatase conjugate for detection as previously described.[12] In this assay, non-overlapping epitopes of a MAb pair results in binding of secondary MAb, which was defined as an absorbance at 405 nm greater than three times background.

Epitope mapping by homolog scanning mutagenesis

Hybrid human/porcine constructs (1 U/ml) were captured on microtiter wells using an immobilized anti-C2 domain MAb, I14. Biotinylated anti-A2 test MAbs were added to a final concentration of 1μg/ml and incubated for 30 minutes at room temperature. Bound MAb was detected using streptavidin-alkaline phosphatase conjugate as described previously.[12] Pymol was used to study the X-ray structure of BDD fVIII (PDB 2R7E) and identify the epitope containing regions of the A2 domain of fVIII.

FVIII inhibitor assay

FVIII inhibitor titers were measured using the Bethesda assay[16] using previously described modifications.[17] Pooled normal human plasma was used as the source of fVIII activity. One Bethesda unit (BU) per mL is defined as the dilution of inhibitor that produces 50% inhibition of fVIII activity. Inhibition curves were fitted by nonlinear least-squares analysis using the 4-parameter logistic equation to estimate the concentration of MAb producing 50% inhibition.

Intrinsic fXase assay

BDD human fVIII (50 nM) was incubated with increasing concentrations of anti-A2 MAbs for 30 min at 37°C. Human thrombin (10 nM) was added and samples were removed 30 s later and placed into SDS-PAGE sample buffer. One minute after addition of thrombin, the sample was diluted 100-fold into a solution containing 1.5 nM factor IXa/20 μM PCPS phospholipid vesicles. Factor X (300 nM) was added 15 s later, followed by removal of aliquots 15, 30, 45 and 60 s thereafter and addition to 0.05 M EDTA to stop factor X activation. Factor Xa activity was measured chromogenically using Spectrozyme Xa as described previously.[14]

Effect of anti-A2 MAbs on thrombin catalyzed proteolytic cleavage of fVIII

Full-length or BDD fVIII (100nM) were incubated for 10 minutes at 37°C with varying concentrations of MAb. In some experiments, fVIII was incubated with VWF (1000 nM) for 10 minutes at 37 °C before addition of the antibodies. Thrombin (0.5 nM) was added and the incubation was continued for an additional 10 minutes. The reaction was stopped with the addition of 10 µl non-reducing SDS-PAGE Sample Buffer (Pierce Biotechnology) followed by heating at 98°C for 5 minutes. Samples were analyzed by 10% SDS-PAGE (Ready Gel, Bio-Rad Life Science) and were stained overnight with GelCode Blue (Pierce). Gels were destained with water and imaged with an Odyssey Imaging System (Licor Biosciences).

Results

The diversity of epitopes recognized by anti-human A2 MAbs

A competition ELISA was used to determine the overlap pattern of 29 murine anti-human A2 MAbs. Full-length fVIII was bound to an immobilized primary (capture) anti-A2 MAb, followed by addition of biotinylated secondary anti-A2 MAb and detection of the secondary MAb using streptavidin alkaline phosphatase. Binding of the secondary MAb indicates that the Ab pair recognizes non-overlapping epitopes.

Each MAb was used in both primary and secondary configurations producing an overlap matrix (Figure 1A). Non-competing and competing MAbs are depicted as color (yellow) or non-color (white) elements, respectively. As expected, the diagonal elements are white, indicating that each MAb competed with itself for binding. The matrix is symmetrical with respect to the diagonal element with the exception of biotinylated G6 and G4 and non-biotinylated B99 and G74, which displayed no binding. Lack of binding of G6 and G4 may have resulted from biotinylation of the critical residues in the Ab paratope. Conversely, lack of binding of B99 and G74 may have resulted from insufficient immobilization of the Ab to the microtiter plate. In total, the competition matrix produced 16 unique epitopes.

The results of this analysis yielded a basis set of 5 MAbs, 4A4, 2-93, G48, 2-54 and 1D4. The basis set is defined as a set of MAbs that do not compete with each other for binding to fVIII but as a group compete with all of the remaining MAbs. The members of the basis set in turn defined five Ab groups, designated A, B, C, D and E. Group A consists of 4A4 and MAbs that compete only with 4A4. Group A MAbs represented the largest group, consisting of MAbs derived from 5 different mice, and include the previously characterized MAb 413.[10, 11] MAbs 2-76, G32, I62, I155, I160, and G119 displayed an overlap pattern identical to 413. Group A MAb 2-105 differs because it overlaps G6 while 413 does not. Group B consists of 2-93 and MAbs that compete only with 2-93. Group C consists of G48 and MAbs that only compete with G48. Group D consists of 2-54 and MAbs that compete only with 2-54. Group E consists of 1D4 and MAbs that compete only with 1D4. Three additional groups were identified based on partial overlaps with the basis set MAbs. Group AB consists of MAbs that compete with 4A4 and 2-93. Group BCD consists of MAbs that compete with 2-93, G48 and 2-54 and Group DE MAbs compete with 2-54 and 1D4. The individual group members are identified as a row header in Figure 1A and in Table 1. By comparing all pairwise overlaps and non-overlaps, a Venn diagram consisting of 16 unique epitopes was produced (Figure 1B).

Epitope mapping by homolog scanning mutagenesis

Most murine anti-human fVIII MAbs cross-react poorly with porcine fVIII.[12] Thus, loss of antigenicity of hybrid human/porcine fVIII constructs created by replacement of human A2 regions with homologous regions of porcine fVIII (Figure 2A) can be used to map regions that contain major epitope determinants.[18] Because human/porcine fVIII molecules have normal procoagulant activity, this method has the advantage of using conformationally intact fVIII molecules, avoiding the possibility of artifactual loss of antigenicity due to protein misfolding. Using this approach, an epitope-containing region was found for 24 of

the 29 MAbs (Table 1). ELISA results from representative MAbs from Groups A through E are shown in Figure 2B and results for the remaining MAbs set are shown in Supplemental Figure S1. For Group A MAb 413, HP9 was the construct that contained the smallest porcine substitution associated with loss of antigenicity. This confirms Arg484-Ile508 as an epitope recognized by this MAb, consistent with earlier results.[11] For Group B MAb 2-93, loss of antigenicity associated with HP2 (porcine substitution at Ala387-Glu604), but not HP4 (porcine substitution at Ala387-Arg541) placed a major determinant of the epitope between residues Arg541-Glu604. For Group C MAb G48, loss of antigenicity associated with HP5 (porcine substitution at Ala387-Ile508), but not HP6 (porcine substitution at Ala387-His444) placed a major determinant of the epitope between residues Glu445-Ile508. For Group D and E MAbs, loss of antigenicity associated with HP1 (porcine substitution at Ala387-Arg740), but not HP2 (porcine substitution Ala387-Glu604) or other HP constructs place a major determinant of the b and epitope between residues Asp605 and Arg740 in the C-terminal cupredoxin-like A2 subdomain. The amino acid sequences that contribute to antibody binding identified by homolog scanning mutagenesis corresponded closely to the overlap groups. For example, 8 of the 9 Group A MAbs mapped to epitopes that include the Arg484-Ile508 segment. The exception was Group A MAb 4A4, which mapped to residues Asp403-His444 yet competed with all other group A MAbs in the competition ELISA. Regions of Asp403-His444 and Arg484-Ile508 are in close proximity in the X-ray structure of fVIII (Figure 3A). In homolog scanning mutagenesis amino acids that constitute major determinants of a MAb's epitope cannot be identified if they are identical in the porcine and human sequences. Therefore, our overlap data suggests that 4A4 and MAbs that map to Arg484-Ile508 recognize part of a shared discontinuous epitope.

Mapping the anti-A2 MAb Venn diagram onto the fVIII surface

Low resolution (3.7 – 4.0 Å) X-ray structures of BDD human fVIII are available (Figure 3A).[19, 20] The polypeptide segments recognized by anti-A2 MAbs obtained from homolog scanning mutagenesis (Table 1) were used to map the Venn diagram (Figure 1B) onto the A2 surface of these models (Figure 3B). Because the Venn diagram delineates only a logical relationship between overlapping epitopes, its rings do not correspond to a physical dimension. However, simultaneous localization of Group A, C and E MAb Venn rings to their corresponding cognate polypeptide segments restricts the size the Venn structure such that the each ring approximates the footprint associated with its associated MAb. Using this approach, it is evident that nearly the entire A2 surface is targeted by the MAbs used in this study.

Inhibitory properties of anti-A2 MAbs

The anticoagulant properties of the anti-A2 MAbs were investigated using the Bethesda assay. Anti-fVIII Abs are classified as Type I or Type II inhibitors based on whether they completely (greater than 90%) or incompletely inhibit fVIII at saturating concentrations.[21] A representative assay is shown for MAbs from Groups A, B, C, D and E in Figure 4A and the results for the entire MAb set is shown in Table 1. All of the Group A MAbs were Type I inhibitors and except for G32, had high specific activity. In contrast to most of the other Group A MAbs, the epitope of G32 included but was not restricted to Arg484-Ile508. Group B and C MAbs displayed little or no inhibition. Group D and Group E MAbs displayed a wide range of inhibitory activity.

Additionally, the inhibition of fVIIIa by anti-A2 MAbs was analyzed in a plasma-free intrinsic fXase assay using purified components. Figure 4B shows results for representative Group A, D and E MAbs that displayed inhibition in the Bethesda assay. Group A MAb 413 and Group E MAb 1D4 completely inhibited factor Xa formation. Group A MAbs 4A4 and G32 and Group E MAb B66 also produced near complete inhibition of factor Xa formation (data not shown). In contrast, Group D MAb 2-54 displayed partial inhibition at saturating concentrations. Thus, the inhibitory properties of the MAbs in the purified intrinsic fXase system are consistent with their anticoagulant properties in the Bethesda assay.

Effect of anti-A2 MAbs on thrombin – catalyzed proteolytic cleavage of fVIII

Because fVIII was allowed to bind antibody before addition of thrombin in the experiment described in Figure 4B, inhibition of factor Xa formation could be due either to direct inhibition of fVIIIa in the intrinsic fXase complex or inhibition of proteolytic cleavages leading to fVIIIa formation. To evaluate the latter possibility, the effect of anti-A2 MAbs on thrombin – catalyzed cleavage of fVIII was evaluated by SDS-PAGE.

The activation of fVIII by thrombin is initiated by rapid heavy chain cleavage at Arg740, which releases the B domain and produces the A1-A2 subunit (Figure 7).[22] This is followed by Arg1689 cleavage at the ap-A3 light chain junction producing the thrombin-cleaved light chain (LC_{IIa}), and Arg372 cleavage at the A1-A2 junction, which produces the A1 and A2 subunits of fVIIIa.

Most of the Group D and E MAbs inhibited thrombin-catalyzed proteolysis of full-length fVIII (Figure 5). Group D MAb 2-54 and Group DE MAb G74 inhibited production of the A1 and A2 subunits, but not the A1-A2 subunit, indicating inhibition of cleavage at Arg372.

Additionally, 2-54 and G74 inhibited light chain cleavage. Analysis of samples taken during the intrinsic fXase experiment described in Figure 4B showed partial inhibition of BDD fVIII heavy chain and light chain cleavage at saturating concentrations of 2-54 (Supplemental Figure S3), consistent with the partial inhibition of factor Xa formation.

Group D MAbs CLB-CAg 9 and GMA-012 also inhibited light chain cleavage, but in contrast to 2-54 and G74, inhibited production of the A2 subunit, but not the A1 subunit. This result is consistent with normal cleavage at Arg372 and inhibition of cleavage at Arg740. Additionally, the inhibition pattern due to CLB-CAg 9 and GMA-012 was associated with the appearance of a novel 60 kDa band (Figure 5, asterisks). The presence of this band is consistent with a secondary thrombin cleavage within the B domain.

Group E MAbs B161, B66 and 1D4 inhibited light chain cleavage, but not heavy chain cleavage (Figure 5). Analysis of samples taken during the intrinsic fXase experiment described in Figure 4B showed complete inhibition of fVIII light chain cleavage at saturating concentrations of 1D4 (Supplemental Figure S3).

In contrast, the non-inhibitory Group E MAbs 4C7 and 2G10 did not inhibit cleavage of fVIII by thrombin. Group A MAb 413 did not affect cleavage of full-length fVIII (Supplemental Figure S2), consistent with its known function as an inhibitor of fVIIIa, not fVIII [94]. The weakly inhibitory Group B MAb 4F4 and Group C MAb B25 also did not affect cleavage of full-length fVIII (Supplemental Figure S2).

FVIII circulates bound to VWF and dissociates following cleavage of the light chain.[5] Cleavage of the fVIII light chain by thrombin is accelerated several-fold when VWF is bound

to either porcine [22] or human fVIII (Supplemental Figure S4). Therefore, the effect of VWF on the inhibition of light chain cleavage by Group D and Group E MAbs was examined (Figure 6). VWF alleviated inhibition of light chain cleavage produced by Group D and Group DE MAbs 2-54, CLB-CAg 9, GMA-012 and G74, but did not affect inhibition of heavy chain cleavages. In contrast, inhibition of light chain cleavage in the presence of Group E MAbs B161, B66 and 1D4 persisted in the presence of VWF (Figure 6 and Supplemental Figure S5.) Because VWF accelerates fVIII light chain cleavage by thrombin, Group E MAbs potentially could act indirectly by inhibiting the binding of fVIII to VWF. However, the binding of fVIII to VWF was not affected by Group E MAbs B161, B66 or 1D4 (Supplemental Figure S6).

Discussion

In this study, we characterized a panel of 26 anti-fVIII A2 domain MAbs that were produced by hemophilia A mice in response to adjuvant-free clinically relevant intravenous doses of human fVIII and three previously described anti-A2 MAbs, 413, GMA-012 and CLB-CAg 9. We used competition ELISA to interrogate all pairwise interactions of the MAb set. Competition ELISA, which was described soon after the advent of monoclonal antibody technology[24], is a powerful method to map overlapping MAb epitopes. The use of Venn diagrams to represent overlapping epitopes (Fig. 1B) was described as early as 1994 [25]. More recently, competition ELISA has been used to map MAb epitopes resulting from the polyclonal response to influenza hemagglutinin [26, 27] , *P. falciparum* [28], norovirus [29] and West Nile virus antigens.[30]

Our analysis identified a basis set of 5 anti-A2 MAbs, 4A4, 2-93, G48, 2-54 and 1D4 (Figures 1A and 1B), defined as MAbs that do not compete with each other, but as a group compete with all other Abs. The 5 basis set groups, A, B, C, D and E, accounted for 26 of the 29 MAbs that were studied. The 3 remaining MAbs fit into more than one group and are designed Group AB, BCD and DE MAbs. The Venn diagram produced by the overlapping epitopes yielded a continuous spectrum of epitopes. Homolog scanning mutagenesis identified antigenic A2 sequences (Figure 2), which allowed physical placement of the Venn map onto the A2 surface. Our results indicate that the overlapping epitopes cover most of the A2 surface and contrast

with our earlier study of the immune response to human fVIII C2 domain in the murine hemophilia A model.[23] In that study, continuously overlapping epitopes were identified that corresponded to only one face of the C2 β-sandwich.
Eight of the 9 Group A MAbs mapped to the extensively characterized Arg484-Ile508 epitope (Table 1).[10, 11] 413 in this group is a noncompetitive inhibitor of the intrinsic fXase complex.[6] Group A MAbs are characterized by specific inhibitory activities that reach ~40,000 Bethesda units per mg of IgG. This corresponds to near stoichiometric inhibition in which every MAb in the system binds and inhibits a fVIIIa molecule.

Groups AB, B, BCD, and C contained 11 of the 29 MAbs. All of these MAbs possessed either weak or undetectable inhibitory activity. Non-inhibitory anti-fVIII antibodies conceivably could contribute to pathogenicity by increasing the clearance rate of fVIII or contributing to Fc receptor – mediated inflammation. Most patients with anti-fVIII antibodies measured by ELISA have positive inhibitor titers as measured by the Bethesda assay[31], indicating that these non-inhibitory antibodies occur as part of an immune response that also contains inhibitory antibodies. Nonetheless, the results of this study suggest that non-inhibitory antibodies make up a significant portion of the immune response. Thus, immune complexes must be considered in the overall pathogenicity of anti-A2 antibodies.

Homolog scanning mutagenesis revealed that Group D and Group E MAbs recognize major epitope determinants in the C-terminal cupredoxin-like A2 subdomain. Our

results are consistent with Western blotting studies of GMA-012 and deletion mapping studies of CLB-CAg, which located the epitopes recognized by these MAbs to the C-terminal A2 subdomain.[32, 33] Most of these MAbs interfere with proteolytic activation cleavages of fVIII catalyzed by thrombin. Our results must be interpreted in light of the complex pathway that leads to fVIII activation. Following rapid removal of the B domain following cleavage at Arg740, two pathways potentially lead to the formation of the A1/A2/A3-C1-C2 fVIIIa heterotrimer, depending on whether cleavage at Arg1689 occurs followed by cleavage at Arg372 or *vice versa*[22] (left and right paths, respectively in Figure 7). In the presence of VWF, which is the physiologically relevant situation, Arg1689 cleavage is accelerated several-fold, suggesting that the right-sided pathway dominates fVIII activation *in vivo*.[22] This VWF cofactor effect is due to the ability of fVIII to bind more tightly to thrombin in the presence of VWF.[22] Arg1689 cleavage results in the dissociation of VWF from fVIII. This event appears to be necessary for normal hemostasis because VWF competes with phospholipid membranes for binding to fVIII[34, 35], preventing assembly of the intrinsic fXase complex in the absence of dissociation of fVIII.

Arg372 cleavage generates the A2 subunit, which is critical for fVIIIa function.[36 37] Group D MAb 2-54, which has a specific activity of 34,000 BU/mg (Table 1), inhibits A2 subunit formation (Figures 5 and 6 and Supplemental Figure S3). 2-54 also inhibits factor Xa formation in a purified intrinsic fXase system (Figure 4B), which correlates well with its degree of inhibition of A2 formation (Supplemental Figure S3). 2-54 also inhibits fVIII light chain cleavage (Figure 5), which, however, is

alleviated in the presence of VWF (Figure 6). This indicates that the binding of fVIII to VWF produces a conformational change that allows thrombin to out compete 2-54 for binding to fVIII. Overall, these results suggest that in the physiologically relevant setting in which VWF is present, inhibitory Group D antibodies function as anticoagulants by blocking cleavage at Arg372. Group D antibodies bind the C-terminal end of the A2 domain, which evidently is remote from Arg372. This suggests that they allosterically regulate thrombin recognition at Arg372.

CLB-CAg 9 and GMA-012 displayed little or no anticoagulant activity consistent with previous reports (Table 1).[33, 38] Both MAbs also inhibited production of the A2 but not the A1 subunit, indicating that they interfere with cleavage at Arg740, but not Arg372. Site-directed mutagenesis of Arg740 produces an activatable fVIII molecule[37], indicating that cleavage at this site is not necessary for fVIII activation. Donath et al. concluded that the inhibition of light chain cleavage by CLB-CAg 9 indicates that the C-terminal A2 subdomain contributes to fVIII activation.[39] However, inhibition of light chain cleavage by CLB-CAg 9 and GMA-012 was alleviated by VWF (Figure 6), which is consistent with the lack of observed anticoagulant activity.

Group E MAbs B66 and 1D4 displayed relatively high specific activities of 4,000 and 7,000 BU/mg, respectively. Both MAbs inhibited light chain cleavage, but not heavy chain cleavage (Figure 5). In contrast to Group D MAbs, this inhibition persisted in the presence of VWF (Figure 6). Thus, inhibition of light chain cleavage, which prevents dissociation of fVIIIa from VWF, appears to contribute to the anticoagulant properties of B66 and 1D4. Additionally, B66 and 1D4 also may inhibit fVIIIa directly. 1D4 completely inhibited factor

Xa formation in a VWF-free intrinsic fXase assay (Figure 4B), which correlated with complete inhibition of fVIII light chain cleavage (Supplemental Figure 3S). A1/A2/LC heterotrimers that lack Arg1689 cleavage have substantial factor IXa cofactor activity.[40, 41] Furthermore, although mutations at Arg1689 cause hemophilia A, dissociation of Arg1689 mutant fVIII/VWF complexes with disulfide-bond reducing agents can produce fVIII procoagulant activity *in vitro*.[42, 43] Thus, cleavage at Arg1689, while necessary for dissociation of fVIII from VWF, is not necessary for significant fVIIIa activation *per se*. These results suggest that B66 and 1D4 possess both VWF-dependent and VWF-independent inhibitory properties.

The Arg484-Ile508 immunodominant epitope that is recognized by MAb413 and other Group A murine MAbs is also recognized by human inhibitors. [6] Murine anti-A2 MAbs potentially can be used as probes to determine whether novel epitopes characterized in the present study are also targeted by the human immune system. For example, we have identified a novel class of human C2 inhibitors by ELISA using biotinylated murine anti-human C2 MAbs as competitive ligands testing human inhibitor plasmas.[44] Recently, murine anti-PF4/heparin MAbs were used to identify epitopes involved in the pathogenesis of heparin-induced thrombocytopenia using a similar approach. [45]

In summary, our results confirm that the Arg484-Ile508 region is an immunodominant fVIII A2 epitope and identify two additional mechanisms by which fVIII inhibitors act, namely inhibition of cleavage at Arg372 and activation of the fVIII cofactor function and inhibition of cleavage at Arg1689 and dissociation of fVIII from VWF. Additionally, our epitope mapping results reveal that the anti-A2 immune response in the murine hemophilia A model is structurally complex.

Acknowledgments

Supported by National Institutes of Health grants U54 HL112309 and K08 HL102262 and Hemophilia of Georgia, Inc. (S.L.M.); and National Institutes of Health grants U54 HL112309, R01 HL082609 and R01 HL040921 and Hemophilia of Georgia, Inc. (P.L.). The authors thank Dr. Jan Voorberg for his generous gift of CLB-CAg 9.

R.C.M., J.F.H. and E.T.P designed and performed research, analyzed data, and co-wrote the paper; S.L.M designed research, analyzed data and co-wrote the paper; P.L. designed research, analyzed data, and co-wrote the paper. **RM** designed experiments, collected data and made the figures for all the figures shown with the exception of 4B.

The authors state they have no conflicts of interest.

References

(1) Lusher JM, Arkin S, Abildgaard CF, Schwartz RS, and the Kogenate Previously Untreated Patient Study Group. Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy, and the development of inhibitors. *N Engl J Med* 1993;328(7):453-459.

(2) Bray GL, Gomperts ED, Courtier S et al. A multicenter study of recombinant factor VIII (Recombinate): Safety, efficacy, and inhibitor risk in previously untreated patients with hemophilia A. *Blood* 1994;83(9):2428-2435.

(3) Lusher JM, Lee CA, Kessler CM, Bedrosian CL, the Refacto Phase 3 Study Group. The safety and efficacy of B-domain deleted recombinant factor VIII concentrate in patients with severe haemophilia A. *Haemophilia* 2003;9(1):38-49.

(4) Ziatseva I, Zaitsev V, Card G et al. The X-ray structure of human serum ceruloplasmin at 3.1 Angstroms: nature of the copper centres. *JBIC* 1996;1:15-23.

(5) Lollar P, Hill-Eubanks DC, Parker CG. Association of the factor VIII light chain with von Willebrand factor. *J Biol Chem* 1988;263(21):10451-10455.

(6) Lollar P, Parker ET, Curtis JE et al. Inhibition of human factor VIIIa by anti-A2 subunit antibodies. *J Clin Invest* 1994;93(6):2497-2504.

(7) Fay PJ, Beattie T, Huggins CF, Regan LM. Factor VIIIa A2 subunit residues 558 to 565 represent a factor IXa interactive site. *J Biol Chem* 1994;269(32):20522-20527.

(8) Lollar P, Parker CG. pH-dependent denaturation of thrombin-activated porcine factor VIII. *J Biol Chem* 1990;265(3):1688-1692.

(9) Prescott R, Nakai H, Saenko EL et al. The inhibitory antibody response is more complex in hemophilia A patients than in most nonhemophiliacs with fVIII autoantibodies. *Blood* 1997;89(10):3663-3671.

(10) Scandella D, Timmons L, Mattingly M, Trabold N, Hoyer LW. A soluble recombinant factor VIII fragment containing the A2 domain binds to some human anti-factor VIII antibodies that are not detected by immunoblotting. *Thromb Haemostas* 1992;67(6):665-671.

(11) Healey JF, Lubin IM, Nakai H et al. Residues 484-508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. *J Biol Chem* 1995;270(24):14505-14509.

(12) Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. The humoral response to human factor VIII in hemophilia A mice*. J Thromb Haemost* 2007;5(3):512-517.

(13) Fay PJ, Haidaris PJ, Smudzin TM. Human factor $VIII_a$ subunit structure: reconstitution of factor $VIII_a$ from the isolated A1/A3-C1-C2 dimer and A2 subunit*. J Biol Chem* 1991;266(14):8957-8962.

(14) Parker ET, Doering CB, Lollar P. A1 subunit-mediated regulation of thrombin-activated factor VIII A2 subunit dissociation. *J Biol Chem* 2006;281(20):13922-13930.

(15) Summers RJ, Meeks SL, Healey JF et al. Factor VIII A3 domain substitution N1922S results in hemophilia A due to domain-specific misfolding and hyposecretion of functional protein. *Blood* 2011;117(11):3190-3198.

(16) Kasper CK, Aledort LM, Counts RB et al. A more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975;34(3):869-872.

(17) Barrow RT, Lollar P. Neutralization of anti-factor VIII inhibitors by recombinant porcine factor VIII. *J Thromb Haemost* 2006;4(10):2223-2229.

(18) Lubin IM, Healey JF, Scandella D, Runge MS, Lollar P. Elimination of a major inhibitor epitope in factor VIII. *J Biol Chem* 1994(12);269:8639-8641.

(19) Shen BW, Spiegel PC, Chang C-H et al. The tertiary structure and domain organization of coagulation factor VIII. *Blood* 2008;111(3):1240-1247.

(20) Ngo JC, Huang M, Roth DA, Furie BC, Furie B. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. *Structure* 2008;16(4):597-606.

(21) Hoyer LW, Gawryl MS, de la Fuente B. Immunochemical characterization of factor VIII inhibitors. In: Hoyer LW, editor. *Factor VIII Inhibitors* New York: Alan R. Liss; 1984. 73-85.

(22) Hill-Eubanks DC, Lollar P. von Willebrand factor is a cofactor for thrombin-catalyzed cleavage of the factor VIII light chain. *J Biol Chem* 1990;265(29):17854-17858.

(23) Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Anti-human factor VIII C2 domain antibodies in hemophilia A mice recognize a functionally complex continuous spectrum of epitopes dominated by inhibitors of factor VIII activation. *Blood* 2007;110(13):4234-4242.

(24) Pierres M, Devaux C, Dosseto M et al. Clonal analysis of B- and T-cell responses to Ia antigens. I. Topology of epitope regions on I-Ak and I-Ek molecules analyzed with 35 monoclonal alloantibodies. *Immunogenetics* 1981;14(6):481-495.

(25) Morris CA, Underwood PA, Bean PA, Sheehan M, Charlesworth JA. Relative topography of biologically active domains of human vitronectin. Evidence from monoclonal antibody epitope and denaturation studies. *J Biol Chem* 1994;269(38):23845-23852.

(26) Corti D, Suguitan AL, Jr., Pinna D et al. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *J Clin Invest* 2010;120(5):1663-1673.

(27) Corti D, Voss J, Gamblin SJ et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* 2011;333(6044):850-856.

(28) de Silva HD, Saleh S, Kovacevic S et al. The antibody response to Plasmodium falciparum Merozoite Surface Protein 4: comparative assessment of specificity and growth inhibitory antibody activity to infection-acquired and immunization-induced epitopes. *Malaria Journal* 2011;10:266.

(29) Parra GI, Abente EJ, Sandoval-Jaime C, Sosnovtsev SV, Bok K, Green KY. Multiple antigenic sites are involved in blocking the interaction of GII.4 norovirus capsid with ABH histo-blood group antigens. *J Virol* 2012;86(13):7414-7426.

(30) Lelli D, Moreno A, Brocchi E et al. West Nile virus: characterization and diagnostic applications of monoclonal antibodies. *Virology Journal* 2012;9:81.

(31) Ling M, Duncan EM, Rodgers SE, Street AM, Lloyd JV. Low detection rate of antibodies to non-functional epitopes on factor VIII in patients with hemophilia A and negative for inhibitors by Bethesda assay. *J Thromb Haemost* 2003(12);1:2548-2553.

(32) Fay PJ, Smudzin TM, Walker FJ. Activated protein C-catalyzed inactivation of human factor VIII and factor VIIIa. Identification of cleavage sites and correlation of proteolysis with cofactor activity. *J Biol Chem* 1991;266(30):20139-20145.

(33) Leyte A, Mertens K, Distel B et al. Inhibition of human coagulation Factor VIII by monoclonal antibodies. Mapping of functional epitopes with the use of recombinant Factor VIII fragments. *Biochem J* 1989;263(1):187-194.

(34) Andersson LO, Brown JE. Interaction of Factor VIII-von Willebrand Factor with phospholipid vesicles. *Biochem J* 1981;200(1):161-167.

(35) Lajmanovich A, Hudry-Clergeon G, Freyssinet JM, Marguerie G. Human Factor VIII procoagulant activity and phospholipid interactions. *Biochim Biophys Acta* 1981;678(1):132-136.

(36) Arai M, Inaba H, Higuchi M et al. Direct characterization of factor VIII in plasma: detection of a mutation altering a thrombin cleavage site (arginine-372 -> histidine). *Proc Natl Acad Sci USA* 1989;86(11):4277-4281.

(37) Pittman DD, Kaufman RJ. Proteolytic requirements for thrombin activation of anti-hemophilic factor (factor VIII). *Proc Natl Acad Sci USA* 1988;85(8):2429-2433.

(38) Fay PJ, Scandella D. Human inhibitor antibodies specific for the factor VIII A2 domain disrupt the interaction between the subunit and factor IXa. *J Biol Chem* 1999;274(42):29826-29830.

(39) Donath MJ, Lenting PJ, van Mourik JA, Mertens K. Kinetics of factor VIII light-chain cleavage by thrombin and factor Xa. A regulatory role of the factor VIII heavy-chain region Lys713-Arg740. *Eur J Biochem* 1996;240(2):365-372.

(40) Hill-Eubanks DC, Parker CG, Lollar P. Differential proteolytic activation of factor VIII-von Willebrand factor complex by thrombin. *Proc Natl Acad Sci USA* 1989;86(17):6508-6512.

(41) Regan LM, Fay PJ. Cleavage of factor VIII light chain is required for maximal generation of fVIIIa activity. *J Biol Chem* 1995;270(15):8546-8552.

(42) Aly AM, Arai M, Hoyer LW. Cysteamine enhances the procoagulant activity of factor VIII-East Hartford, a dysfunctional protein due to a light chain thrombin cleavage site mutation (arginine-1689 to cysteine). *J Clin Invest* 1992;89(5):1375-1381.

(43) Arai M, Higuchi M, Antonarakis SE et al. Characterization of a thrombin cleavage site mutation (Arg 1689 to Cys) in the factor VIII gene of two unrelated patients with cross-reacting material-positive hemophilia A. *Blood* 1990;75(2):384-389.

(44) Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Non-classical anti-C2 domain antibodies are present in patients with factor VIII inhibitors. *Blood* 2008;112(4):1151-1153.

(45) Sachais BS, Litvinov RI, Yarovoi SV et al. Dynamic antibody-binding properties in the pathogenesis of HIT. *Blood* 2012;120(5):1137-1142.

Table 1. Properties of Anti-A2 MAbs

	MAbs	BU/mg IgG	Type	Isotype	Group	Epitope
1	4A4	40,000	I	IgG2aκ	A	Asp403-
2	2-76	38,000	I	IgG2aκ	A	Arg484-
3	G32	3,000	I	IgG2aκ	A	His444-
4	I62	15,000	I	IgG2aκ	A	Arg484-
5	I155	40,000	I	IgG1κ	A	Arg484-
6	I160	20,000	I	IgG2aκ	A	Arg484-
7	413	21,000	I	IgG1κ	A	Arg484-
8	G119	10,000	I	IgG1κ	A	Arg484-
9	2-105	40,000	I	IgG1κ	A	Arg484-
10	B157	<1	N/A†	IgG1κ	AB	Indeterminat
11	2-93	4	II	IgG1κ	B	Arg541-
12	B107	Indeterminat	II	IgG2aκ	B	Arg541-
13	4F4	330	I	IgG2aκ	B	Indeterminat
14	G139	<1	N/A†	IgG1κ	B	Arg541-
15	B94	Indeterminat	II	IgG1κ	B	Arg541-
16	G6	NI	N/A†	IgG2aκ	B	His444-
17	G4	9	I	IgG1κ	B	Arg541-
18	B99	11	I	IgG1κ	BCD	Indeterminat
19	G48	5	II	IgG2aκ	C	His444-
20	B25	100	I	IgG1κ	C	His444-
21	2-54	34,000	II	IgG1κ	D	Glu604-
22	CLB-CAg 9	Indeterminat	II	IgG1κ	D	Glu604-
23	GMA-012	<1	N/A†	IgG1κ	D	Glu604-
24	G74	1,500	I	IgG1κ	DE	Glu604-
25	1D4	7,000	I	IgG2aκ	E	Glu604-
26	B66	4,000	I	IgG2aκ	E	Glu604-
27	B161	150	I	IgG2aκ	E	Glu604-
28	4C7	<1	N/A†	IgG2aƙ	E	Indeterminat
29	2G10	Indeterminat	II	IgG2aƙ	E	Indeterminat

* Residual fVIII activity at saturating concentrations of antibody was greater than 50%. †

Not applicable to non-inhibitory MA

Figure Legends

Figure 1. ELISA competition matrix and overlap pattern of anti-A2 MAbs. (A) Competition matrix compiling the results of competition ELISA between 29 anti-A2 MAbs. Rows represent primary MAbs and columns represent biotinylated secondary MAbs. Yellow and white squares represent binding and no binding (no overlap and overlap), respectively, of secondary MAbs. (B) Venn diagram representing overlaps produced by the competition matrix. Each MAb was assigned a group based on its overlap with a basis set of 5 MAbs 4A4, 2-93, G48, 2-54 and 1D4 that are underlined and italicized. Colors depict the 8 groups of MAbs defined by their overlap with basis set MAbs: dark red, group A; red, group AB; orange, group B; yellow, group C; green, group BCD; blue, group D; light blue, group DE; and purple, group E.

Figure 2. Epitope mapping of anti-A2 MAbs by homolog scanning mutagenesis. (A) BDD hybrid human/porcine fVIII constructs. Shaded areas represent porcine fVIII substitutions. (B) Binding of anti-A2 MAbs to human/porcine fVIII. FVIII was captured on a microtiter plate using immobilized anti-C2 MAb, followed by addition of biotinylated anti-A2 MAb and detection using streptavidin alkaline phosphatase. Bars represent the range of duplicate determinations.

Figure 3. Map of overlapping MAb epitopes onto the A2 surface. (A) X-ray structure of BDD fVIII (PDB 2R7E) showing regions recognized by Group A MAbs (Asp403-His444 and Arg484-Ile508) and Group E MAbs (Glu604-Cys711). A2 residues Asp712-Arg740 were not identified in the structure. (B) Regions with the

A2 polypeptide chain identified by homolog scanning mutagenesis were used to anchor the Venn diagram onto the A2 surface.

Figure 4. Anticoagulant properties of anti-A2 MAbs. (A) Residual fVIII activity was measured by one-stage coagulation assay following incubation of normal human plasma with varying concentrations of MAbs for 2 hours at 37 ºC. Data represent sample means and sample standard deviations. The curves represent least-squares fits to the data. The inhibitor titer in Bethesda units (BU) per ml was obtained by determining the dilution of MAb producing 50% inhibition and converted to BU/mg using the MAb concentration. (B) BDD human fVIII (50 nM) was incubated with the indicated concentrations of anti-A2 MAbs for 30 min and then thrombin for 60 s, followed by sample dilution into factor IXa/ PCPS phospholipid vesicles, addition of factor X and measurement of factor Xa as described under "Intrinsic fXase assay" in "Methods". Results are presented as percent of factor Xa formed in the absence of MAb.

Figure 5. Effect of Group D and E anti-A2 MAbs on thrombin – catalyzed proteolytic cleavage of full-length fVIII. FVIII (100 nM) was incubated with 0.5 nM thrombin for 10 min at 37 ºC in the presence of the indicated MAb concentrations. Proteolytic cleavages were analyzed by SDS-PAGE as described in "Methods" under "Effect of anti-A2 MAbs on thrombin catalyzed proteolytic cleavage of fVIII". "Ctrl", fVIII control not exposed to thrombin or anti-A2 MAbs. Molecular weight standards ("Stds") are 250, 150, 100, 75, 50, 37, and 25 kDa.

Figure 6. Effect of Group D and E anti-A2 MAbs on thrombin – catalyzed proteolytic cleavage of full-length fVIII/VWF complex. FVIII (100 nM) was incubated for 10 min at 37 ºC with VWF (1000nM monomer equivalents) and then incubated for an additional 10 min in the presence of increasing MAb concentrations. Thrombin (0.5 nM) was added for 10 min and the reaction products were analyzed by SDS-PAGE as described in "Methods" under "Effect of anti-A2 MAbs on thrombin catalyzed proteolytic cleavage of fVIII". "Ctrl", FVIII control not exposed to thrombin or anti-A2 MAbs. Molecular weight standards ("Stds") are 250, 150, 100, 75, 50, 37, and 25 kDa.

Figure 7. Inhibition of thrombin-catalyzed activation of fVIII by Group D and E MAbs. The activation of full-length fVIII (A1-A2-B/*ap*-A3-C1-C2) is associated with proteolytic cleavages catalyzed by thrombin. The fastest cleavage occurs at Arg740 in the A1-A2-B heavy chain, producing the A1-A2 fragment and the *ap*-A2-C1-C2 light chain. Cleavage then occurs at Arg372 or Arg1689. Cleavage at Arg372 is necessary for the development of fIXa cofactor activity of fVIIIa, whereas cleavage at Arg1689 is necessary for the dissociation of fVIII from VWF. Cleavage at Arg740 is not necessary for either fVIIIa formation or the dissociation of fVIII from VWF. The effects of anti-A2 MAbs on fVIII cleavages are described in the text.

Figures

Figure 1. ELISA competition matrix and overlap pattern of anti-A2 MAbs

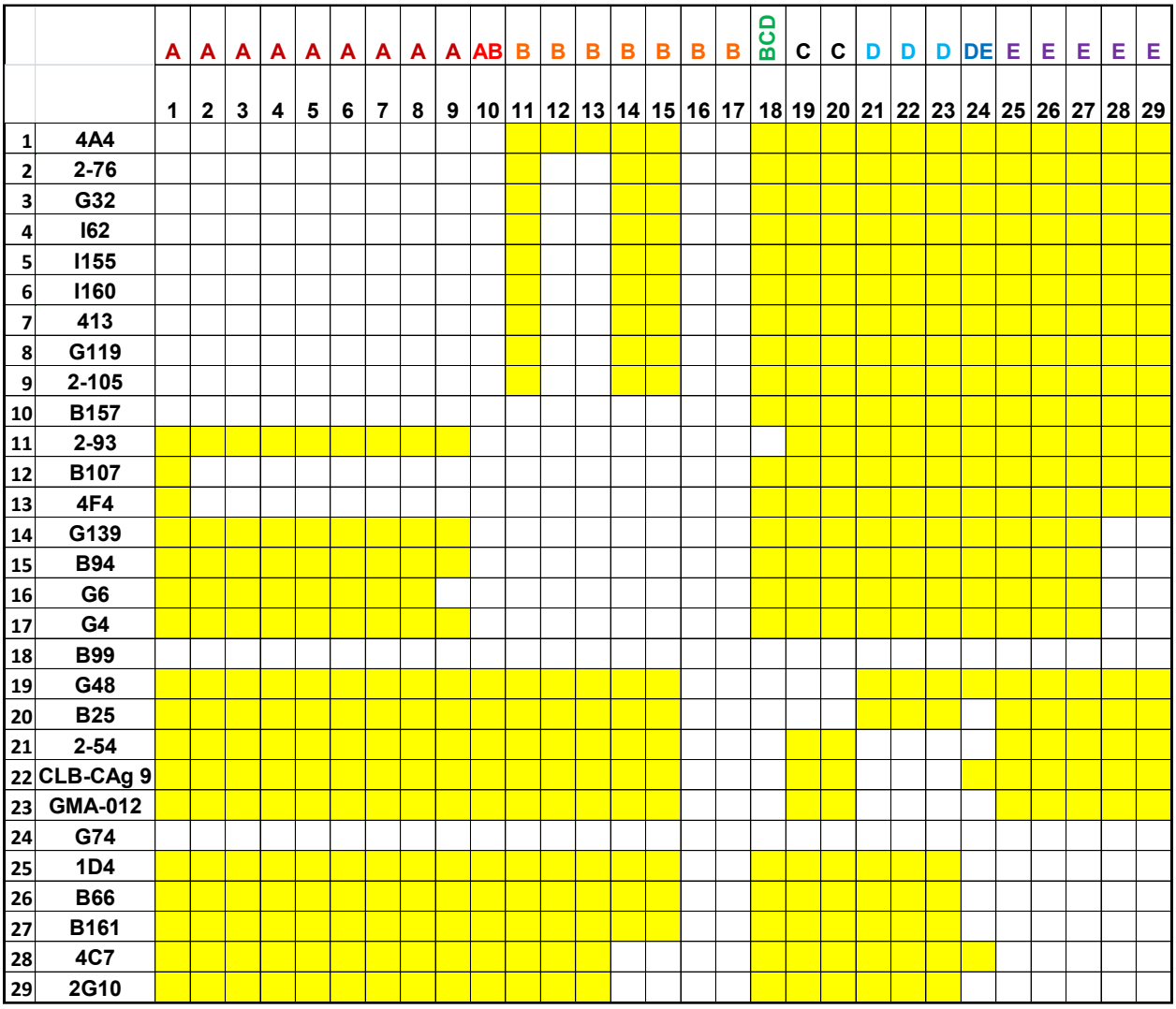


Figure 1B.

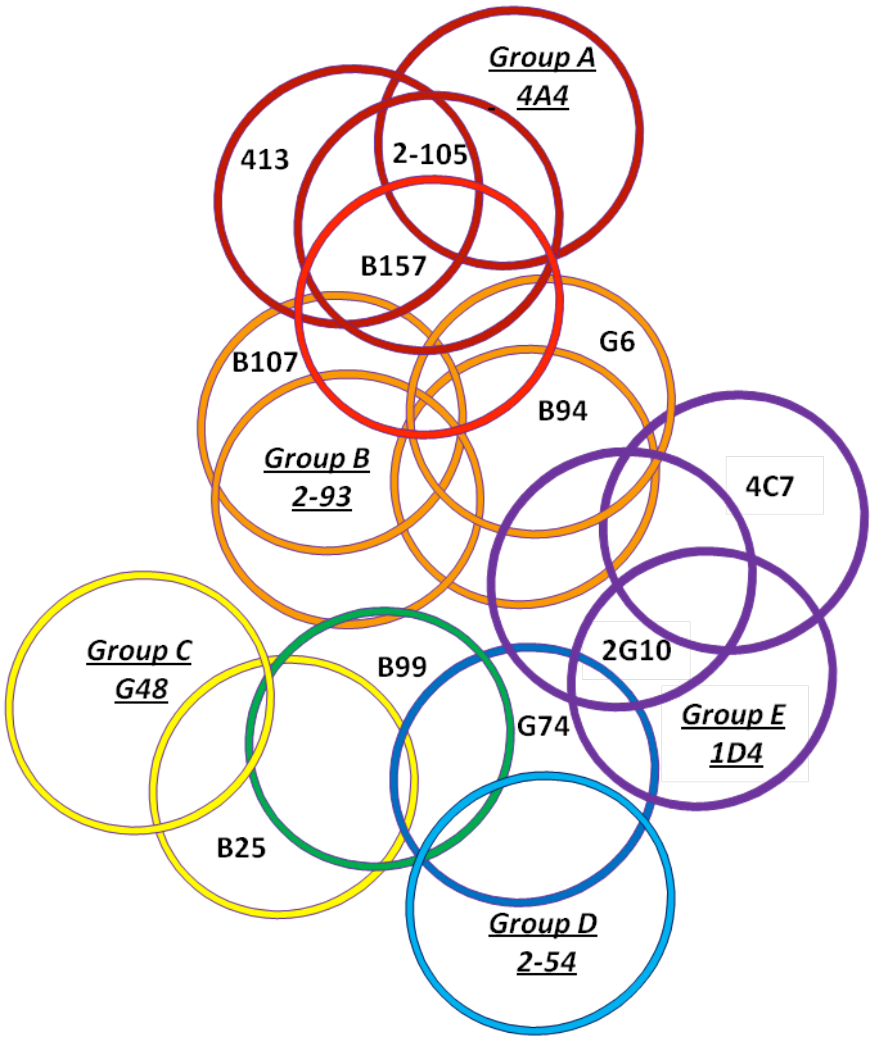

Group A
4A4
413
2-105
B157
B107
G6
B94
Group B
2-93
4C7
Group C
G48
B99
2G10
G74
Group E
1D4
B25
Group D
2-54

Figure 2. Epitope Mapping of Anti-A2 MAbs

by Homolog Scanning Mutagenesis

2A.

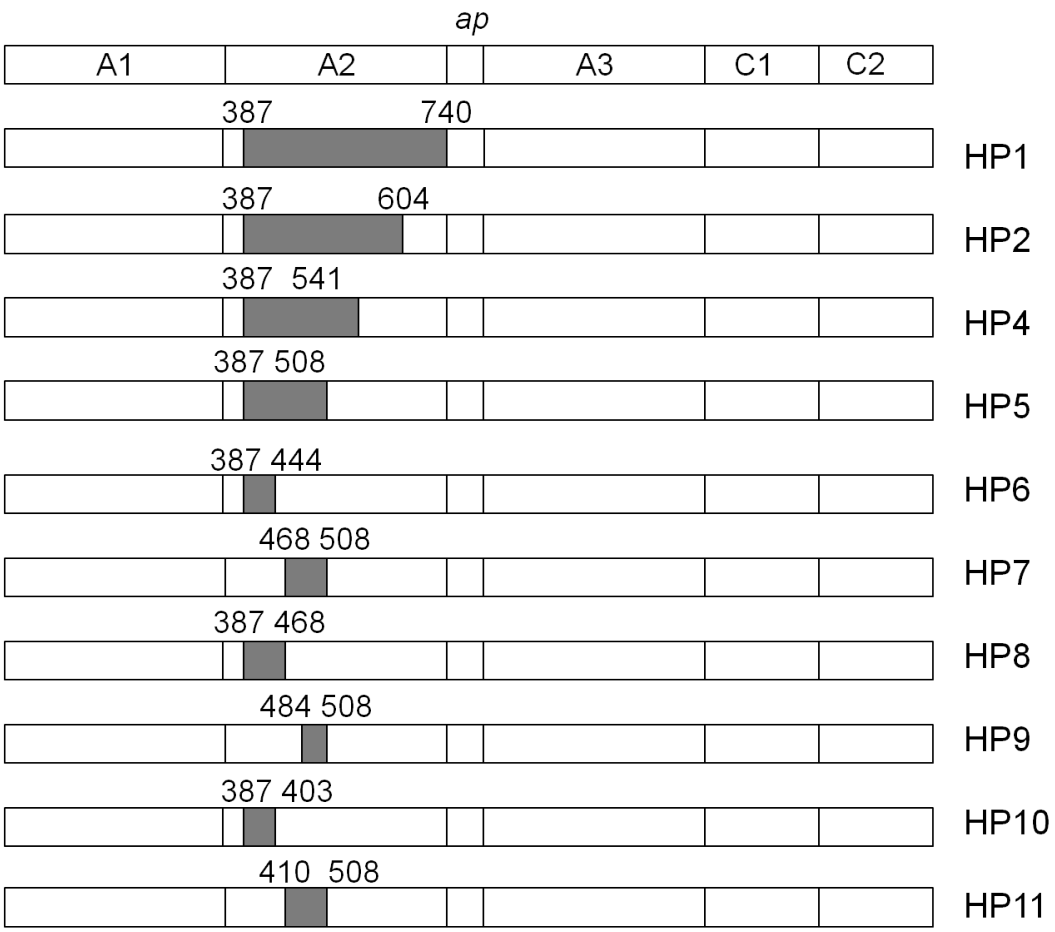
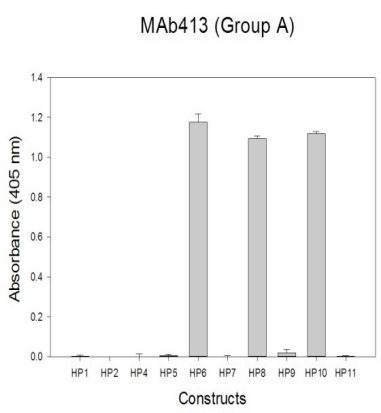
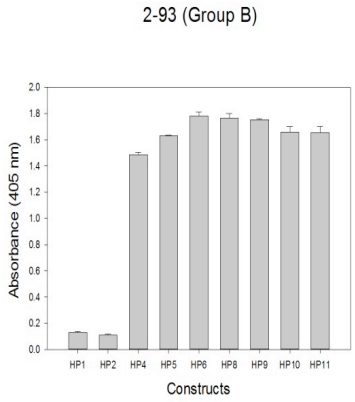
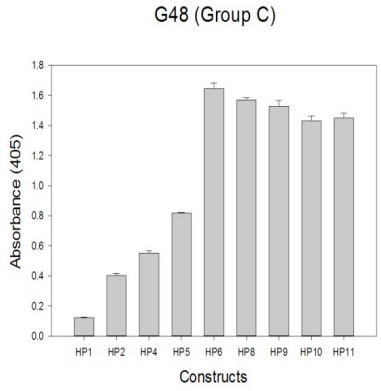
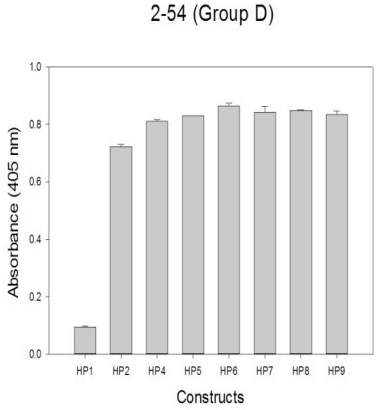


Figure 2B.


MAb413 (Group A)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP7 HP8 HP9 HP10 HP11


2-93 (Group B)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10 HP11


G48 (Group C)
Absorbance (405)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10 HP11


2-54 (Group D)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP7 HP8 HP9

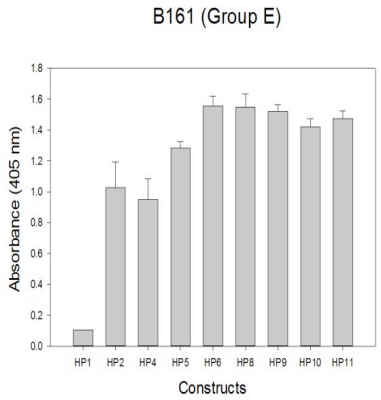

B161 (Group E)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10 HP11

Figure 3. Map of overlapping MAb epitopes onto the A2 surface

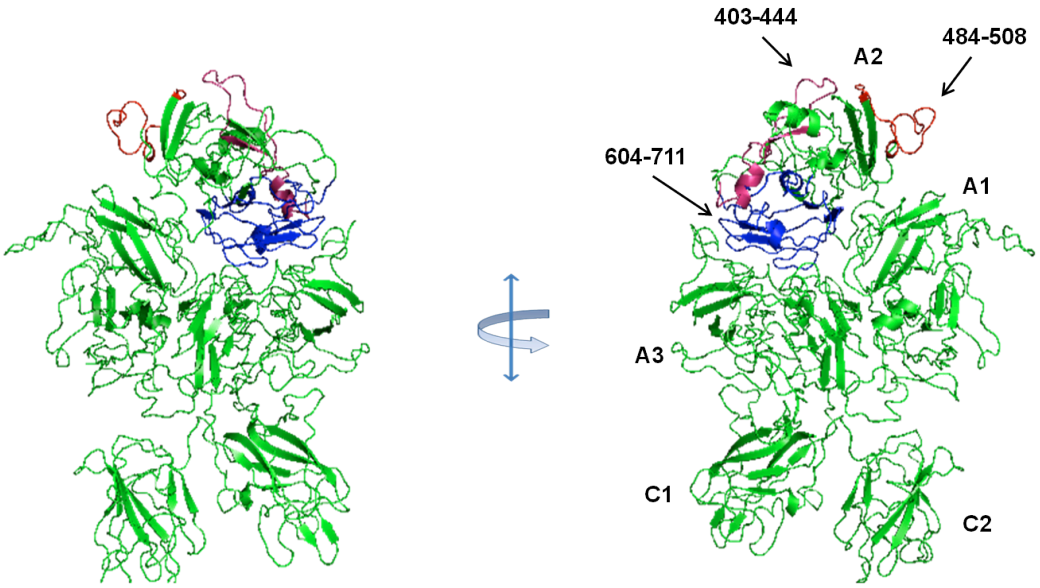


Figure 3B.

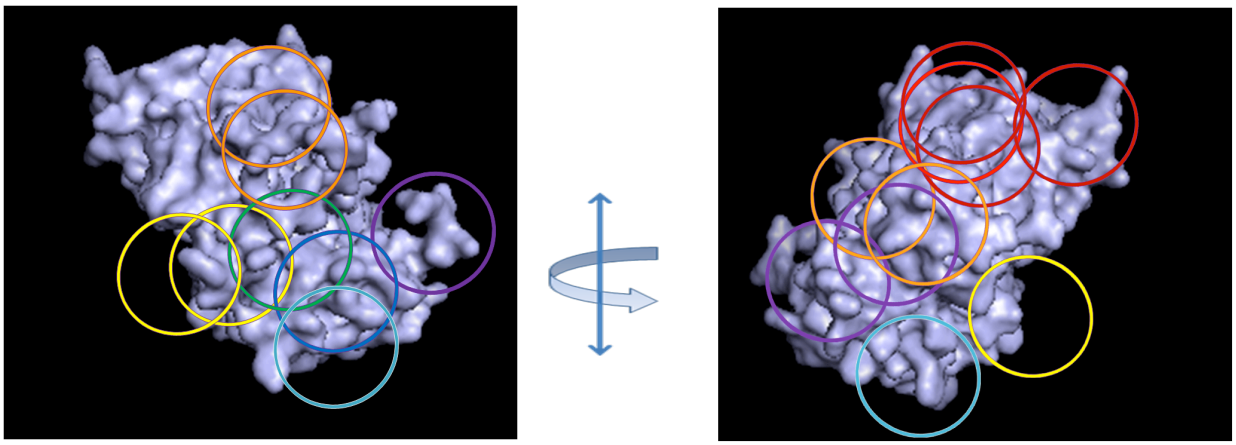
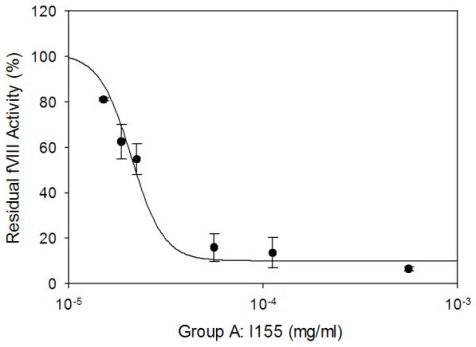
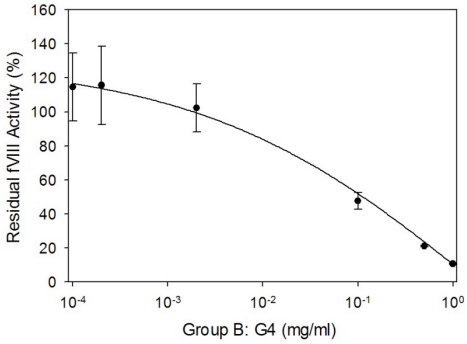
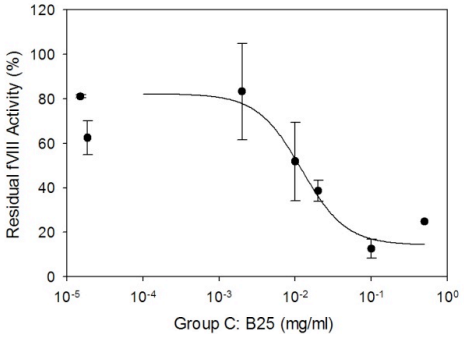
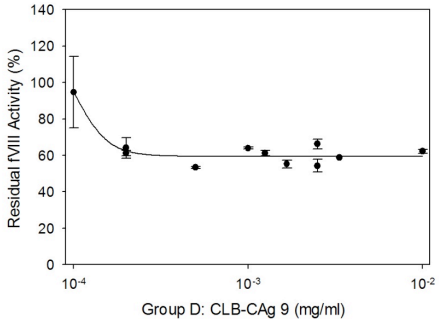


Figure 4. Anticoagulant properties of anti-A2 MAbs









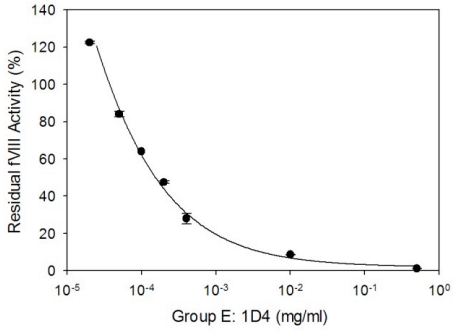


Figure 4B.

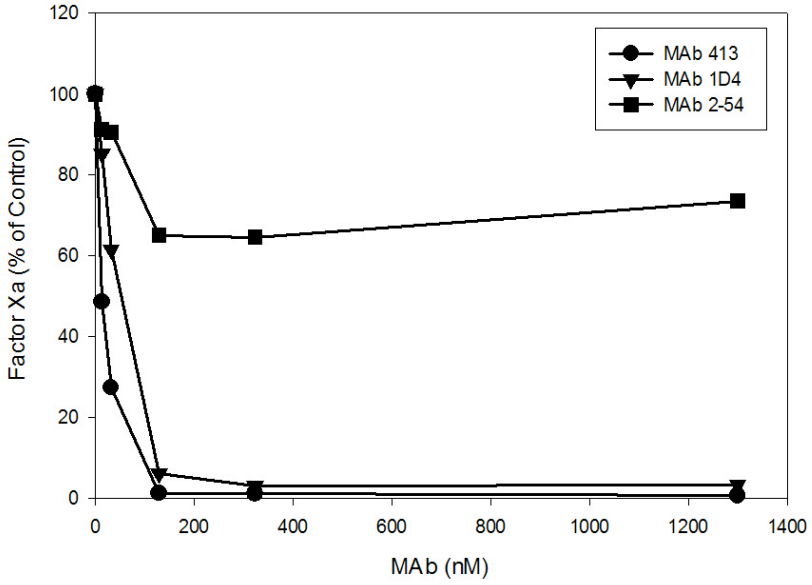

120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Factor Xa (% of Control)
MAb (nM)
MAb 413
MAb 1D4
MAb 2-54

Figure 5. Effect of group D and E anti-A2 MABs on thromin-catalyzed proteolytic cleavage of full-length fVIII

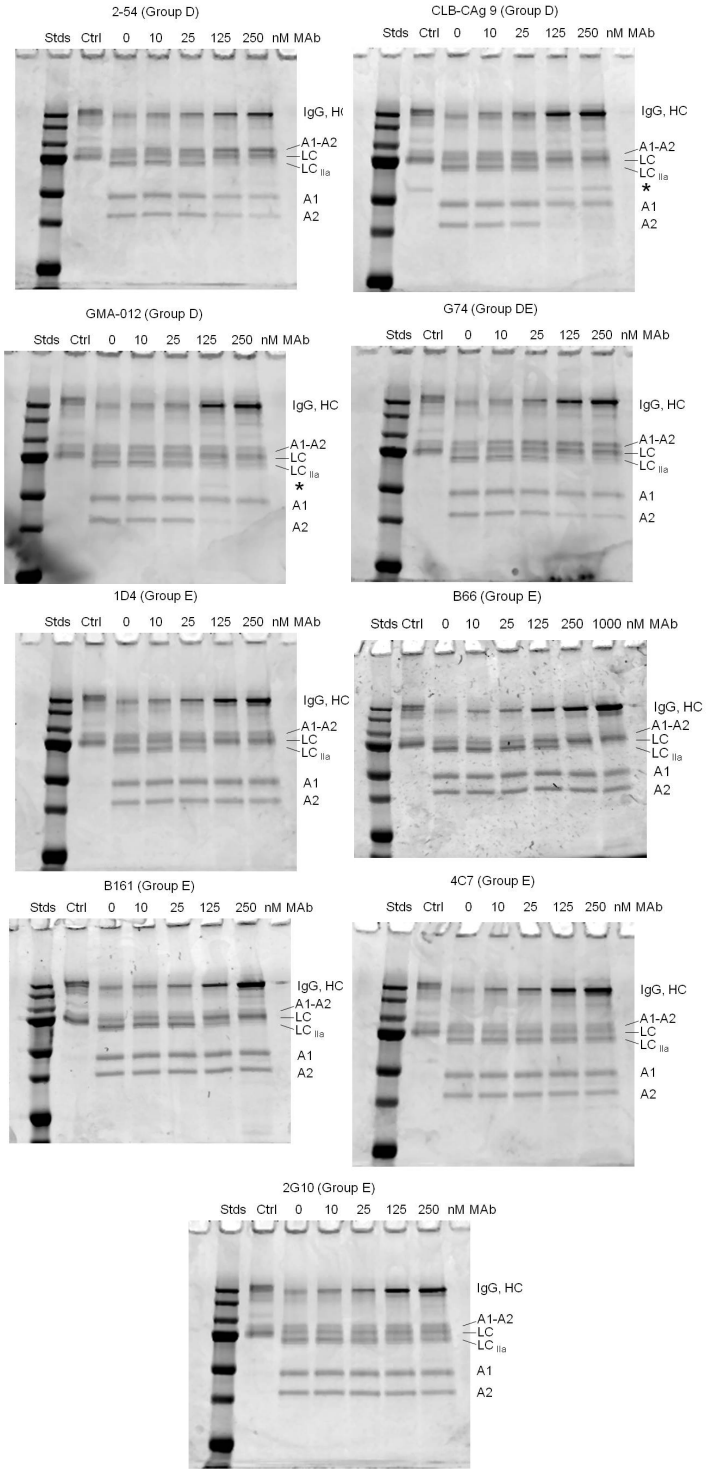


Figure 6. Effect of group D and E anti-A2 MAbs on thrombin-catalyzed proteolytic cleavage of full-length fVIII/VWF complex

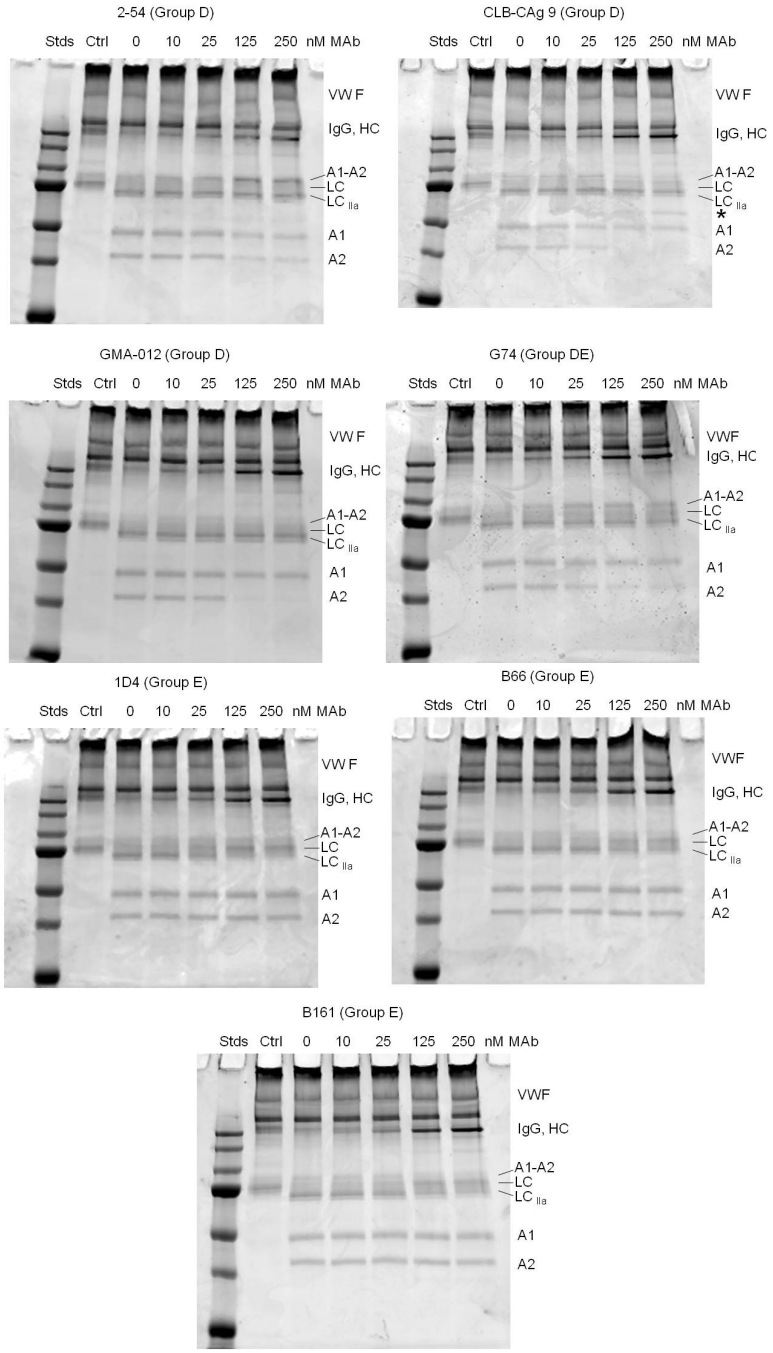
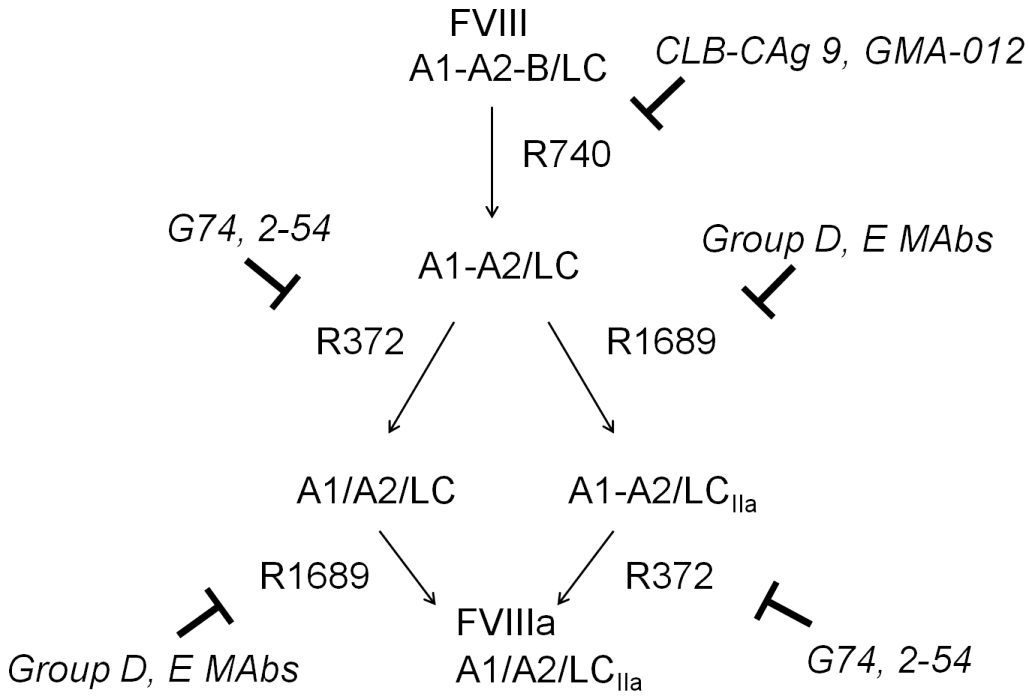


Figure 7. Inhibition of thrombin-catalyzed activation of fVIII by group D and E MAbs



High Titer Type I but not Type II Anti-Factor VIII A2 Domain Antibodies Are Pathogenic in a Murine *in vivo* Bleeding Model

Short title: Pathogenicity of Anti-Factor VIII A2 Domain Antibodies

Joshua Eubanks, B.A.[1], W. Hunter Baldwin, B.S.[2], Rebecca Markovitz, B.A.[1], Ernest T Parker, B.A.[2] and Shannon L. Meeks, MD[2]

1 Emory University, Atlanta, GA; 2 Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta/Emory University, Atlanta, GA

.

Key Words: Hemophilia A, Factor VIII, Inhibitor, Antibody

Corresponding author: Shannon L. Meeks, M.D., Emory Children's Center, Room 442, 2015 Uppergate Drive, Atlanta, GA 30322; e-mail: shannon.meeks@choa.org; phone: 404-727-1608; fax: 404-727-4859

Abstract word count: 240

Word count: 3376

Grant support: Supported by grants from the National Institutes of Health (HL XXXX) and Hemophilia of Georgia (S.L.M.).

Abstract

Background: The primary immunogenic epitopes of factor VIII (fVIII) are in the A2 and C2 domains. Within the C2 domain antibody epitope and kinetics are more important than inhibitory titer in prediction pathogenicity in a murine *in vivo* bleeding model. *Objective:* The pathogenicity of a diverse panel of anti-human fVIII A2 domain monoclonal antibodies (MAbs) was tested in the murine model. *Methods:* MAbs were injected into hemophilia A mice to a peak plasma concentration of 65 nM, followed by injection of human B domain-deleted fVIII at 180 U/kg, producing peak plasma concentrations of ~2.5 nM. At 2 hours, blood loss following a 4 mm tail snip was measured. The following anti-A2 MAbs were tested: 1) 4A4 (group A), 2-76 (group A), 1D4 (group E), high titer type I inhibitors, 2) 2-54 (group D), high titer type II inhibitor, 3) B94 (group B), type II inhibitor with maximum inhibition of ??%, and 4) GMA-012 (group D), 4C7 (group E), and B25 (group C), non-inhibitory MAbs. *Results:* All high titer type I MAbs produced blood loss that was significantly greater than control mice injected with fVIII alone while all non-inhibitory MAbs produced blood loss that was similar to control mice. The type II MAbs were not pathogenic despite 2-54 having an inhibitory titer of 34,000 BU/mg IgG. *Conclusions:* The discrepancy between inhibitory titer and bleeding phenotype combined with similar findings in the C2 domain stress the importance of inhibitor properties not detected in the standard Bethesda assay in predicting response to fVIII therapy.

Introduction

The immune response to fVIII currently is the most significant complication in the management of patients with hemophilia A. Approximately 30% of patients with severe hemophilia A develop detectable anti-factor VIII (fVIII) antibodies in response to infusions of fVIII [1-4]. In addition, autoimmune antibodies to fVIII can develop in non-hemophiliacs, producing acquired hemophilia A, which frequently produces life- or limb-threatening bleeding.

FVIII contains a domain sequence designated A1-A2-B-*ap*-A3-C1-C2. It circulates as an A1-A2-B/*ap*-A3-C1-C2 heterodimer bound to von Willebrand factor (VWF). During the activation of fVIII by thrombin, the B domain and the light chain activation peptide, *ap*, are released, and cleavage between the A1 and A2 domains occurs, producing an A1/A2/A3-C1-C2 heterotrimer[5]. Within the active heterotrimer the A2 domain is necessary for fVIIIa binding to fIXa in the membrane-bound intrinsic Xase complex (fIXa-fVIIIa). It has been reported that fIXa binds to amino acids 558-565 in the A2 subunit of fVIII. [6, 7] Following thrombin activation, the A2 domain spontaneously dissociates resulting in a loss of cofactor activity.[8]The binding of fVIIIa to fIXa stabilizes the heterotrimeric form of fVIIIa by decreasing the rate of spontaneous dissociation of the A2 domain. In either congenital or acquired hemophilia A, the majority of inhibitory antibodies are directed at either the 40-kDa A2 or the 15-kDa C2 domains of fVIII[9]. Congenital hemophilia patients typically have a polyclonal response consisting of antibodies to both the A2 and C2 domain, whereas acquired hemophilia patients typically have a more limited B cell epitope response and either have anti-A2 or anti-C2 antibodies but not both[9]. FVIII inhibitors can either inhibit

fVIII completely or incompletely at saturating concentrations, corresponding to type I and type II behavior, respectively[10].

We have characterized the diversity of a large panel of anti-A2 and anti-C2 MAbs that were produced in a murine hemophilia A immunogenicity model[11, 12]. For the C2 antibodies five groups of structural B-cell epitopes were defined based on patterns of overlapping epitopes. Group A, AB and B antibodies correspond to classical anti-C2 antibodies that inhibit the binding of fVIII and fVIIIa to phospholipid and VWF. Group BC antibodies are the most frequent and are type II inhibitors with inhibitory titers usually greater than 10,000 Bethesda units per mg IgG. These antibodies inhibit the activation of fVIII by thrombin and factor Xa in the presence and absence of VWF. Group C antibodies, which are rare, are represented by the well characterized commercial MAb, ESH8, which blocks the release of VWF from fVIII following thrombin activation[13]. Non-classical Group BC/C antibodies are present in the plasmas of most human fVIII inhibitor patients[14]. Group BC antibodies have inhibitory titers on an equimolar basis that are usually at least 10-fold higher than classical anti-C2 antibodies. However, at saturating concentrations they produce residual fVIII levels of 20 – 40%. Because fVIII levels in this range in the absence of inhibitory antibodies are sufficient for normal hemostasis, the pathogenicity of anti-C2 antibodies was assessed in a murine *in vivo* bleeding model. Type I and type II anti-C2 MAbs were pathogenic in this model but higher doses of fVIII overcame the pathogenicity of the high-titer type II non-classical antibodies but not the lower titer classical C2 antibodies or a single high titer type I anti-A2 antibody, 4A4[15]. Thus, within the C2 domain it was shown that epitope specificity and inhibitor kinetics was more important than inhibitory titer in predicting response to high dose fVIII therapy.

Anti-A2 antibodies were placed into groups A, AB, B, BCD, C, D, DE, and E based on the pattern of overlap on the B-cell epitopes in a competition ELISA. Most Group A MAbs recognized a previously described epitope bounded by Arg484-Ile508 in the N-terminal A2 subdomain, resulting in binding to activated fVIII and noncompetitive inhibition of the intrinsic fXase complex. Group B and C MAbs displayed little or no inhibitory activity. Group D and E MAbs recognized epitopes in the C-terminal A2 subdomain. A subset of Group D MAbs inhibited the activation of fVIII by interfering with thrombin-catalyzed cleavage at Arg372 at the A1-A2 domain junction. Other Group D MAbs displayed indeterminate or no inhibitory activity despite inhibiting cleavage at Arg740 at the A2-B domain junction. Group E MAbs inhibited fVIII light chain cleavage at Arg1689[16]. In this study, we compared the pathogenicity of a diverse panel of anti-A2 MAbs in a murine *in vivo* bleeding model.

Materials and Methods

Materials:

Pooled citrated normal plasma (FACT) and fVIII deficient plasma were obtained from George King Biomedical (Overland Park, KS). Isoflurane (VetOne, Boise, ID) and 0.9% sterile saline (Hospira, Lake Forest, IL) were obtained from the Emory University Department of Animal Resources. All other materials were reagent grade or are described in the cited literature.

Recombinant fVIII

A recombinant B domain deleted (rBDD) human fVIII was expressed from a baby hamster kidney derived cell line as previously described[17, 18] and was purified from conditioned serum-free cell culture media using a two-step ion-exchange chromatography procedure as previously described[19]. Fractions were analyzed by one-stage coagulation assay, by absorbance at 280nm, and by sodium dodecyl sulfate polyacrylamide gel electrophoresis as previously described[20]. The purity of the IgGs was judged to be greater than 90% by SDS-PAGE. The amino acid content was used to estimate the molar extinction coefficient and then the absorbance at 280nm was used to estimate the concentration of fVIII that was then used to calculate the specific activity in a one-stage coagulation assay[21].

Hemophilia A Mice

Exon 16 disrupted hemophilia A mice in a C57BL/6 background were originally obtained from Dr. Leon Hoyer (American Red Cross, Holland Lab) and a breeding colony was established[22, 23]. In these experiments 8-12 week old male and female mice were used.

Anti-fVIII Monoclonal Antibodies from Anti-fVIII Hybridomas

Murine anti-fVIII A2 domain MAbs were isolated as previously described[11, 12]. IgG concentrations were calculated using an extinction coefficient at 280 nm of 1.37 (mg/ml)-1 cm-1. All inhibitory titers were calculated using the Bethesda assay with buffered pooled normal plasma as the human fVIII source[11, 24]. *In vitro* characteristics of all anti-A2 MAbs were previously described and are summarized in Table 1[16]. MAbs 4A4 and 2-76 are very high titer type I group A inhibitors. 4A4 blocks the activation of factor X by the intrinsic pathway factor X activation complex and has an inhibitory titer of 40,000 Bethesda units (BU)/mg IgG [15]. MAb 2-76 has an inhibitory titer of 38,000 BU/mg IgG. Group E MAb, 1D4, is also a high titer type I anti-A2 inhibitor with a titer of 7,000 BU/mg IgG. B94 and 2-54 are both type II inhibitors: Neither MAb is able to completely inhibit fVIII at saturating concentrations of MAb. Bethesda titer for B94 cannot be determined because it does not reduce fVIII activity beyond 50% at saturating concentrations. MAb 2-54 has a high titer of 34,000 BU/mg IgG. GMA-012 and 4C7 are non-inhibitory MAbs with titers less than 1 BU/mg IgG.[16] B25 is a type I very low titer MAb with a titer of 100 BU/mg IgG; however, B25, as dosed in this study, has a titer of <1 BU/mL plasma and as such is considered to be non-inhibitory.

Methods:

In vivo Bleeding Model

This bleeding model was performed as previously described with changes as follows[15]: Mice were injected using retro-orbital injections instead of tail vein injections and both injections were set at a volume of 120 µL. Briefly, hemophilia A mice were weighed and anesthesia was induced using 3% isoflurane at a flow of 1000 mL/min using an RC2 Rodent Circuit Controller (VetEquip®, Pleasanton, CA). Mice were then placed on a heating pad with 3% isoflurane at a flow rate of 500mL/min delivered via nose cone. Mice were injected with 120 µL of saline or 120 µL of 0.5 mg/kg MAb diluted in saline. Next, mice were injected with 120 µL of either 180 U/kg or 360 U/kg (about 0.02 mg/kg and 0.04 mg/kg, respectively) of human rBDD fVIII in sterile saline. The peak plasma concentration of MAb *in vivo* was approximately thirteen-fold higher than the peak concentration of the higher dose of fVIII: Peak plasma concentration of MAb was approximately 65 nM, while fVIII was approximately 2.5 nM and 5 nM respectively. Ten minutes prior to tail transection, mice were induced and tails warmed. At 120 minutes after fVIII injection, 4mm of the distal tail was transected using a No. 15 blade scalpel and the proximal tail placed into a new, pre-weighed 15 mL conical tube of normal saline. At the time of tail snip, isoflurane was reduced to 1.75% at a flow rate of 500 mL/min. Mice tails were removed from their tubes at 40 minutes after tail transection, or at time of death. The amount of blood loss was calculated by measuring the change in tube weight in grams with evaporative loss added back. This amount was recorded as mg of blood loss per g of body weight.

In vivo recovery of fVIII

Cardiac puncture was performed on some mice instead of tail transection. At 120 minutes after fVIII injection, these mice were euthanized using carbon dioxide and a cardiac puncture was performed using a 22-gauge needle with syringe. Between 0.5 and 1.0 mL of blood was collected to measure fVIII activity using the one-stage coagulation assay[20]. rBDD human fVIII diluted into E16 mouse plasma was the source of fVIII for the standard curve in these assays.

FVIII inhibitor assays

FVIII inhibitor titers were also measured with the Coatest® SP FVIII kit (Chromogenix, Lexington, MA). Briefly, 25 µl of buffered normal pooled human plasma was incubated for 2 hours at 37°C with 25 µl of MAb diluted in 0.15 M NaCl, 20 mM HEPES, 0.05% (w/v) Tween 80, pH 7.4 (HBS/Tween) or with 25 µl of HBS/Tween as the control. A standard curve based on serial dilutions of pooled normal plasma was used. FVIII levels were measured in triplicate for each sample using the methods of the Coatest® SP FVIII kit.

Statistics

Comparisons between groups were made using the Mann-Whitney U test and Prism 6.0 (GraphPad Software Inc., La Jolla, CA). A P value of less than 0.05 was considered statistically significant.

Approval

Approval for the use of animals in this study and approval of study methods was granted by the Emory University Institutional Animal Care and Use Committee (IACUC). The Emory University School of Medicine Division of Animal Resources (DAR) provided training for the proper handling and euthanasia of animals.

Results

High titer type I anti-A2 antibodies are pathogenic in a murine bleeding model

An *in vivo* bleeding model was established in which blood loss following a 4 mm tail-snip was used to determine the bleeding phenotype in hemophilia A mice injected with BDD human fVIII and various anti-fVIII MAbs. In this model retro-orbital injections were used instead of the previous reported intravenous tail vein injections with similar results in both the positive and negative control animals (data not shown). A total of 131 E16 hemophilia A mice aged between 8-12 weeks were injected retro-orbitally with anti-A2 MAb or normal saline, followed fifteen minutes later by injection of fVIII or normal saline. A total of 11 mice were excluded from analysis due to missed injections or death prior to tail snip. There were between 5 and 10 mice in each group. Mean blood loss in saline control mice was 37.6 mg/g body weight (Table 2, Fig. 1). In contrast, mice that received no MAb and 180 U/kg fVIII had blood loss of 2.5 mg/g body weight. There was a significant decrease in bleeding between the mice that received fVIII compared to the saline control group ($p = 0.001$).

Negative control mice were given two injections of normal saline and had a mean blood loss of 37.6 mg/g body weight and a median blood loss of 42.3 mg/g body weight. Positive control mice were given a single injection of normal saline followed by either a low dose (180U/kg, 2.5 nM) or a high dose (360U/kg, 5.0 nM) of fVIII; both groups of mice had significantly less bleeding than negative controls ($p = 0.001$ and $p < 0.001$, respectively, by Mann-Whitney test).

Experimental mice received MAb at saturating concentrations (peak plasma concentration approximately 65 nM) followed by either low or high dose fVIII. The group A high titer type I inhibitors 4A4 and 2-76 and the group E high titer type I inhibitor 1D4. Mice injected with 4A4 and low-dose fVIII had a median blood loss of 39.7 mg/g body weight. Mice injected with 2-76 and low-dose fVIII had a median blood loss of 40.3 mg/g body weight. Mice injected with 1D4 and low-dose fVIII had a median blood loss of 40.7 mg/g body weight (Table 2). Similar results were seen for the high-dose fVIII groups for all three of these MAbs (Figure 1).

Type II anti-A2 inhibitors are not pathogenic in a murine bleeding model

Group D MAb 2-54 and Group B MAb B94 are both type II inhibitors. They have maximum inhibition of 80% and 40% respectively at saturating concentrations of MAb. In the Bethesda assay B94 never reaches 50% inhibition and thus by definition it cannot be assigned an inhibitory titer while 2-54 has titer of 34,000 BU/mg IgG. Despite the high inhibitory titer, 2-54 had no significant bleeding at either high dose or low dose of fVIII. Mice treated with 2-54 and low dose fVIII had median blood loss of 3.3 mg/g body weight ($p = 0.232$). Mice treated with 2-54 and high dose fVIII had median blood loss of 2.1 mg/g ($p = 0.369$; Table 2). B94 also was not pathogenic at either low or high doses of fVIII. Mice injected with B94 and low-dose fVIII had median blood loss of 3.1 mg/g body weight, and with high-dose fVIII had median blood loss of 7.7 mg/g body weight (Table 2).

Non-inhibitory antibodies are not pathogenic in the murine bleeding model

4C7 and GMA-012 are non-inhibitory anti-A2 antibodies with titers less than 1 BU/mg IgG. B25 is a group C very low titer type I inhibitor (100 BU/mg IgG) with estimated inhibitory titer of <1 BU/ml plasma under the experimental conditions. Mice treated with 4C7 and low dose fVIII had a median blood loss of 0.0 mg/g (mean of 0.3 mg/g; p = 0.273). MAb GMA-012 produced a median blood loss of 0.0 mg/g (mean of 6.3 mg/g; p = 0.838) when administered with low dose fVIII. When administered with high dose fVIII, median blood loss was 1.1 mg/g (mean 1.3 mg/g; p = 0.767; Table 2). Mice treated with B25 and low dose fVIII had no significant bleeding, with median blood loss of 0.55 mg/g body weight (p = 0.714). Because B25 and 4C7 produced very little blood loss at low doses of fVIII, they were not tested at high doses of fVIII (Table 2).

Residual fVIII activity at the time of tail snip correlates with bleeding phenotype

Residual fVIII levels were evaluated for hemophilia A mice injected with saturating concentrations of MAb followed by fVIII at doses ranging from 0-720 U/kg (0-10 nM) (Figure 2). 2-76 and 1D4 completely inhibited fVIII activity throughout the dose range as 4A4 did in a previous study[15]. In contrast, there was a dose-dependent increase in fVIII recovery in mice treated with 2-54, B94, and GMA-012. These MAbs all produced residual fVIII levels of at least 0.4 U/mL, which is comparable to a 40% residual fVIII activity level.

Discussion

In this study we found that within the A2 domain of fVIII only the high titer type I inhibitors (35, 190, and 200 BU/ml) were pathogenic at both 180 U/kg and 360 U/kg fVIII in a murine bleeding model. These results were consistent with the residual fVIII activity at time of injury. Mice injected with these MAbs had residual fVIII activity levels similar to mice given only saline despite injections of up to 720 U/kg fVIII. The non-inhibitory MAbs did not cause bleeding. In addition the type II inhibitors B94 and 2-54 did not cause bleeding. B94 is a relatively week inhibitor of fVIII. It inhibits 40% of fVIII activity in the Bethesda assay at concentrations of 0.01 mg/ml. 2-54 is a type II inhibitor with a titer of 34,000 BU/mg IgG (170 BU/ml in this experimental design) that has maximum inhibition of 80%.

The discrepancies seen in this bleeding model between inhibitory titer and bleeding phenotype are also seen in patients with inhibitors. The Bethesda assay, as originally described, is based on the one-stage coagulation assay and is a sensitive and specific test in the diagnosis of clinically significant fVIII inhibitors[24]. Although the Bethesda assay is useful for the detection of clinically significant fVIII inhibitors, the inhibitor titer and the residual fVIII activity in the plasma are not necessarily good predictors of clinical severity[25]. Patients with acquired hemophilia often have type II inhibitors and in a recent observational study of these patients in United Kingdom, fVIII levels and inhibitor titers at presentation were not predictive of the severity of bleeding events[26]. In that study, the median fVIII level and inhibitory titers were nearly identical for patients with fatal bleeding events compared to those who did not require treatment for their bleeding symptoms. In

addition, it has also been reported that patients with high-titer inhibitors have had a clinical response to high doses of fVIII despite being given significantly less fVIII than would be needed to bind all circulating anti-fVIII antibody[27, 28].

In our studies, not only did the bleeding phenotype differ between type I and type II inhibitors, it also differed among the type II inhibitors based on whether the MAb was directed to the A2 or the C2 domain. 2-54, anti-A2 group D, has similar inhibitory titer and kinetics in the one stage coagulation assay as the non-classical C2 antibodies 2-77 (25,000 BU/mg IgG or 125 BU/ml). In addition both 2-54 and 2-77 have only minimal inhibition in another *in vitro* assay, the thrombin generation assay[29]. However in this murine bleeding model 2-54 does not cause bleeding while 2-77 causes bleeding at 180 U/kg fVIII; however, this bleeding phenotype is overcome by giving a higher dose of fVIII. These anti-fVIII MAbs inhibit fVIII by different mechanisms. The anti-A2 MAb, 2-54, inhibits thrombin cleavage of both the heavy chain and light chain of fVIII while the anti-C2 MAb, 2-77, appears to inhibit the activation of fVIII. [11, 15, 16] This suggests that differences in mechanism of action of inhibitors to different fVIII epitopes may not be detected in the classic *in vitro* assays but are important to the *in vivo* bleeding phenotype.

Currently, treatment of inhibitor patients is based primarily on the titer of the fVIII inhibitory antibody as measured by the Bethesda assay. Bleeding episodes for patients with inhibitory titers greater than 10 BU/ml are typically managed with bypassing agents—recombinant activated factor VII (fVIIa) and/or activated prothrombin complex concentrate (aPCC)—while regular exposure to fVIII is used for immune tolerance. Although the use of bypassing agents has significantly improved the treatment of bleeding episodes in hemophilia A patients who develop inhibitors, some patients—for unknown reasons—have

a poor hemostatic response to bypass therapy[30]. In the C2 domain the pathogenicity of high titer non-classical anti-C2 inhibitors can be overcome by increasing the dose of fVIII. In the A2 domain the high titer group E antibody 2-54 is not pathogenic. In contrast classical C2 antibodies with 10-20 fold lower inhibitory titers were pathogenic. The results from our A2 and C2 studies show that antibody epitope differences may explain why clinical studies have shown that response to fVIII therapy and bleeding phenotype do not necessarily correlate with the degree of inhibition measured in the Bethesda assay. Mapping of inhibitor epitopes may help better predict which high titer inhibitor patients will benefit from fVIII therapy. Further clinical studies are needed.

Acknowledgements

This project was supported by National Institutes of Health (NIH) grant U54 HL112309 and Hemophilia of Georgia, Inc. (S.L.M.).

Authorship

JE, SLM designed research, performed research, analyzed data and wrote the manuscript; WHB, RM, ETP performed research and analyzed data.

RM helped collect data for table 1.

Disclosure of Conflicts of Interest

The authors state that they have no conflict of interest.

Figures

Table 1

Name	Domain	Group	Epitope	BU/mg IgG	BU/ml plasma*	Type
2-76	A2	A	Arg484-Ile508	38,000	190	I
4A4	A2	A	Asp403-His444	40,000	200	I
B94	A2	B	Arg541-Glu604	Indeterminate‡	N/A	II
B25	A2	C	His444-Gln468	100	<1	I
2-54	A2	D	Glu604-Arg740	34,000	170	II
GMA-012	A2	D	Glu604-Arg740	<1	<1	N/A
4C7	A2	E		<1	<1	N/A
1D4	A2	E	Glu604-Arg740	7,000	35	I

* Calculated using estimated plasma volume and antibody concentration

‡ Residual fVIII activity at saturating concentrations of MAb was >50%

Table 1. Characteristics of anti-A2 MAbs

Antibody	Median Blood Loss (mg/g of body weight)		Mean Blood Loss (mg/g of body weight)	
	Low Dose	High Dose	Low Dose	High Dose
No fVIII control	42.3		37.6	
No MAb control	0.9	0.8	2.5	7.0
2-76	40.3*	38.1*	39.1	38.2
4A4	39.7*	31.2*	38.2	32.1
B94	3.1	7.7	8.5	14.6
B25	0.6	-	1.4	-
2-54	3.3	2.1	11.2	14.2
GMA-012	0.0	1.1	6.3	1.3
4C7	0.0	-	0.3	-
1D4	40.7*	41.3*	36.1	35.1

* ($P < 0.05$, Mann-Whitney U test)

Table 2. Antibody-dependent blood loss in hemophilia A mice as a function of fVIII dose

Figure 1. Bleeding produced by anti-A2 MAbs in a murine tail snip model

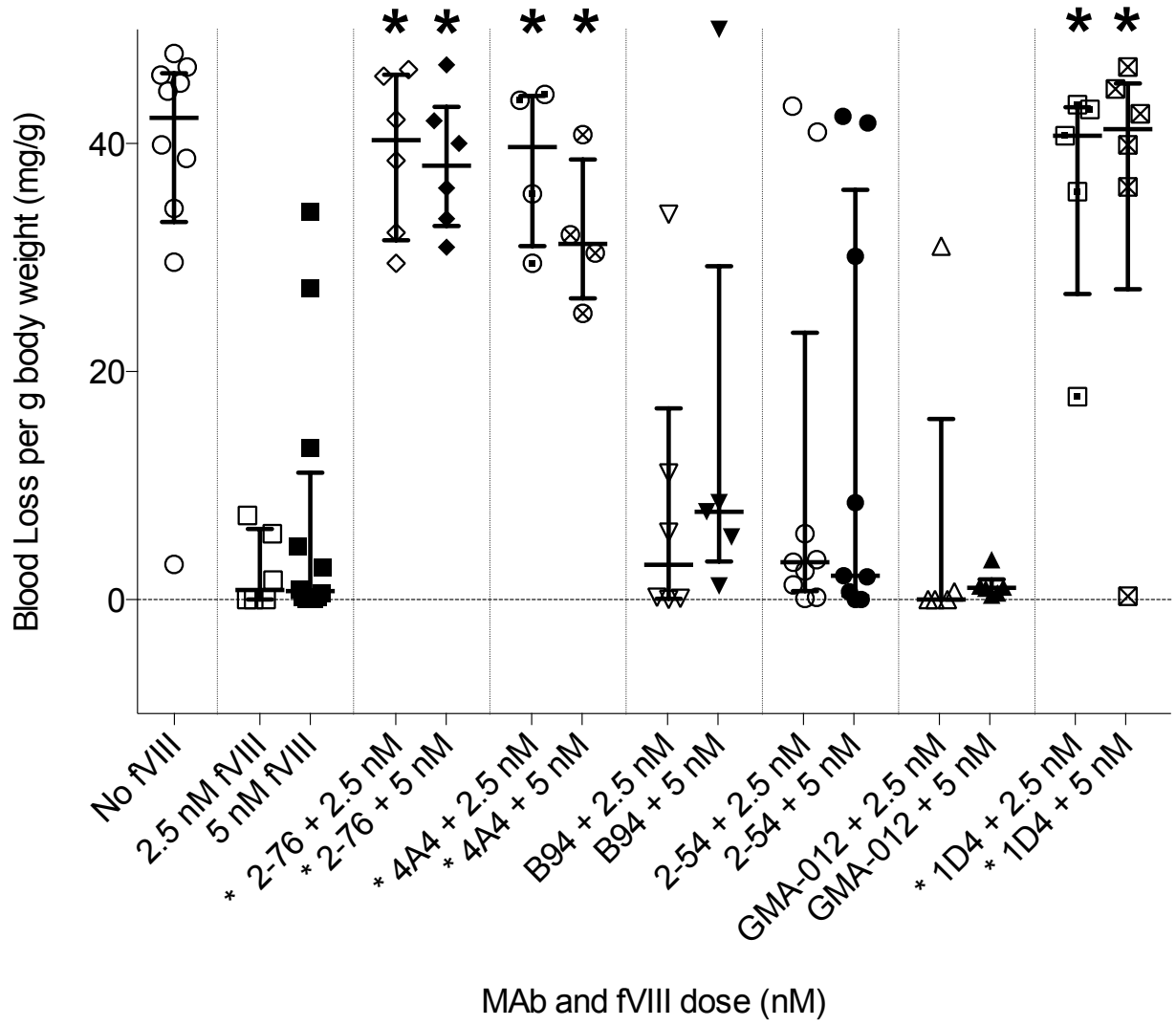


Figure 1 Legend: **Bleeding produced by anti-fVIII MAbs in a murine tail snip model. Hemophilia A mice were injected with 0.5 mg/kg MAb followed either 180 U/kg (2.5 nM) or 360 U/kg (5.0 nM) rBDD fVIII.** Error bars represent median with interquartile

Figure 2. *In vivo* recovery of fVIII in the presence of saturating concentrations of anti-fVIII MAbs

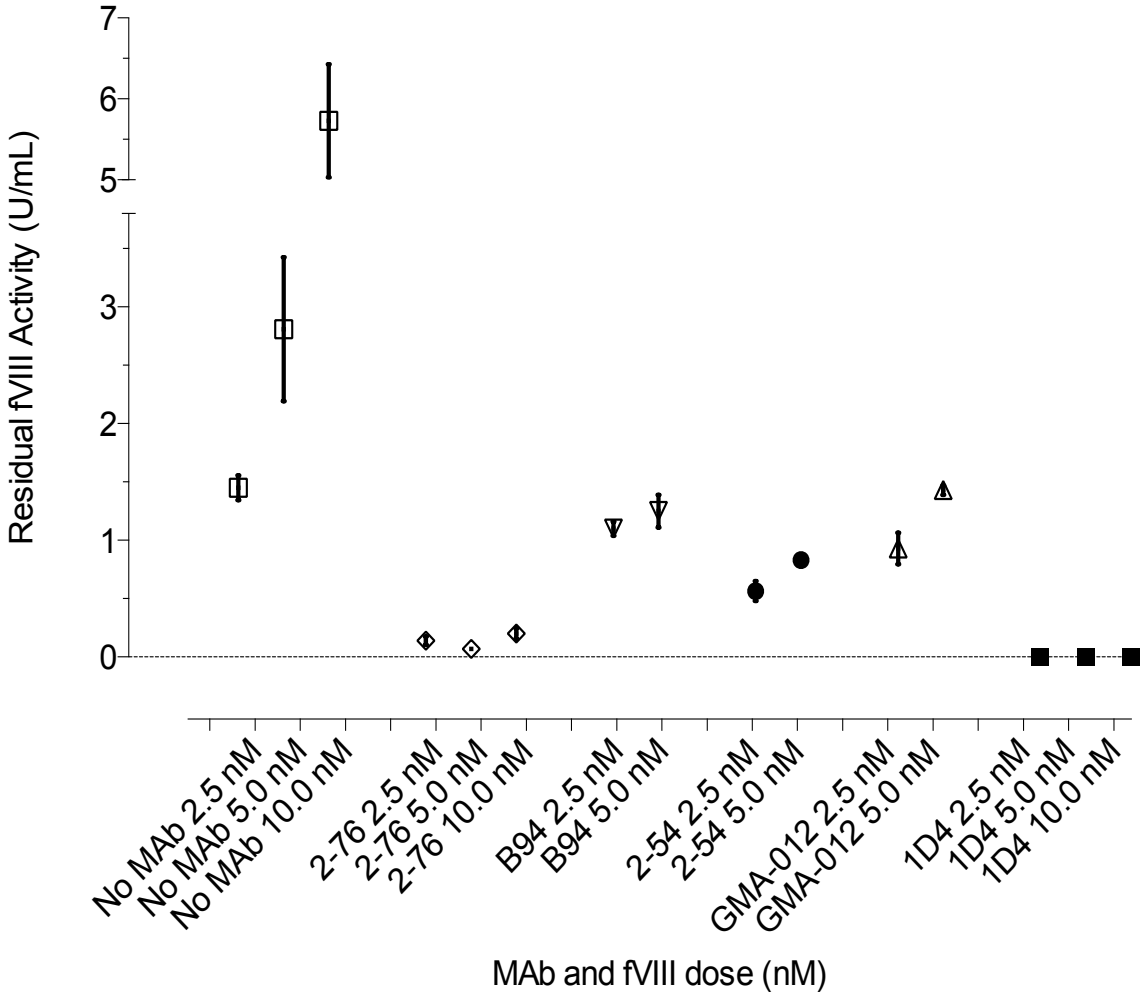


Figure 2. Blood was obtained by cardiac puncture 120 min after injection of rBDD fVIII to the indicated peak plasma concentrations in the presence of saturating concentrations (~65 nM) of the indicated MAb. FVIII recovery was determined by one-stage coagulation assay. Error bars represent SEM (each group n=2 or 3).

References

1. Bray GL, Gomperts ED, Courter S, Gruppo R, Gordon EM, Manco-Johnson M*, et al.* A multicenter study of recombinant factor VIII (recombinate): safety, efficacy, and inhibitor risk in previously untreated patients with hemophilia A. The Recombinate Study Group. *Blood* 1994,**83**:2428-2435.
2. Kreuz W, Ettingshausen CE, Zyschka A, Oldenburg J, Saguer IM, Ehrenforth S*, et al.* Inhibitor development in previously untreated patients with hemophilia A: a prospective long-term follow-up comparing plasma-derived and recombinant products. *Semin Thromb Hemost* 2002,**28**:285-290.
3. Lusher JM, Arkin S, Abildgaard CF, Schwartz RS. Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy, and development of inhibitors. Kogenate Previously Untreated Patient Study Group. *N Engl J Med* 1993,**328**:453-459.
4. Lusher JM, Lee CA, Kessler CM, Bedrosian CL. The safety and efficacy of B-domain deleted recombinant factor VIII concentrate in patients with severe haemophilia A. *Haemophilia* 2003,**9**:38-49.
5. Lollar P, Parker CG. Subunit structure of thrombin-activated porcine factor VIII. *Biochemistry* 1989,**28**:666-674.
6. Fay PJ, Koshibu K. The A2 subunit of factor VIIIa modulates the active site of factor IXa. *The Journal of biological chemistry* 1998,**273**:19049-19054.
7. Jenkins PV, Dill JL, Zhou Q, Fay PJ. Contribution of factor VIIIa A2 and A3-C1-C2 subunits to the affinity for factor IXa in factor Xase. *Biochemistry* 2004,**43**:5094-5101.
8. Lollar P, Parker ET. Structural basis for the decreased procoagulant activity of human factor VIII compared to the porcine homolog. *The Journal of biological chemistry* 1991,**266**:12481-12486.
9. Prescott R, Nakai H, Saenko EL, Scharrer I, Nilsson IM, Humphries JE*, et al.* The inhibitor antibody response is more complex in hemophilia A patients than in most nonhemophiliacs with factor VIII autoantibodies. Recombinate and Kogenate Study Groups. *Blood* 1997,**89**:3663-3671.
10. Gawryl MS, Hoyer LW. Inactivation of factor VIII coagulant activity by two different types of human antibodies. *Blood* 1982,**60**:1103-1109.
11. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Antihuman factor VIII C2 domain antibodies in hemophilia A mice recognize a functionally complex continuous spectrum of epitopes dominated by inhibitors of factor VIII activation. *Blood* 2007,**110**:4234-4242.
12. Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. The humoral response to human factor VIII in hemophilia A mice. *J Thromb Haemost* 2007,**5**:512-519.
13. Saenko EL, Shima M, Gilbert GE, Scandella D. Slowed release of thrombin-cleaved factor VIII from von Willebrand factor by a monoclonal and a human

antibody is a novel mechanism for factor VIII inhibition. *J Biol Chem* 1996,**271**:27424-27431.
14. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Nonclassical anti-C2 domain antibodies are present in patients with factor VIII inhibitors. *Blood* 2008,**112**:1151-1153.
15. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Non-classical anti-factor VIII C2 domain antibodies are pathogenic in a murine in vivo bleeding model. *J Thromb Haemost* 2009,**7**:658-664.
16. Markovitz RC, Healey JF, Parker ET, Meeks SL, Lollar P. The diversity of the immune response to the A2 domain of human factor VIII. *Blood* 2013,**121**:2785-2795.
17. Barrow RT, Healey JF, Gailani D, Scandella D, Lollar P. Reduction of the antigenicity of factor VIII toward complex inhibitory antibody plasmas using multiply-substituted hybrid human/porcine factor VIII molecules. *Blood* 2000,**95**:564-568.
18. Healey JF, Barrow RT, Tamim HM, Lubin IM, Shima M, Scandella D*, et al.* Residues Glu2181-Val2243 contain a major determinant of the inhibitory epitope in the C2 domain of human factor VIII. *Blood* 1998,**92**:3701-3709.
19. Lollar P, Fay PJ, Fass DN. Factor VIII and factor VIIIa. *Methods Enzymol* 1993,**222**:128-143.
20. Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. High level expression of recombinant porcine coagulation factor VIII. *J Biol Chem* 2002,**277**:38345-38349.
21. Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 1995,**4**:2411-2423.
22. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH, Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* 1995,**10**:119-121.
23. Bi L, Sarkar R, Naas T, Lawler AM, Pain J, Shumaker SL*, et al.* Further characterization of factor VIII-deficient mice created by gene targeting: RNA and protein studies. *Blood* 1996,**88**:3446-3450.
24. Kasper CK, Aledort L, Aronson D, Counts R, Edson JR, van Eys J*, et al.* Proceedings: A more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975,**34**:612.
25. Hay CR. Acquired haemophilia. *Baillieres Clin Haematol* 1998,**11**:287-303.
26. Collins PW, Hirsch S, Baglin TP, Dolan G, Hanley J, Makris M*, et al.* Acquired hemophilia A in the United Kingdom: a 2-year national surveillance study by the United Kingdom Haemophilia Centre Doctors' Organisation. *Blood* 2007,**109**:1870-1877.
27. Negrier C. Inhibitors to factor VIII: treatment of acute bleeds. In: *Textbook of Hemophilia*. Edited by Lee CA, Berntorp, E.E., Hoots, W.K., editors: Blackwell Publishing Ltd.; 2005. pp. 80-85.
28. Luna-Zaizar H, Esparza-Flores MA, Lopez-Guido B, Aguilar-Lopez LB, Cortes Alvarez CR, Jaloma-Cruz AR. Kinetics of factor VIII:C inhibitors and treatment response in severe hemophilia A patients. *Int J Lab Hematol* 2009,**31**:673-682.

29. Doshi BS, Gangadharan B, Doering CB, Meeks SL. Potentiation of thrombin generation in hemophilia A plasma by coagulation factor VIII and characterization of antibody-specific inhibition. *PLoS One* 2012,**7**:e48172.
30. Astermark J, Santagostino E, Keith Hoots W. Clinical issues in inhibitors. *Haemophilia* 2010,**16 Suppl 5**:54-60.

Expanding the Ortholog Approach for Hemophilia Treatment Complicated by Factor VIII Inhibitors

P.M. Zakas[1], K. Vanijcharoenkarn[2], R.C. Markovitz[3], S.L. Meeks, MD[4] and C.B. Doering, PhD[4**]

[1] Graduate Program in Molecular and Systems Pharmacology, Laney Graduate School, Emory University, Atlanta, GA

[2] School of Medicine, Emory University, Atlanta, GA

[3] Graduate Program in Immunology and Molecular Pathogenesis, Laney Graduate School, Emory University, Atlanta, GA

[4]Aflac Cancer and Blood Disorders Center, Emory University/Children's Healthcare of Atlanta, Atlanta, GA

**Corresponding Author: Christopher B. Doering, PhD, Emory Children's Center 2015 Uppergate Drive, Rm 450, Atlanta, Georgia 30322, cdoerin@emory.edu, (404) 727-7988

This work was supported by grants from the National Institute of Health: C.B.D. – HL092179; C.B.D. and S.L.M. – HL112309, P.M.Z. – 5T32GM008602, and from Hemophilia of Georgia: S.L.M.

Background: The formation of neutralizing antibodies (inhibitors) directed against human coagulation factor VIII (hFVIII) is a life-threatening pathogenic response that occurs in 20–30% of severe congenital hemophilia A patients and 0.00015% of remaining population (i.e. acquired hemophilia A). Interspecies amino acid sequence disparity among FVIII orthologs represents a promising strategy to mask FVIII from existing inhibitors while retaining procoagulant function. Evidence for the effectiveness of this approach exists in clinical data obtained for porcine FVIII products, which have demonstrated efficacy in the setting of congenital and acquired hemophilia.

Objectives: In the current study, recombinant (r) ovine FVIII (oFVIII), was evaluated for antigenicity and procoagulant activity in the context of human patient-derived and murine model-generated FVIII inhibitors.

Methods: The antigenicity of roFVIII was assessed using i) inhibitor patient plasma samples, ii) murine anti-FVIII MAbs, iii) immunized murine hemophilia A plasmas, and iv) an in vivo model of acquired hemophilia A

Results: Overall, roFVIII demonstrated reduced reactivity to, and inhibition by, anti-hFVIII immunoglobulin in patient plasmas. Additionally, several hFVIII epitopes were predicted and empirically shown not to exist within roFVIII. In a murine hemophilia A model designed to mimic clinical inhibitor formation, it was demonstrated that inhibitor titers to roFVIII were significantly reduced compared to the orthologous immunogens, rhFVIII or rpFVIII. Furthermore in a murine model of acquired hemophilia A, roFVIII administration conferred protection from bleeding following tail transection.

Conclusion: These data support the investigation of FVIII orthologs as treatment modalities in both the congenital and acquired FVIII inhibitor settings.

Keywords: Hemophilia A, congenital; Hemophilia A, acquired; Factor VIII; Antibody Specificity

Introduction

Factor VIII (FVIII) is a procofactor in the intrinsic pathway of blood coagulation. Deficiency of FVIII activity resulting from genetic mutation of the X-chromosome-linked *F8* gene presents as a bleeding disorder, termed hemophilia A, that has a reported prevalence of 1 in 7,800 males [1]. Treatment consists of lifelong protein replacement via intravenous infusions of recombinant (r) or plasma-derived (pd) human (h) FVIII products. Upon repeated exposure, approximately 20-30% of severe hemophilia A patients develop inhibitory anti-hFVIII alloantibodies (inhibitors). In countries where replacement therapy is available, the immune response to hFVIII is the most significant complication affecting the management of patients with hemophilia A. Additionally, autoantibodies to hFVIII develop in non-hemophiliacs at a rate of 1.48/million/year producing an autoimmune condition termed acquired hemophilia A, which frequently results in life- or limb-threatening bleeding. [2-5]

On the molecular level, FVIII displays a domain structure A1-A2-B-*ap*-A3-C1-C2 where the A and C domains are defined by internal sequence homology and the heavy and light chains are separated by an activation peptide (*ap*) [6]. Although antibodies targeting each of the hFVIII domains can be found in patient plasmas, the A2 and C2 domains appear to contain the dominant immunogenic and inhibitory epitopes [7-9]. Epitopes, mechanisms of action, and kinetics have been defined for a large collection of anti-A2 and C2 domain murine monoclonal antibodies (MAbs)

demonstrating that the murine hemophilia A model recapitulates many features of the anti-FVIII immune response observed in humans [10-12]. Recently, high resolution structural data of anti-hFVIII MAbs in complex with the FVIII C2 domain using small angle x-ray scattering, x-ray crystallography, and hydrogen-deuterium exchange mass spectrometry was obtained and has brought the understanding of inhibitor mechanism of action to the atomic level [13-15].

Treatment options for hemophilia A patients with inhibitors are limited in terms of availability and efficacy. For example, in Immune Tolerance Induction (ITI) studies, the frequent administration of FVIII product at doses as high as 200 IU/kg/day, is effective at eradicating inhibitors in up to 70% of patients [16]. However, due to FVIII product supply constraints and expense, ITI is not an option for the majority of patients with hemophilia A. Aside from eradicating inhibitors, acute and frequently life-threatening bleeding can be treated in this setting using FVIII bypassing agents (e.g. activated prothrombin complex concentrate or activated recombinant factor VII). Plasma-derived porcine FVIII (pd-pFVIII) products also have been utilized although they no longer are available. However, a recombinant pFVIII (rpFVIII) product is under clinical development for acquired hemophilia A. The rationale for use of pd- or rpFVIII products stems from the presence of non-conserved amino acid sequence differences that confer reduced antigenicity and inhibition. Furthermore, it has been shown that there are several species-specific differentials in non-immunological properties between rh- and rp-FVIII [17, 18] and preclinical evidence exists to support the benefit of utilizing FVIII orthologs, i.e. proteins from

different species that evolved from a common ancestral gene, or hybrid FVIII molecules engineered to possess sequences from multiple orthologs, in gene therapy applications [19-23].

A naturally occurring ovine model of severe hemophilia A has been identified and the responsible genetic lesion and disease phenotype was characterized [24]. Additionally, the ovine FVIII (oFVIII) ortholog was generated in recombinant form thereby facilitating biochemical characterization. B-domain-deleted (BDD) recombinant ovine FVIII (roFVIII) has been shown to display greater specific activity, prolonged half-life following activation by thrombin, functionality in a hFVIII-deficient plasma bioassay and efficacy in a murine hemophilia A tail-transection bleeding model [25]. As the rationale for clinical use of pFVIII is based on reduced antigenicity achieved through differential amino acid sequence, herein we sought to investigate the potential therapeutic utility of roFVIII which also contains a distinct repertoire of non-conserved amino acids, but still possesses procoagulant function in human plasma. Outside of the B-domain, ovine and porcine FVIII share 86 and 83% amino acid identity to human FVIII, respectively [24, 26]. We hypothesized that roFVIII would be less antigenic than hfVIII in plasma from human patients and hemophilia A mice with inhibitors and that there would be inter-patient differentials in the antigenicity to each FVIII ortholog. To test these ideas, the antigenicity of roFVIII was assessed using i) inhibitor patient plasma samples, ii) a collection of well-characterized murine anti-FVIII MAbs that mimic human inhibitors [10-12], iii) plasmas from hemophilia A mice immunized with

either rhFVIII or rpFVIII, and iv) an in vivo bleeding challenge assay designed to model designed to model acquired hemophila A.

.

Materials and Methods

Materials

Pooled citrated normal plasma (FACT) and FVIII-deficient plasma were purchased from George King Biomedical (Overland Park, KS). Automated APTT reagent was purchased from Trinity Biotech (Wicklow, Ireland). Inhibitor patient plasmas were drawn and banked in accordance with Emory University IRB protocol no. IRB00006290. Acquired patient samples were generously donated by Dr. David Green (Feinberg School of Medicine of Northwestern University, Chicago, IL). Patients were selected for inclusion if inhibitor titers against human exceeded 5 BU/ml and sufficient plasma was available. Streptavidin-alkaline phosphate conjugate was purchased from Jackson Immuno Research (West Grove, PA). Goat anti-mouse IgG-alkaline phosphatase conjugate and alkaline phosphatase substrate kit (AP pNPP) was purchased from Bio-Rad (Hercules, CA). Dimethyl pimelimidate was purchased from Thermo Scientific (Rockford, IL) and Protein A/G Plus was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Domain specific monoclonal antibodies were generated and purified as previously described [12]. MAb ESH-8 was purchased from American Diagnostica (Stamford CT). MAbs 413 and CLB-Cag 9 were gifts from American Red Cross (Rockville, MD) and Dr. Jan Voorberg (Sanquin-AMC Lamdsteiner Laboratory, Amsterdam, The Netherlands). BDD roFVIII, rpFVIII, and rhFVIII were generated and purified as described previously [25, 27]. Full-length rhFVIII was a generous gift from Hemophilia of Georgia. OTII C57BL/6 mice were purchased from The Jackson Laboratory (Bar

Harbor, Maine) and kept in accordance with Emory IACUC. Exon 16-disrupted hemophilia A mice have been previously described [28].

Inhibitor plasma and monoclonal competition ELISA

Competition ELISAs were performed as previously described [10, 11, 29]. Controls for each MAb against the A2 or C2 domain were replicated 11 and 16 times, respectively. Competition was defined as a reduction of signal greater than 2 standard deviations (SD) from the control mean. Only patient plasmas that displayed predominantly A2 and/or C2 specificity, as determined by homolog-scanning ELISA using human/porcine FVIII hybrids revealed (data not shown), were selected for competition ELISA analysis.

Cross-reactivity of inhibitor plasma and MAbs

An indirect ELISA was performed using plates containing adsorbed rhFVIII, rpFVIII, and roFVIII to which serial dilutions of patient plasma or MAbs were added followed by detection using goat anti-mouse AP-conjugated secondary antibody. ELISA titration curves were fitted to the 4-parameter logistic equation. The dilution of inhibitor plasma required to produce A_{405} of 0.3 was calculated by interpolation and compared across orthologs. The absorbance threshold was set as an arbitrary point in which colorimetric signal is approximately three times background while substrate remains in excess. MAb interactions that did not achieve an absorbance at 405 nm of 0.3 at the lowest dilution (10-fold molar excess) were designated as non-reactive or below the limit of detection.

FVIII inhibitor titer assays: patient plasma, mouse plasma, and MAbs

FVIII inhibitor titers against rhFVIII, rpFVIII, and roFVIII were measured using a modified Bethesda assay previously described [30]. For determination of the ortholog titers, pooled citrated FVIII-deficient plasma was combined with 0.8-1.2 units/ml of rpFVIII or roFVIII and buffered with 100 mM imidazole. Due to limited availability of plasma, not all patients could be fully screened. MAb inhibitor titer was calculated similarly using dilutions of MAbs at known concentrations and reported in BU/mg IgG.

Immunization of hemophilia A E16 F8$^{-/-}$ mice

Mice were immunized with BDD rhFVIII or rpFVIII as described previously [31]. Mice received six tail vein injections of 10 μg/kg FVIII at 7-day intervals followed by a final injection of 25 μg/kg FVIII two weeks after the sixth dose. Subsequently, terminal plasma collections were performed and these samples were used to determine inhibitor titer by modified Bethesda assay.

Murine acquired hemophilia A hemostatic challenge

In a blinded study, 8-12 week old C57BL/6 OTII mice received 10ug anti-A2 domain MAb 4A4 via intraperitoneal (IP) injection. After 15 minutes, the mice were administered 9 units ofrecombinant fVIII or saline via intravenous tail vein injection. Hemostatic challenge was performed 2 hours afterrecombinant fVIII

administration by tail transection at 2mm diameter and total blood loss over 40 minutes was recorded as previously described [25, 32].

Anti-oFVIII MAb generation, and domain specificity immunoprecipitations

Three 9-12 week old E16 $F8^{-/-}$ mice were injected with 1μg roFVIII each diluted in 100μl saline via tail vein each week for 7 weeks. Inhibitor titers were taken at week 8 via ELISA and Bethesda assay as described above. Two weeks after the last injection, mice were administered 1.25 μg roFVIII via tail vein. Three days after final immunization, the mouse with the highest ELISA and Bethesda titer was sacrificed and MAbs were generated and purified as previously described [12]. The 9 resulting MAbs were used to immunoprecipitate roFVIII following activation by thrombin and visualized by SDS-PAGE as previously described [25].

Results

RoFVIII displays reduced IgG binding and inhibition in inhibitor patient plasmas

As an expansion of the ortholog approach to hemophilia A treatment in the context of FVIII inhibitors, roFVIII was investigated for binding to IgG present in inhibitor patient plasmas (i.e. antigenicity) and resistance to inhibition conferred by the same. An indirect ELISA-based screen of 26 congenital and 10 acquired hemophilia A patient plasmas demonstrated reduced reactivity of plasma IgG to both roFVIII and rpFVIII compared to hFVIII with median values of 35.6 and 49.3%, respectively (Figure 1A). No significant difference was observed between roFVIII and rpFVIII (P = 0.097; Mann-Whitney U test). Of the 36 plasmas tested, 32 displayed reduced reactivity to both roFVIII and rpFVIII and of these 22 demonstrated less reactivity to roFVIII compared to rpFVIII.

To measure inhibitor titers, a modified Bethesda assay utilizing the three FVIII orthologs was implemented. This analysis revealed that inhibitory titers against both roFVIII and rpFVIII were statistically reduced compared to hFVIII ($P < 0.05$) although they were not distinguishable from each other ($P > 0.05$; Kruskal-Wallis One Way ANOVA) with median titers of 7.25 (roFVIII), 4.4 (rpFVIII), and 34 BU/mL (rhFVIII) (Figure 1B). Clinical experience shows that patients with inhibitor titers less than 5 often respond to high dose hFVIII replacement therapy while patients with inhibitor titers >10 BU/ml generally are not considered candidates for hFVIII infusion therapy [33]. Twenty-nine of the patient plasmas studied possessed

inhibitor titers above 10 BU/mL against hFVIII and of those, 21 had <10 BU/mL titers against rpFVIII or roFVIII. Furthermore, 5 of the plasma samples assayed harbored comparatively lower titers against roFVIII than rpFVIII and 2 of these plasmas had titers >10 BU/ml against both rhFVIII and rpFVIII suggesting that roFVIII exclusively might be effective in certain populations of inhibitor patients. Due to limited availability of certain patient plasmas, 2 patient plasmas could not be tested for inhibitor titer and an additional sample (from patient 17) could not be tested for rpFVIII inhibitor titer. Significant correlations were observed between the ELISA and Bethesda titers determined for rpFVIII and roFVIII (P = 0.0028, and 0.0003, respectively, Student's two-tailed t distribution), but no significant correlation was observed for rhFVIII (*P* = 0.4913; Figure 1C). Correlation coefficients for rhFVIII, rpFVIII, and roFVIII are 0.0145, 0.354, and 0.3827 respectively. These data demonstrate that hFVIII titers are not predictive of each other given that similar inhibitor titers spanned two orders of magnitude of ELISA titer. Inhibitor titers against rpFVIII or roFVIII were consistently refined within only one order of magnitude.

Distribution of A2 and C2 epitopes targeted by inhibitor patient plasmas

Inhibitor bank plasmas were screened for domain specificity by homolog-scanning ELISA incorporating single domain human/porcine hybrid molecules as described previously [12] (data not shown). Twenty patient plasmas of the initial 36 were shown to contain anti-hFVIII antibodies predominantly against the A2, C2, or both domains (Table 1). For 18/20 patients, there was sufficient plasma available to

interrogate the targeted A2 and C2 epitopes by competition with panels of MAbs known to recognize non-overlapping epitopes in these domains (Figure 2) [10, 11]. Due to limited plasma availability, a single MAb was used to represent each inhibitor group. Additional A2 – A and C2 – BC MAbs were added because of their clinical prevalence and inhibitor potency and efficacy. Successful competition with at least one of the A2 and/or C2 domain targeting MAbs was demonstrated for 15/18 patient plasmas. Furthermore, at least one patient plasma competed with each group of MAbs within the C2 domain but only 3/7 groups within the A2 domain. Within the C2 domain, 12/14 patient plasmas competed with group BC MAbs followed by 8/14 with groups A and C, 4/14 with group B, and 3/14 with group AB. In contrast, only 3/7 groups of A2 domain targeting MAbs were competed by the patient plasmas. Overall, the A2 epitopes targeted by the patient plasmas appeared to overlap primarily with those targeted by A2 – A (6/10 plasmas) followed by A2 – E (2/10 plasmas) and then A2 – B (1/10 plasmas) with no overlap/competition observed with A2 – BCD, – C, – D or – DE MAbs. Of note, MAb groups A2 – A and C2 – BC contain the most potent inhibitory MAbs and are the most prevalent groups with which patient plasma ELISA competition was observed.

Inhibitory MAbs display reduced reactivity to orthologous FVIII molecules

To determine if the reduced reactivity and inhibition of rpFVIII and roFVIII was due to the absence of inhibitory epitopes, we tested the ability of anti-hFVIII A2 and C2 domain specific MAbs known to possess varying inhibitor titers and kinetics to bind and inhibit roFVIII via indirect ELISA. Within the A2 domain, cross-reactivity only

was observed with two MAbs, 4F4 and G48, representing inhibitor groups A2 – B and A2 – C, respectively (Figure 3). These antibodies previously were shown to have low human FVIII specific inhibitory activities of 330 and 5 BU/mg IgG respectively. To confirm that the diminished ELISA binding observed correlated with decreased inhibitor titers, specific inhibitory titers were measured for each MAb against roFVIII and rpFVIII (Table 2). Both MAb 4F4 and G48 demonstrated no detectable titer against roFVIII or rpFVIII. None of the high specific inhibitory activity hFVIII A2 domain targeting MAbs demonstrated cross-reactivity to either FVIII ortholog.

Cross-reactivity within the C2 domain, however, revealed moderate roFVIII-specific cross-reactivity with 3 inhibitors, I14, B75, and I55, at 30 – 50% of hFVIII reactivity. MAbs I14, B75, and I55 are characterized by specific inhibitory activities of 44,000, <1, and 10,000 BU/mg IgG against hFVIII, respectively. Of these, only I14 possessed an inhibitor titer to roFVIII with a specific inhibitory activity between 100 – 1,000 BU/mg IgG. An additional 3 MAbs, D102, G99, and 3G6, cross-reacted with roFVIII at 5 – 15% of the hFVIII level however, inhibitor titers against the orthologs were again nominal. Cross-reactivity against rpFVIII was observed only with two MAbs, 2-117 and D102, and the percent reactivity was between 5 – 10% that of the reactivity to hFVIII.

Due to the selection bias against identification of cross-reactive hFVIII C2 domain targeting MAbs, a similar pool of MAbs, this time generated against rpFVIII, were tested for cross-reactivity with rhFVIII and roFVIII. Ten MAbs with measurable

inhibitor titers against rpFVIII were selected and tested by indirect ELISA. MAbs targeting the A1 or A2 domains of rpFVIII did not show any cross-reactivity to hFVIII or roFVIII, however all anti-rpFVIII light chain (A3 – C1 – C2) MAbs demonstrated cross-reactivity to roFVIII exceeding rpFVIII for all four C2 domain targeting MAbs (Figure 4). However, measurement of the specific inhibitory activities of these MAbs revealed near zero inhibition of either hFVIII or roFVIII (data not shown).

Pre-immunized hemophilia A mice display reduced inhibitor titer to roFVIII

To study the reactivity of anti-rFVIII immune plasma to roFVIII, samples from hemophilia A mice immunized with rhFVIII (n = 6) or rpFVIII (n = 10) were obtained from a previous study [31]. All mice displayed inhibitor titers to the specific immunogen of ≥ 10 BU/ml. However when tested for inhibition of roFVIII, all mice demonstrated reduced inhibitor titers against roFVIII as compared to the FVIII immunogen with mean reduction of 22 and 31 fold (P = 0.021 and 0.007, respectively for rhFVIII and rpFVIII; Paired t-test; Figure 5).

RoFVIII restores procoagulant function in an in vivo acquired hemophilia A model

As each of the previous studies utilized in vitro surrogate assays for prediction of hemostatic function, an in vivo assay was developed to assess roFVIII functionality in vivo in a model of acquired hemophilia A. Autoimmunity against endogenous

FVIII develops unpredictably in individuals resulting in a transient but rapid development of inhibitors. Although the hyper-immune state can correct without intervention, affected individuals are high risk for loss of life or limb and in these cases immune tolerance induction is not recommended. The high potency, type I kinetics and prevalence of A2-group A inhibitors in patient plasma provided support for their use for modeling this condition. Because C2 domain inhibitors possess type II kinetics, the residual activity even in saturating concentrations of inhibitor has empirically corrected a bleeding phenotype in mice. Therefore, *F8*$^{+/+}$ mice were administered MAb 4A4 at levels empirically shown to completely inhibit endogenous murine FVIII and predicted to neutralize infused rhFVIII. Subsequently, each animal received 9 units of onerecombinant fVIII ortholog, to achieve near 100% normal murine FVIII activity levels, or saline only and then were challenged by tail transection bleeding assay. Wild type mice were selected to more accurately recapitulate the acquired disorder due to continuous biosynthesis and secretion of endogenous FVIII into circulation. Mean blood loss of roFVIII treated mice was significantly reduced compared to saline and rhFVIII (10.0, 32.1, and 30.3 mg/g body weight, respectively; $P = 0.005$ and 0.007 respectively, Student's t-test) and no significant difference was observed between rpFVIII and roFVIII ($P = 0.421$, Mann Whitney *U*-test) (Figure 6). Administration of rhFVIII did not significantly reduce bleeding over saline only ($P = 0.771$; Student's t-test).

RoFVIII produces high titer inhibitor titer mice with predominant A2 specificity

The previous findings of this study have all addressed the antigenicity of roFVIII with respect to existing anti-human or anti-porcine inhibitors. To address the issue of immunogenicity briefly, we sought to generate monoclonal antibodies against oFVIII. Following 8 injections of roFVIII, the paired ELISA/inhibitor titers of the 3 immunized *F*8$^{-/-}$ mice were 7,500/900, 2,500/71, and 700/35 (arbitrary units/BU per ml) respectively. Following purification of 9 anti-oFVIII MAbs, immunoprecipitation of activated roFVIII revealed a predominant A2 domain specificity with 6 of the 9 precipitating the A2 domain and 3 MAbs precipitating the cleaved light chain (cLC). Although there is no data regarding the inhibitory status of these MAbs, these data suggest that immunogenic regions within the A2 are highly conserved in oFVIII.

Discussion

The development of anti-hfVIII inhibitors remains the most challenging complication of FVIII replacement therapy, which otherwise is effective at achieving and maintaining hemostasis in individuals with hemophilia A. While the development of humoral immunity to a protein replacement product is not unique, the doses (2 – 4 μg/kg) of FVIII needed to elicit this response are comparatively low [34]. Although knowledge regarding the antigen uptake, presentation, costimulatory signals, and predisposing genetic factors associated with FVIII inhibitor development is rather sparse, recent studies have described the epitopes, tertiary structures, mechanism of inhibition, and frequency of inhibitors in both human patients and FVIII-immunized murine models of hemophilia A [10, 11, 13, 29, 31, 35]. Well in advance of these high-resolution molecular studies, FVIII had been crudely isolated from animal plasmas (e.g. bovine and porcine) [36] and shown to control bleeding in hemophilia A patients with and without FVIII inhibitors [37, 38]. The clinical successes demonstrated using these animal FVIII preparations in inhibitor patients (both congenital and acquired) supported the commercial development of a highly-purified pd-pFVIII product (Hyate:C, formerly Speywood/Ipsen) in 1980 [39]. A decade later, Lollar and colleagues cloned the pFVIII cDNA [26] and began defining the major inhibitory epitopes present in hFVIII, but lacking in pFVIII, using hybrid human/porcine FVIII constructs and

inhibitor patient plasmas [8, 9, 40-42]. This work also provided a scientific foundation and supported the development of a commercial rpFVIII product (OBI-1, Baxter International Inc.). However outside of the rpFVIII (OBI-1) and original bovine plasma FVIII studies, no further development of the interspecies antigenicity differential has been pursued despite the obvious success of the approach, which is supported by the clinical utility of both pd- and r-pFVIII products.

Although the antigenicity and immunogenicity characteristics of FVIII orthologs largely have been ignored, their study has provided a platform for identifying structure/function relationships as well as interspecies differentials in biosynthetic, biochemical, and pharmacological properties that are thought to be exploitable for the rational design of improvedrecombinant fVIII therapeutics and hemophilia A gene therapy applications. To date, FVIII orthologs from pigs, dogs, mice, monkeys (Dr. Pete Lollar, personal communication), and sheep have been generated and studied in recombinant form [25, 27, 43, 44]. Despite the interspecies differentials described above, each FVIII ortholog displays effective procoagulant activity in a human FVIII-deficient plasma bioassay and binds tightly to human von Willebrand factor, which is necessary for stabilization in plasma circulation. Furthermore, each of the FVIII orthologs displays unique biochemical properties in areas such as cellular secretion efficiency (rpFVIII > roFVIII > rhFVIII ≥ rmFVIII), decay rate following thrombin activation (rmFVIII > rpFVIII ≥ roFVIII > rhFVIII), and specific procoagulant activity (roFVIII > rpFVIII > rhFVIII > rmFVIII). The current study

represents a continuation of this line of pursuit to identify FVIII sequences/molecules that can better address the clinical FVIII inhibitor problem.

Four key observations/findings were made in the current study. First, the inhibitor titers to roFVIII were significantly lower in most patient plasmas. Second, utilization of the ovine and porcine FVIII orthologs enabled further refinement of inhibitor epitopes within the A2 and C2 domains of hFVIII as well as determination of the inhibitor-epitope targeting frequencies within an existing patient population. Third, murine anti- rh- and rp-FVIII inhibitor plasmas both demonstrate lower inhibitor titers against roFVIII suggesting its potential utility as a tertiary treatment for patients with inhibitors formed against pFVIII in addition to hFVIII. Fourth, roFVIII retains procoagulant activity and restores hemostatic protection *in vivo* in an acquired A2 domain specific hemophilia A murine model. It was reported in a previous study that A2 domain – group A inhibitors map to residues 484-508 [8]. Alignment of human, porcine, and ovine FVIII A2 domains within this region reveals that oFVIII and pFVIII share non-conserved residues R484S, Y487H, R489G, and F501M. RoFVIII also contains unique residues at L491F and I508V. In the current study, it was observed that group A inhibitors are present within several patient plasmas and A2–A MAbs do not cross-react with, or inhibit, either rpFVIII or roFVIII. In contrast to the A2 domain findings, for patient plasmas that displayed inhibitory titers above 10 BU against roFVIII and rpFVIII, there tended to be an abundance of polyclonal anti-C2 domain IgG that are predicted to bind conserved functional epitopes. Thus it can be concluded that in the absence of a significant C2 domain

inhibitor population, shared non-conserved residues within the A2 domains of rpFVIII and roFVIII are responsible for retained activity.

The present study provides evidence for reduced antigenicity of roFVIII in the context of human inhibitor patients. While we acknowledge that roFVIII displayed no overall significant difference from rpFVIII, this study demonstrates response to exogenous FVIII orthologs is not universal. Due to inter-patient variation in inhibitor epitope specificity as well as inter-species differentials in epitope targeting efficiency, it seems reasonable to conclude that there would be distinct advantages to having multiple FVIII ortholog-based products in the clinical hemophilia A armamentarium. The current results also support the general investigation of orthologous biomolecules not only as an approach to understanding structure/function, but also for the development of improved biotherapeutics. Given the ever-advancing push towards personalized medicine and the established clinical Bethesda assay for inhibitor detection, the case-by-case identification of the least antigenic and inhibited FVIII molecule may become the status quo.

Authorship Contributions: P.M.Z., K.V. and R.C.M. designed and performed research, analyzed data, and co-wrote the manuscript; S.L.M. and C.B.D. provided reagents, designed research, analyzed data, and co-wrote the manuscript. **RM** helped design experiments for figure 2 and collect data for table 2.

Acknowledgements

This work was supported by grants from the National Institute of Health: C.B.D. – HL092179; C.B.D. and S.L.M. – HL112309, P.M.Z. – 5T32GM008602, and from Hemophilia of Georgia: S.L.M.

Disclosure of Conflicts of Interest: The authors declare no competing financial interests.

Figures

Tables

Table 1. Inhibitor patient plasma cross-reactivity and inhibitor titers of patient plasmas

		ELISA Cross Reactivity (%)		Bethesda Titer (BU/mL)		
Patient	**Domain**	**Porcine**	**Ovine**	**Human**	**Porcine**	**Ovine**
10	A2	3	11	59.0	0.4	1.0
14	A2	50	50	29.0	2.0	1.8
19	A2	23	28	12.3	0.4	0.4
20	A2	17	62	9.7	0.5	7.8
1	C2	76	54	187.6	37.5	28.3
3	C2	49	41	119.5	7.0	18.7
5	C2	56	34	76.0	33.8	39.6
7	C2	51	24	68.4	10.3	3.7
9	C2	55	43	59.3	60.7	60^
13	C2	48	38	42.0	6.6	16.7
21	C2	68	78	8.0	3.9	6.2
22	C2	60	42	8.0	7.7	6.3
23	C2	43	37	5.7	0.4	1.8
6	A2 + C2	62	47	73.8	43.9	83.1
2	A2 + C2	11	9	124.0	1.0	0.3
11	A2 + C2	24	36	55.0	10.5	3.0
12	A2 + C2	83	28	45.0	0.4	4.5
16	A2 + C2	0	0	27.9	0.4	1.0
17	A2 + C2	54	26	19.3	NT	1.0
A1	A2 + C2	13	37	900.0	24.0	225.0

NT: Not tested

^ : Value shown is a minimal approximation

Table 2. Inhibitor titers of anti-human fVIII MAbs against ovine and porcine

Inhibitor	Domain	Kinetics	Group	HumanTiter (BU/mg)	Porcine Titer (BU/mg)	Ovine Titer (BU/mg)
4A4	A2	I	A	25,500	< 8	< 8
413	A2	I	A	61,000	< 1	< 1
4F4	A2	I	B	330	< 1	< 1
B25	A2	I	C	18	< 4	< 1
1D4	A2	I	E	51,000	5	6
G48	A2	II	C	*	*	*
2-54	A2	II	D	****	*	*
3E6	C2	I	A	11	< 1	< 4
I109	C2	I	AB	3,100	< 1	< 1
D102	C2	I	B	8,600	10	5
3D12	C2	I	B	3,800	< 1	< 1
I14	C2	II	BC	****	*	**
B75	C2	II	BC	*	*	*
3G6	C2	II	BC	****	*	*
G99	C2	II	BC	****	*	*
2-77	C2	II	BC	****	*	*
I55	C2	II	BC	****	*	*
ESH-8	C2	II	C	****	*	*
2-117	C2	II	C	*	*	*

*: 0-100 BU/mg; **: 100-1,000 BU/mg; ***: 1,000-10,000 BU/mg; ****: > 10,000 BU/mg

Figure 1. Antigenicity and inhibitor titers for inhibitor patient plasmas

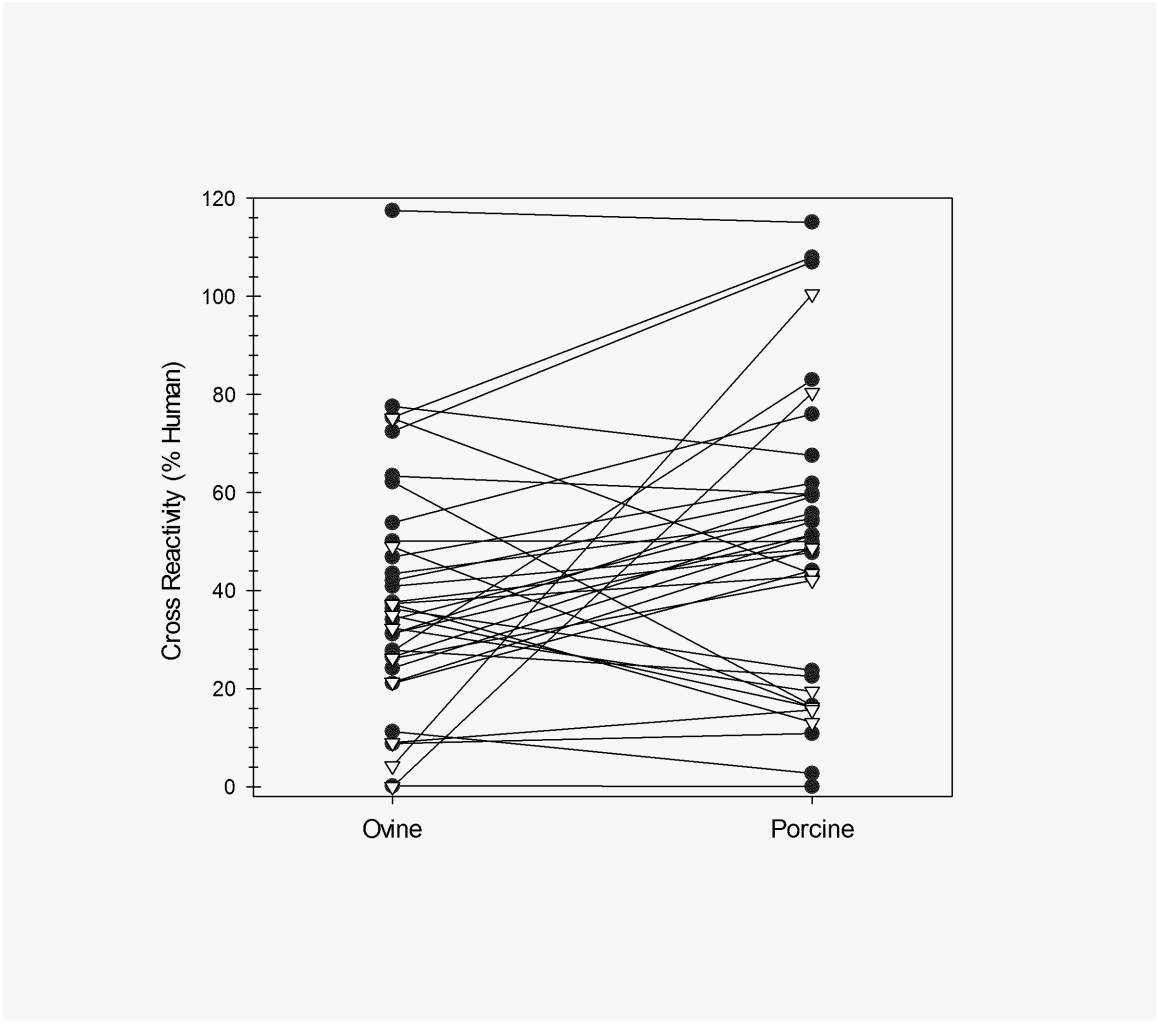


Figure 1B.

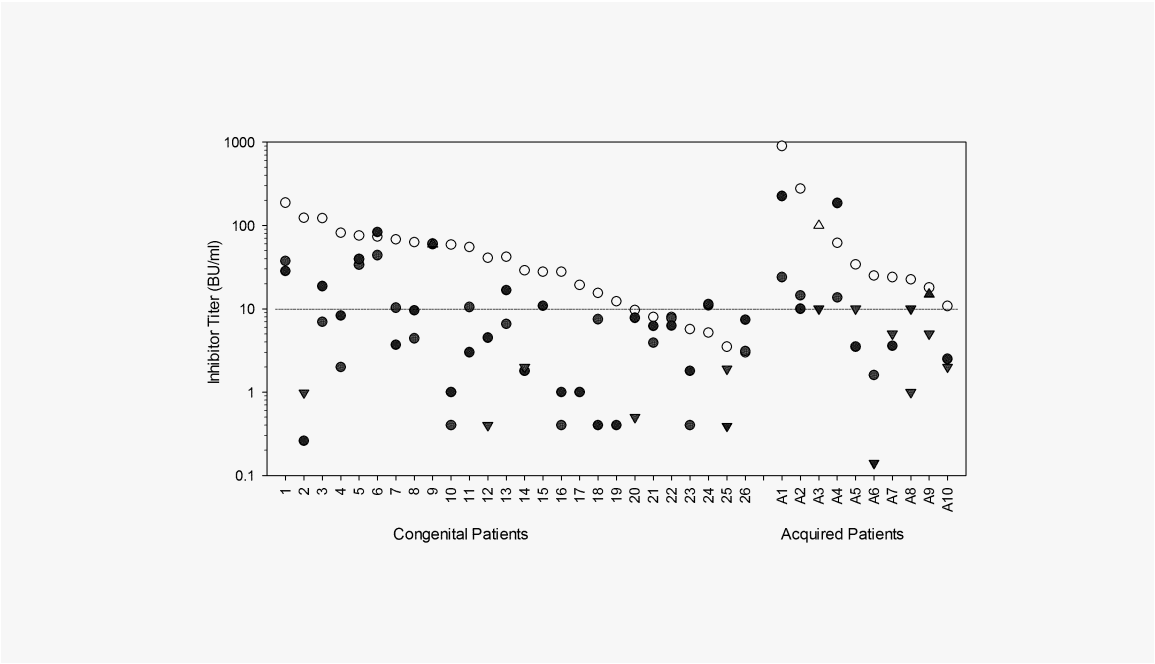

Inhibitor Titer (BU/ml)
1000
100
10
1
0.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10
Congenital Patients
Acquired Patients

Figure 1C.

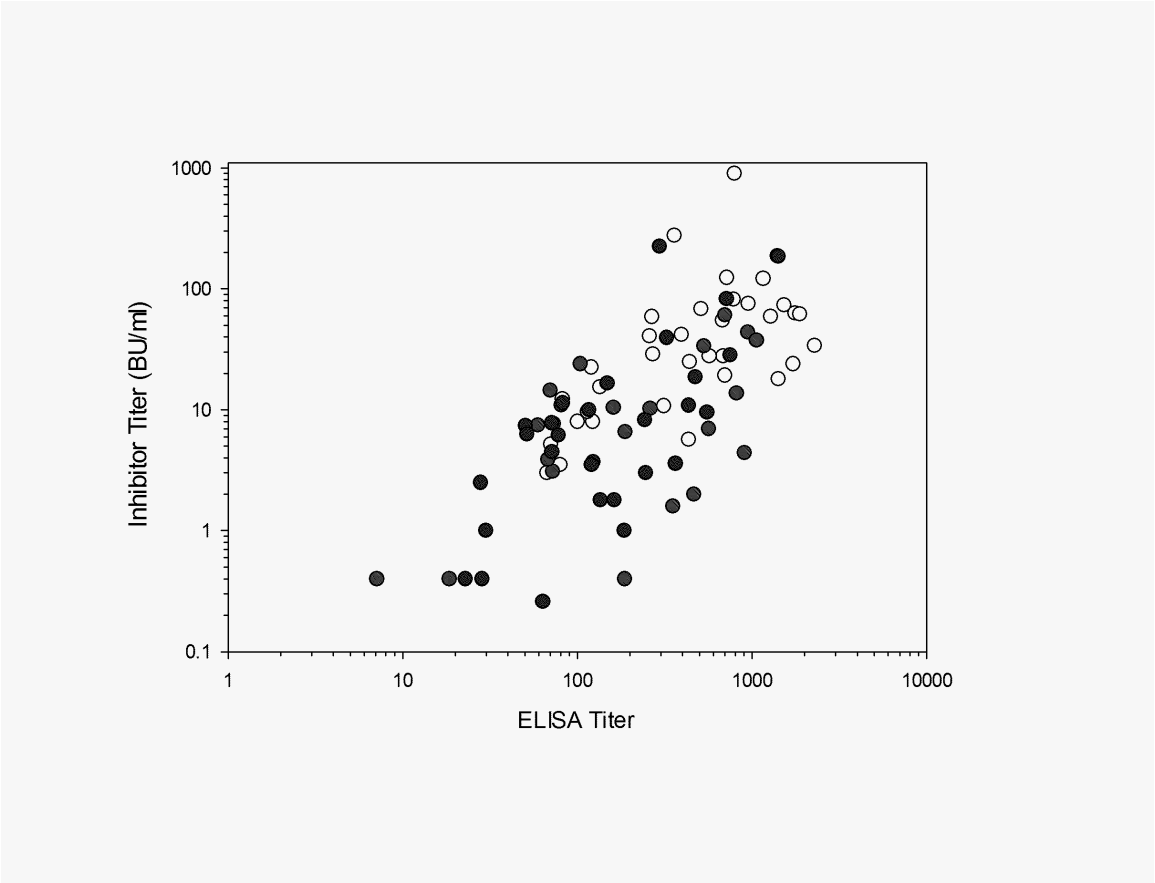

Inhibitor Titer (BU/ml)
1000
100
10
1
0.1
1
10
100
1000
10000
ELISA Titer

Figure 2. Identification of A2 and C2 domain epitopes targeted by patient plasmas

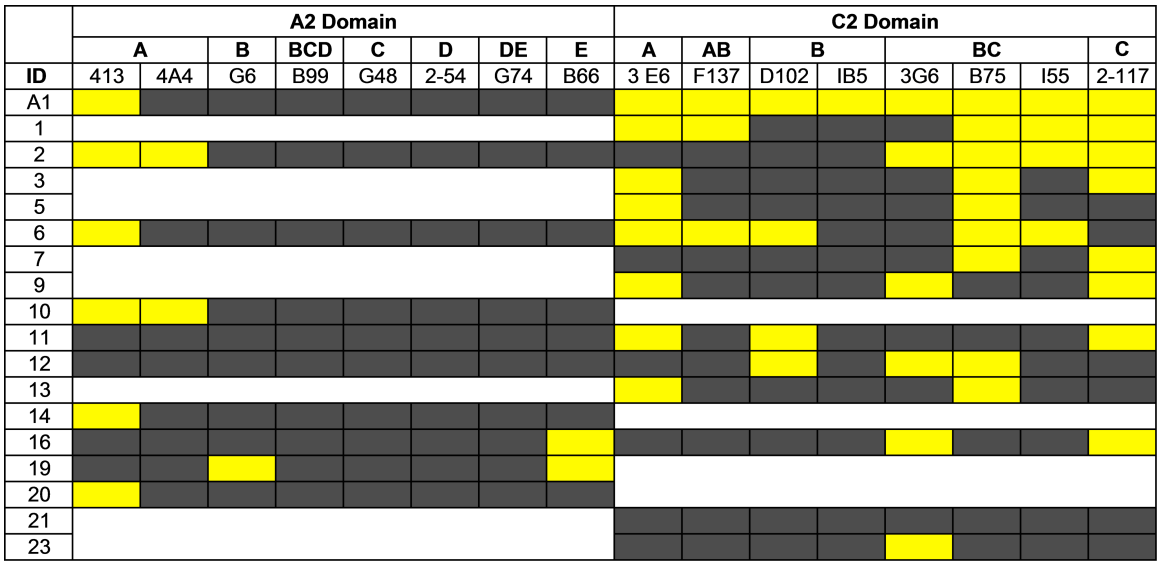


Figure 3. Reactivity of anti-hFVIII A2 and C2 domain MAbs with FVIII orthologs

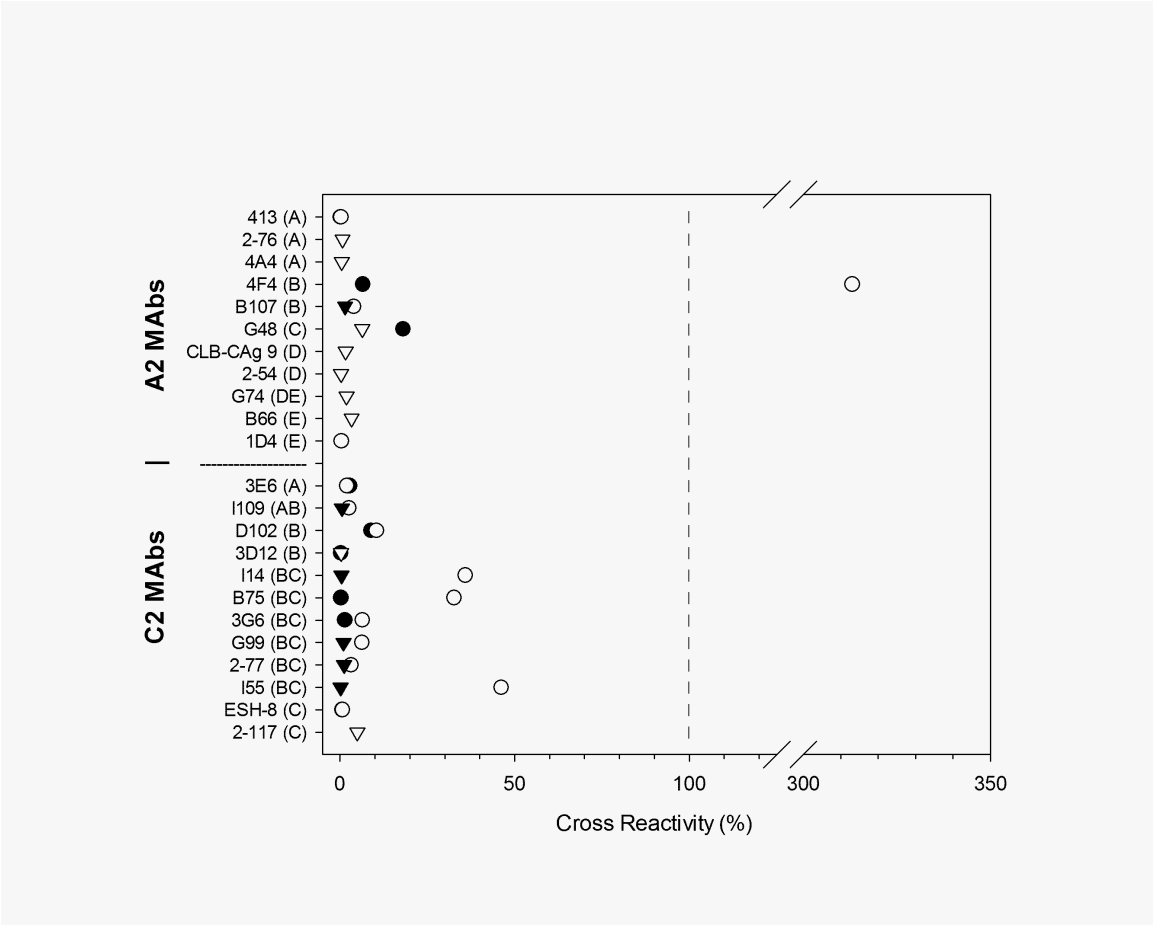


Figure 4. Reactivity of anti-rpFVIII MAbs with roFVIII

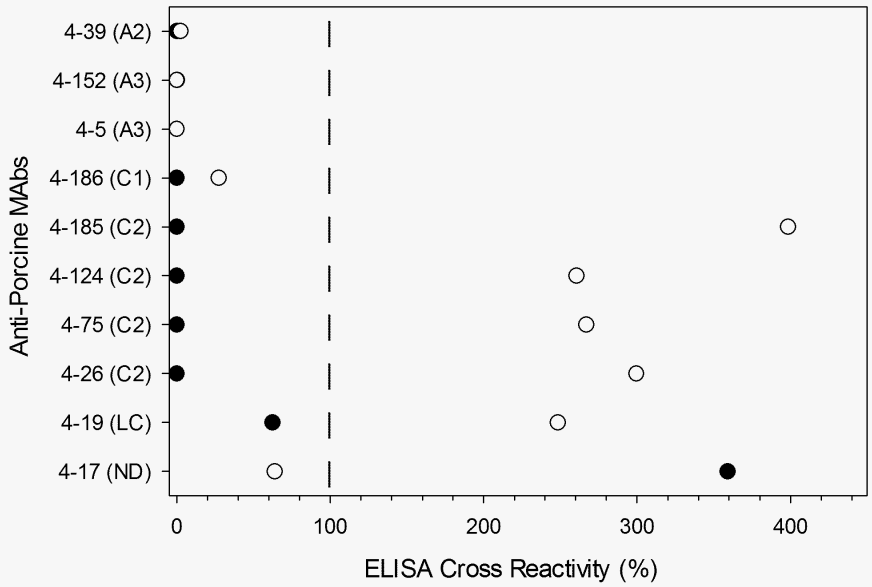


Figure 5. Inhibitor titers to FVIII ortholog in pre-immunized murine hemophilia A plasmas

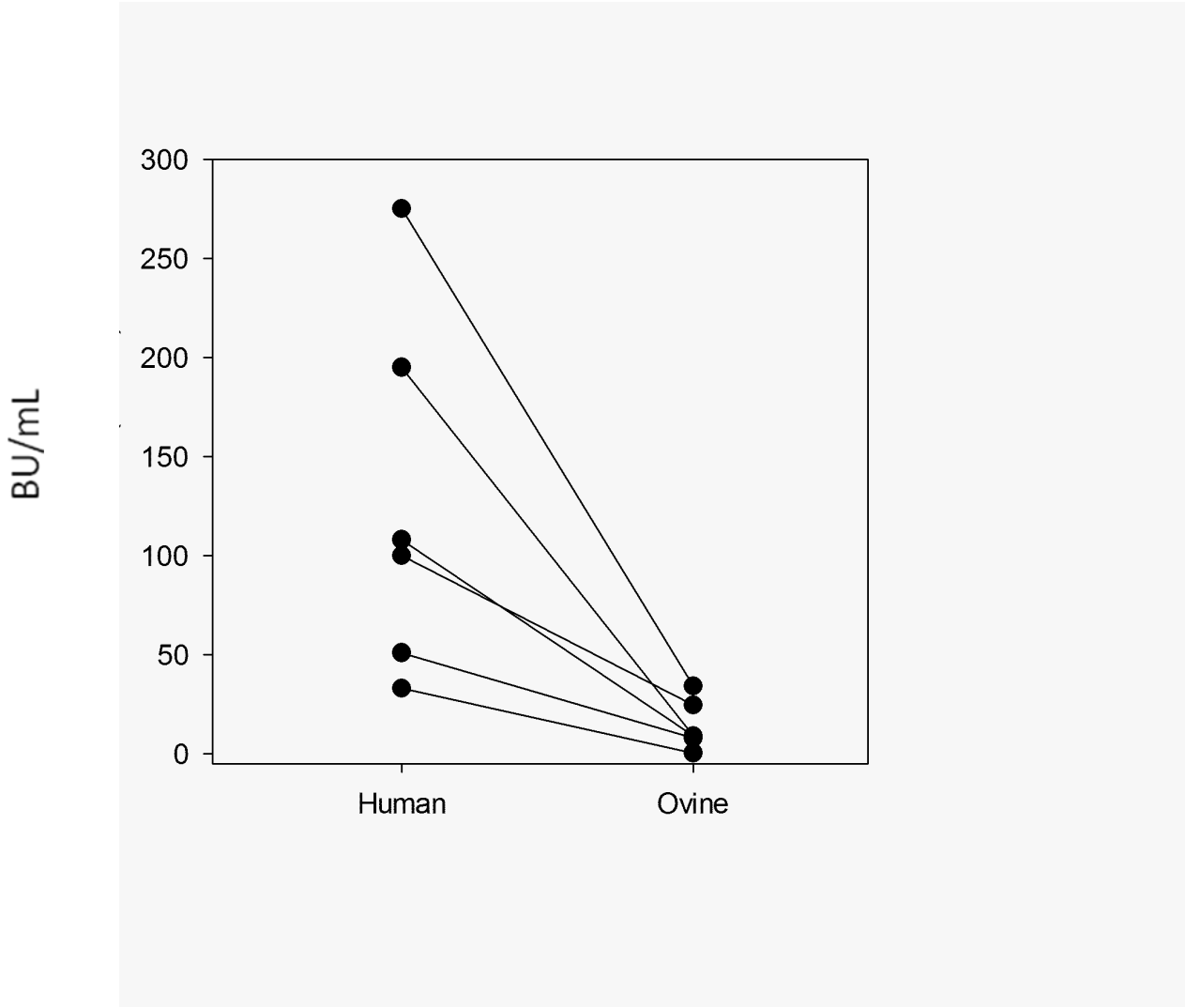


Figure 5B.

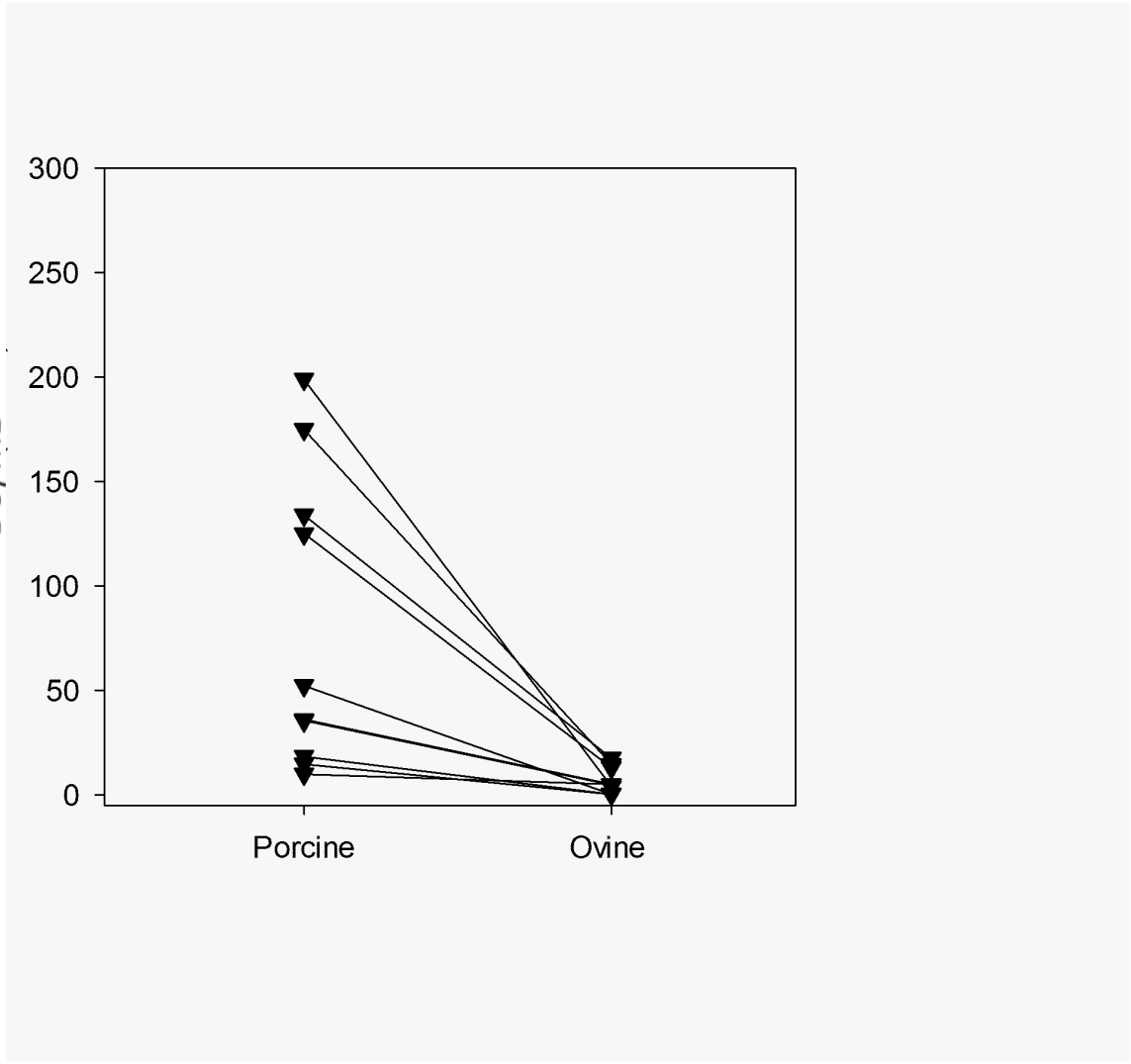
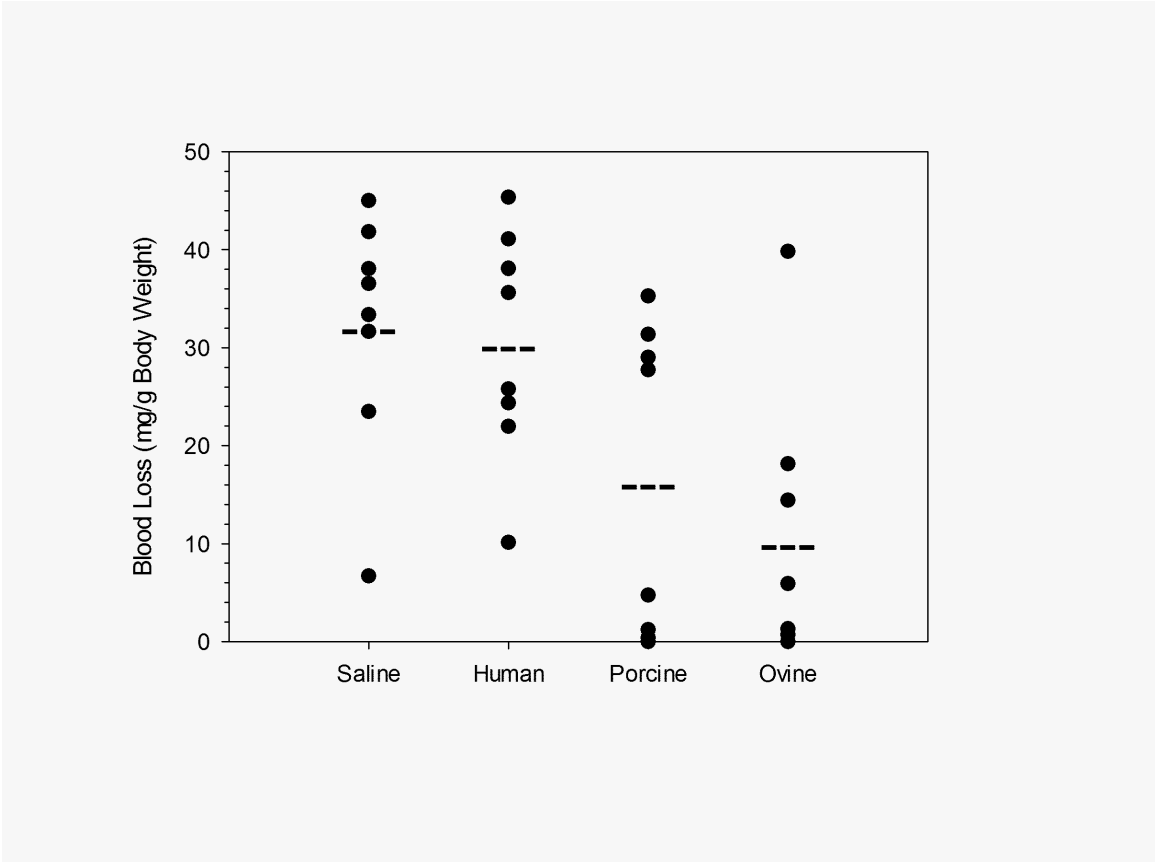


Figure 6. In vivo testing ofrecombinant fVIII orthologs in a murine model of acquired hemophilia A



References

1. Stonebraker JS B-MP, Soucie JM, Walker I, Brooker M. A study of variations in the reported haemophilia A prevalence around the world. *Haemophilia* 2010,**16**:20-32.
2. Collins PW HS, Baglin TP, Dolan G, Hanley J, Makris M, Keeling DM, Liesner R, Brown SA, Hay CR; UK Haemophilia Centre Doctors' Organisation. Acquired hemophilia A in the United Kingdom: a 2-year national surveillance study by the United Kingdom Haemophilia Centre Doctors' Organisation. *Blood* 2007,**109**.
3. Lusher JM, Arkin S, Abildgaard CF, Schwartz RS. Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy, and development of inhibitors. Kogenate Previously Untreated Patient Study Group. *N.Engl.J.Med.* 1993,**328**:453-459.
4. Bray GL, Gomperts ED, Courter S, Gruppo R, Gordon EM, Manco-Johnson M*, et al.* A multicenter study of recombinant factor VIII (recombinate): safety, efficacy, and inhibitor risk in previously untreated patients with hemophilia A. The Recombinate Study Group. *Blood* 1994,**83**:2428-2435.
5. Lusher JM LC, Kessler CM, Bedrosian CL. The safety and efficacy of B-domain deleted recombinant factor VIII concentrate in patients with severe haemophilia A. *Haemophilia* 2003,**9**:38-49.
6. Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH*, et al.* Characterization of the human factor VIII gene. *Nature* 1984,**312**:326-330.
7. Prescott R NH, Saenko EL, Scharrer I, Nilsson IM, Humphries JE, Hurst D, Bray G, Scandella D. The inhibitor antibody response is more complex in hemophilia A patients than in most nonhemophiliacs with factor VIII autoantibodies. Recombinate and Kogenate Study Groups. *Blood* 1997,**89**:3663-3671.
8. Healey JF, Lubin IM, Nakai H, Saenko EL, Hoyer LW, Scandella D*, et al.* Residues 484-508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. *J.Biol.Chem.* 1995,**270**:14505-14509.
9. Barrow RT, Healey JF, Gailani D, Scandella D, Lollar P. Reduction of the antigenicity of factor VIII toward complex inhibitory antibody plasmas using multiply-substituted hybrid human/porcine factor VIII molecules. *Blood* 2000,**95**:564-568.
10. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Antihuman factor VIII C2 domain antibodies in hemophilia A mice recognize a functionally complex continuous spectrum of epitopes dominated by inhibitors of factor VIII activation. *Blood* 2007,**110**:4234-4242.
11. Markovitz RC HJ, Parker ET, Meeks SL, Lollar P. The diversity of the immune response to the A2 domain of human factor VIII. *Blood* 2013,**121**:2785-2795.

12. Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. The humoral response to human factor VIII in hemophilia A mice. *J Thromb Haemost* 2007,**5**:512-519.
13. Walter JD WR, Brison CM, Cragerud RK, Healey JF, Meeks SL, Lollar P, Spiegel PC Jr. Structure of the factor VIII C2 domain in a ternary complex with 2 inhibitor antibodies reveals classical and nonclassical epitopes. *Blood* 2013,**122**:4270-4278.
14. Walter JD WR, Polozova MS, Pohlman J, Healey JF, Meeks SL, Lollar P, Spiegel PC Jr. Characterization and solution structure of the factor VIII C2 domain in a ternary complex with classical and non-classical inhibitor antibodies. *J Biol Chem.* 2013,**288**:9905-9914.
15. Sevy AM HJ, Deng W, Spiegel PC, Meeks SL, Li R. Epitope mapping of inhibitory antibodies targeting the C2 domain of coagulation factor VIII by hydrogen-deuterium exchange mass spectrometry. *J Thromb Haemost* 2013.
16. Hay CR DDIITS. The principal results of the International Immune Tolerance Study: a randomized dose comparison. *Blood* 2012,**119**:1335-1344.
17. Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. High level expression of recombinant porcine coagulation factor VIII. *J Biol Chem* 2002,**277**:38345-38349.
18. Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. Identification of porcine coagulation factor VIII domains responsible for high level expression via enhanced secretion. *J Biol Chem* 2004,**279**:6546-6552.
19. Gangadharan B, Parker ET, Ide LM, Spencer HT, Doering CB. High-level expression of porcine factor VIII from genetically modified bone marrow-derived stem cells. *Blood* 2006,**107**:3859-3864.
20. Ide LM, Gangadharan B, Chiang KY, Doering CB, Spencer HT. Hematopoietic stem-cell gene therapy of hemophilia A incorporating a porcine factor VIII transgene and nonmyeloablative conditioning regimens. *Blood* 2007,**110**:2855-2863.
21. Doering CB, Gangadharan B, Dukart HZ, Spencer HT. Hematopoietic stem cells encoding porcine factor VIII induce pro-coagulant activity in hemophilia A mice with pre-existing factor VIII immunity. *Mol Ther* 2007,**15**:1093-1099.
22. Dooriss KL, Denning G, Gangadharan B, Javazon EH, McCarty DA, Spencer HT, *et al.* Comparison of factor VIII transgenes bioengineered for improved expression in gene therapy of hemophilia A. *Hum Gene Ther* 2009,**20**:465-478.
23. Doering CB, Denning G, Dooriss K, Gangadharan B, Johnston JM, Kerstann KW, *et al.* Directed Engineering of a High-expression Chimeric Transgene as a Strategy for Gene Therapy of Hemophilia A. *Mol Ther* 2009.
24. Porada CD, Sanada C, Long CR, Wood JA, Desai J, Frederick N, *et al.* Clinical and molecular characterization of a re-established line of sheep exhibiting hemophilia A. *J Thromb Haemost* 2010,**8**:276-285.
25. Zakas PM GB, Almeida-Porada G, Porada CD, Spencer HT, Doering CB. Development and characterization of recombinant ovine coagulation factor VIII. *PLoS One* 2012,**7**:e49481.

26. Healey JF, Lubin IM, Lollar P. The cDNA and derived amino acid sequence of porcine factor VIII. *Blood* 1996,**88**:4209-4214.
27. Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. High-level expression of recombinant porcine coagulation factor VIII. *J.Biol.Chem.* 2002,**277**:38345-38349.
28. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH, Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat.Genet.* 1995,**10**:119-121.
29. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Nonclassical anti-C2 domain antibodies are present in patients with factor VIII inhibitors. *Blood* 2008,**112**:1151-1153.
30. Barrow RT, Lollar P. Neutralization of antifactor VIII inhibitors by recombinant porcine factor VIII. *J Thromb Haemost* 2006,**4**:2223-2229.
31. Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. The comparative immunogenicity of human and porcine factor VIII in haemophilia A mice. *Thromb Haemost* 2009,**102**:35-41.
32. Spencer HT, Denning G, Gautney RE, Dropulic B, Roy AJ, Baranyi L*, et al.* Lentiviral vector platform for production of bioengineered recombinant coagulation factor VIII. *Mol Ther* 2011,**19**:302-309.
33. Hay CR. The epidemiology of factor VIII inhibitors. *Haemophilia* 2006:Suppl 6:23-28; discussion 28-29.
34. Porter S. Human immune response to recombinant human proteins. *J Pharm Sci* 2001,**90**:1-11.
35. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Non-classical anti-factor VIII C2 domain antibodies are pathogenic in a murine in vivo bleeding model. *J Thromb Haemost* 2009,**7**:658-664.
36. Bidwell E. The purification of antihaemophilic globulin from animal plasma. *Br J Haematol.* 1955,**1**:386-389.
37. Macfarlane RG MP, Witts LJ, Bidwell E, Biggs R, Fraenkel GJ, Honey GE, Taylor KB. Surgery in haemophilia; the use of animal antihaemophilic globulin and human plasma in thirteen cases. *Lancet* 1957,**273**:251-259.
38. Biggs R MR. *Treatment of Haemophilia and Other Coagulation Disorders*. Oxford: John Wiley and Sons Ltd; 1966.
39. Kernoff PB TN, Lilley PA, Matthews KB, Goldman E, Tuddenham EG. Clinical experience with polyelectrolyte-fractionated porcine factor VIII concentrate in the treatment of hemophiliacs with antibodies to factor VIII. *Blood* 1984,**63**:31-41.
40. Lollar P. Mapping factor VIII inhibitor epitopes using hybrid human/porcine factor VIII molecules. *Haematologica* 2000,**85**:26-28.
41. Healey JF, Barrow RT, Tamim HM, Lubin IM, Shima M, Scandella D*, et al.* Residues Glu2181-Val2243 contain a major determinant of the inhibitory epitope in the C2 domain of human factor VIII. *Blood* 1998,**92**:3701-3709.
42. Lollar P. Analysis of factor VIII inhibitors using hybrid human/porcine factor VIII. *Thromb.Haemost.* 1997,**78**:647-651.

43. Doering C, Parker ET, Healey JF, Craddock HN, Barrow RT, Lollar P. Expression and characterization of recombinant murine factor VIII. *Thromb Haemost* 2002,**88**:450-458.
44. Sabatino DE FC, Toso R, Santos A, Merricks EP, Kazazian HH Jr, Nichols TC, Camire RM, Arruda VR. Recombinant canine B-domain-deleted FVIII exhibits high specific activity and is safe in the canine hemophilia A model. *Blood* 2009,**114**:4562-4565.

Figure Legends

Figure 1. Antigenicity and inhibitor titers for inhibitor patient plasmas

(A) An ELISA was performed on 26 congenital (black circle) and 10 acquired (white triangle) hemophilia A inhibitor patient plasmas using rhFVIII, roFVIII, or rpFVIII as the capture antigen. Data are presented as the relative cross-reactivity to that observed with rhFVIII. (B) The inhibitor titer of each patient plasma against hFVIII (white circle), rpFVIII (red circle), and roFVIII (blue circle) was measured by modified Bethesda assay as described in Methods. Due to limited plasma availability, triangles depict maximum/minimum approximations corresponding with their orientation. For example, an inverted triangle represents a value less than the position of the triangle on the y-axis. (C) Patient plasma ELISA versus inhibitor titers against human (white), porcine (red), and ovine (blue) FVIII orthologs were plotted and analyzed for correlation. Significant non-zero correlations were observed with *P* values of 0.0028, and 0.0003 for p-, and o-FVIII while *P* = 0.4913 for hFVIII.

Figure 2. Identification of A2 and C2 domain epitopes targeted by patient plasmas

A competition ELISA was performed with anti-A2 and -C2 domain MAbs competing against human inhibitor IgG for binding to hFVIII. HFVIII first was blocked with patient plasma and then incubated with individual biotinylated MAbs. Patient ID is listed along the y-axis in descending order of the Bethesda titers measured for each

sample and individual MAbs ID/group are listed across the top. Absence of competition is represented by black shading, and white shading designates data not determined. Competition is defined as a reduction of kinetic signal outside 2 standard deviations of control kinetic rates and is represented as yellow shading.

Figure 3. Reactivity of anti-hFVIII A2 and C2 domain MAbs with FVIII orthologs

Panels of anti-A2 and -C2 domain targeting MAbs were assayed for cross-reactivity via indirect ELISA. Binding to roFVIII (white) and rpFVIII (black) is displayed as percent hFVIII binding as calculated by titration curve analysis. MAbs are listed on the y-axis by name with the inhibitor group classification in parenthesis. Triangles represent the maximal cross-reactivity percentages that could be determined experimentally with the available plasma.

Figure 4. Reactivity of anti-rpFVIII MAbs with roFVIII

Murine MAbs isolated from hemophilia A mice immunized with rpFVIII were screened for cross-reactivity to hFVIII (black) and roFVIII (white). The data presented are normalized to rpFVIII binding. MAbs are listed in the y-axis with the FVIII domain epitope specificity in parentheses. LC: Light chain; ND: not determined.

Figure 5. Inhibitor titers to FVIII ortholog in pre-immunized murine hemophilia A plasmas

Mice were immunized with either rhFVIII (A) or rpFVIII (B), respectively. Following the 7th injection, plasma was collected via terminal cardiac puncture and assayed for inhibitor titer to the original immunogen and roFVIII via modified Bethesda assay. Lines connect the intramouse inhibitor titers recorded for the two FVIII orthologs. N = 6 and 10 respectively.

Figure 6. In vivo testing of rFVIII orthologs in a murine model of acquired hemophilia A.

C57BL/6 mice were administered 10μg MAb 4A4 IP followed by 9 units rFVIII or saline only (n = 8). Blood loss over 40 minutes following tail transection was recorded. Dashes denote mean blood loss values for each experimental cohort.

Conclusion

Summary

Inhibitory Abs against fVIII are the most significant complication in the treatment of hemophilia A. ITI therapy, the gold standard for treating patients with inhibitors, involves a long and expensive treatment period and fails for 30-40% of individuals. The work presented in this thesis first aims to better characterize the immune response against the A2 domain, one of the two most immunogenic regions of fVIII[1].We first studied the epitopes, anti-coagulant properties, and inhibitory mechanisms of anti-A2 MAbs. Based on previous work with anti-C2 MAbs, we hypothesized that the target epitope, rather than the inhibitory titer, would determine the bleeding phenotype in a murine bleeding tail snip model[2], and this hypothesis was confirmed as reported in Chapter II.

Patients with anti-fVIII Abs are classified based on their Bethesda titer and are treated accordingly. It appears that if information beyond that offered by the Bethesda titer were known, patients could be treated more effectively. For example, findings from our group and others suggest that contrary to common clinical practice many patients with high titer inhibitors may benefit from treatment with hfVIII[3, 4].

The anti-A2 MAbs were also used to characterize an ofVIII that had been suggested as a possible alternative to hfVIII treatment(Chapter III.) The MAbs allowed for a comparison of the antigenicity of this product and hfVIII with recombinant porcine fVIII (pfVIII), currently in development for clinical use[5]. Finally, we aimed to decrease or eliminate the immunogenicity of fVIII by depleting

fVIII-specific naïve and memory B cells using a targeted toxin. We introduced the fVIII-saporin conjugate to naïve mice to test if it would block the formation of inhibitors, as well as into mice pre-immunized with fVIII, to test if it could deplete fVIII-specific naïve and memory B cells.

Key Results from the Anti-A2 MAb Studies

This study began by characterizing the diversity of the immune response against the A2 domain of fVIII. We created and characterized monoclonal A2 anti-human fVIII Abs. We identified their epitopes and investigated their inhibitory characteristics and mechanisms. We identified novel inhibitory mechanisms of anti-fVIII MAbs, examined the role of VWF in the immune response to fVIII, and more fully characterized the immunodominant epitope within the A2 domain. Eight of the 29 MAbs mapped to the extensively characterized A2 epitope Arg484-Ile508, which is recognized by human and murine inhibitors[6].

This epitope has previously been mutated, resulting in a significant decrease in immunogenicity[7]. MAb 413, a high titer MAb in this group of MAbs is a noncompetitive inhibitor of the intrinsic Xase complex[7]. All but one of the eight MAbs had a high titer, consistent with previous findings that this is an immunodominant region. In addition, we found that the majority of inhibitory anti-A2 MAbs interfere with the proteolytic activation of fVIII catalyzed by thrombin. Thus, we identified two novel mechanisms of inhibition of A2 MAbs: first, cleavage at Arg372 and second, cleavage at Arg1689. The first inhibits thrombin's activation of fVIII cofactor function while the second inhibits fVIII's dissociation from VWF.

The presence or absence of VWF affected the inhibitory properties of a

group of these MAbs. One group inhibited fVIII's activation in the physiologically relevant situation while another group's inhibition of activation was alleviated in the presence of VWF. The role of VWF in the antigenicity of fVIII is still being determined; therefore, its effect on the inhibition of A2 MAbs is notable.

Debate continues about the role of VWF in the immunogenicity and antigenicity of fVIII. Therefore, showing the role of VWF in the anti-coagulant properties of these anti-A2 MAbs represents one of the key findings of this study. MAbs such as 2-54 inhibited light chain cleavage, but this inhibition was alleviated by the presence of VWF. Similarly, CLB-CAg 9 and GMA-012 inhibited cleavage at Arg740 that was alleviated in the presence of VWF.

Based on their epitopes, kinetics, and mechanism of inhibition, we were able to make successful predictions regarding the *in vivo* pathogenicity of the anti-A2 MAbs. Our group's work with the C2 domain suggested that the high titer type I inhibitors would be pathogenic *in vivo.* Separately, we hypothesized that the high titer type II inhibitors and low titer type I inhibitors would not cause bleeding[2]. Type I inhibitors display second-order kinetics thus fully inactivating fVIII. Type II inhibitors display complex kinetics and do not fully inactive fVIII[8]. Our results fit this paradigm as MAbs such as 2-54, an anti-A2 high titer type II MAb caused no bleeding while there was significant bleeding with the introduction of the type I high titer inhibitor 2-76. Highlighting the importance of epitope, in contrast to 2-54, the anti-C2 high titer type II MAb 2-77 caused bleeding when injected with a low dose of fVIII. This suggests that epitope and kinetics of inhibitions affect pathogenicity[2].

This is consistent with the finding that bleeding phenotype in patients with inhibitors does not always correlate with their Bethesda titer[9].

Findings such as these led to the hypothesis that some patients with high titer inhibitors, dependent upon their kinetics and inhibitor epitopes, may benefit from treatment with fVIII. This has been investigated, first, in a pre-clinical study by our group using selected A2 and C2 MAbs, and then, in a study with hemophilia A patients with high titer inhibitors. Doshi et al. demonstrated an improvement in thrombin generation in the presence of two of three high titer MAb inhibitors when the bypassing agent rfVIIa was supplemented with fVIII. This improvement was seen in the presence of all type II MAbs[4].

This study was followed by a report demonstrating that treating high titer inhibitor patients with fVIII along with rfVIIa achieved 90% hemostasis and decreased the number of bleeding episodes. Before treating patients, studies were made allowing the creation of individualized regimens[3]. Cumulatively, these studies suggest that the ability to dissect the specifics of a patient's humoral immune response to fVIII could result in more effective therapy. As illustrated by the Doshi et al. and Livnat et al., and running contrary to the common practice of treating patients with high titer inhibitors with only bypassing agents, many patients could potentially be treated with fVIII and bypassing agents with substantial benefit.

Combining bypassing agents and hfVIII may be an option for some high titer patients, but another option outlined in Chapter III involves using ofVIII, an ortholog of fVIII, to treat patients with inhibitors. Based on data from pfVIII which is

currently in clinical trials, Zakas and colleagues hypothesized that ofVIII would also have reduced antigenicity in human inhibitor plasma while retaining procoagulant activity. They evaluated this hypothesis using human patient-derived inhibitors and murine anti-human A2 and C2 from our previous studies. After evaluating 36 patient plasmas, they found that 32 had decreased reactivity to pfVIII and ofVIII. They evaluated whether this could be due to loss of inhibitory epitopes within ofVIII and pfVIII, and found that high titer A2 MAbs did not inhibit pfVIII nor ofVIII, while C2 MAbs showed moderate cross-reactivity. They suggested that ofVIII could be a good therapy option for a number of patients with inhibitors refractory to hfVIII treatment. These studies combine the extensive characterization of the humoral immune response that our group has undertaken with pre-clinical and clinical studies to better tailor the individual patient's inhibitor response to his or her therapy.

FVIII-Saporin *In vivo* Experiments

In the Appendix to this work, we describe a strategy that as oppose to improving care for patients with inhibitors aims to inhibit the formation of anti-fVIII Abs. By conjugating fVIII to a toxin, saporin, we hypothesized that we could target fVIII-specific cell surface immunoglobulins and selectively delete fVIII-specific naïve and memory B cells. We hypothesized that we would decrease inhibitor production by targeting the cells that are both precursors to fVIII specific plasma cells and crucial APCs. The results from our adoptive transfer experiment and from our first naïve mouse experiment both suggest that our fVIII-saporin conjugate depleted

fVIII-specific naïve and memory B cells and inhibited the formation of fVIII-specific memory B cells. Below we discuss in greater detail the conflicting results from the first vs. second naïve mouse experiment, giving consideration to whether the negative results from the second experiment reflect experimental error or a flawed hypothesis in targeting naïve and memory B cells.

Our preliminary findings that the fVIII-saporin conjugate holds promise for the treatment of patients with inhibitors are consistent with case studies reporting success with inhibitor patients using rituximab, a pan anti-B cell MAb. These findings are in agreement with the results of Macauley et al. who induced apoptosis of fVIII- specific B cells using nanoparticles expressing fVIII and glycan ligands for the inhibitory coreceptor CD22, resulting in tolerance in hemophilia A mice[10]. Both of these approaches deplete B cells, but rituximab targets all B cells with the exception of plasma cells, while the nanoparticle approach is antigen-specific. Thus, both of these studies suggest that depleting B cells reduces inhibitors and can induce tolerance to fVIII.

One of the mechanisms hypothesized for ITI is the targeting of fVIII-specific naïve and memory B cells. The results of our adoptive transfer experiment, as well as from the first naïve mouse experiment, are consistent with that hypothesis. On the other hand, in pre-clinical studies, many groups have shown that Tregs can induce tolerance to fVIII. Thus, this cell type has also been hypothesized to play an important role in ITI. If that were true, then targeting fVIII-specific B cells may not be the most efficient strategy to inducing tolerance. APCs, B cells, and effector T cells are ultimately all involved in the immune response, therefore, targeting any of them

may lead to some reduction in the humoral immune response to fVIII. Nonetheless, we hypothesize that memory B cells are the best targets for inhibiting anti-fVIII Abs due to their avidity and affinity for fVIII along with their roles as both APCs and as precursors for plasma cells.

As there are other therapies farther along in development for targeting B cells, what special utility would our fVIII-saporin conjugate provide? Rituximab targets all B-cells, making it an unlikely candidate for prophylaxis treatment. We hypothesize that based on the LD_{50} of saporin, we can ultimately optimize our conjugate or, potentially, a recombinant fVIII-saporin protein, to determine a dose that will deplete B cells in an antigen-specific manner. Having a targeted toxin available for testing in pre-clinical studies has utility, as targeted toxins are widely used. Saporin itself has been tested in clinical trials and was found to have only limited toxicity. If we demonstrate that the fVIII-saporin conjugate is consistently depleting fVIII-specific naïve and memory B cells *in vivo*, then we may hypothesize that this saporin conjugate could be more effective in our context due to probable lower burden of disease in a patient with inhibitors compared to a refractory cancer patient.

Our preliminary results demonstrate that we have significant work to do with our conjugate to demonstrate that we are reproducibly depleting fVIII-specific naïve and memory B cells. Our first naïve mouse experiment showed a significant difference between mice initially treated with fVIII vs. those treated with fVIII-saporin after four subsequent injections of fVIII. After six injections of fVIII, significance was lost ($p=.059$) but it appeared that the conjugate was preventing the

formation of anti-fVIII Abs (n=4). The second naïve mouse experiment found that fVII-specific naïve B cells were not depleted by our conjugate, and anti-fVIII Abs formed in response to infusions of fVIII at levels similar to fVIII infusions in mice that did not receive the conjugate. In contrast, a one-time intravenous injection of saporin inhibited the formation of anti-fVIII Abs after six subsequent injections of fVIII. The conjugate contained free saporin but the difference in response could be due to the lower dose of free saporin in the conjugate. Also, the conjugate contained free fVIII that may have stimulated fVIII memory B cells to differentiate into plasma cells, competing with the killing abilities of saporin.

In the first naïve mouse experiment, the group of mice that received saporin had inhibitor levels of about half those receiving just fVIII. In the second naïve mouse experiment, the difference between the two groups reached statistical significance. As previously mentioned, the results of the group initially treated with fVIII compared to the group treated with the conjugate differed between the two naïve mouse experiments. This discrepancy could be explained by a number of factors, but suspects that come to mind include possible experimental error in our second naïve mouse experiment experimental set-up e.g., incorrect dosing, or flaws in our hypothesis that targeting fVIII-specific naïve B cells inhibits the formation of anti-fVIII Abs. This inconsistency highlights the need to improve our system for testing conjugates, both for stability and for killing ability before *in vivo* testing.

Experimental error could explain the discrepant results, suggesting that when intact and functional, our conjugate does deplete naïve and memory B cells by decreasing anti-fVIII Abs. The conjugate appeared to deplete naïve and memory B

cells in the adoptive transfer and first naïve mouse experiment; only in a later experiment did it fail to decrease levels of Abs. This could reflect changes in the conjugate over time. On the other hand, we made aliquots of the conjugate and froze them at -80°C in an effort to protect the integrity of the proteins and the disulfide bond between them. In addition, we took out each individual aliquot immediately before using it in the experiment in an effort to preserve the conjugate.

Future Directions

However, we did not test the integrity of the conjugate before injecting it into the mice. For future experiments, we plan to test conjugates before using them in our *in vivo* models. Before initiating *in vivo* experiments, we will first run out the conjugate on SDS-PAGE under non-reducing and reducing conditions to determine whether or not the disulfide bond in our conjugate is intact. If the bond in our conjugate becomes reduced, the fVIII would no longer be attached to saporin. Thus, fVIII would no longer target saporin to the fVIII-specific naïve and memory B cells, explaining the disparity in our results. In addition, if the conformation of the active site of saporin, the residues Tyr72, Tyr120, Glu176, Arg179 and Trp208[11], has become denatured, it could have decreased or inhibited saporin's ability to kill. Finally, if the fVIII conjugated to the saporin had become denatured, its B cell epitopes could have been lost, making it difficult for the conjugate to bind to the immunoglobulins on fVIII-specific B cells. Thus, the targeting capacity of the conjugate would have been lost. We plan to test the killing ability of the first and

future conjugates using an *in vitro* assay and an ELISA to test the integrity of B cell epitopes.

To further test our strategy *ex-vivo*, we are currently developing a protocol in which we take spleens from hemophilia A mice, deplete the plasma cells, and treat the cells with saporin, fVIII, vehicle, or the conjugate. For these experiments, a new conjugate will be used from which free saporin and fVIII are removed. In addition, the last aliquot of the conjugate used in the previous experiments will be used. The cells are cultured with various concentrations of the B cell mitogens LPS or CpG with fVIII, and antibody production assessed at day six[12]. This protocol closely follows the protocol successfully used by Hausel et al. to stimulate fVIII memory B cells *in vitro*[13]. Using this protocol, we can determine whether our original conjugate is able to inhibit the production of anti-fVIII Abs *in vitro.* We will also repeat the *in vitro* experiments after first enriching for B cells so as to more directly determine the effect of our conjugate on B cells. If the conjugates inhibit the formation of anti-fVIII Abs after enriching for B cells, it would demonstrate that the mechanism of action of the conjugate is B cell specific. We will also look for fVIII-specific B cells using flow cytometry to determine the success of our protocol. Due to the low frequency of memory B cells, we will use a modification of a protocol used by Pape et al. to enrich for B cells[14].

We will use this system to further examine the ability of saporin to inhibit the formation of anti-fVIII Abs. We will test the hypothesis that saporin is entering cells through LRP receptors by treating cells with saporin with and without an anti-LRP MAb. FVIII and VWF have been shown to bind the LRP receptor[15-17].

Furthermore, the LRP receptor has been shown to be involved in the clearance of VWF by macrophages. Numerous groups have hypothesized that the LRP receptor could be involved in the endocytosis of fVIII by APCs and its subsequent presentation but, to date, studies suggest the LRP receptor is not involved in antigen presentation [16, 18, 19]. Therefore, if saporin is entering cells through the LRP receptor, then current data suggests that it would co-localize with cells such as macrophages responsible for VWF, and fVIII, catabolism. This could possibly explain a specific reduction in anti-fVIII Abs with the low dose of saporin used in our experiments. To further test the specificity of both the conjugate and, potentially, saporin, we are presently optimizing an OVA immunization protocol. We are immunizing our experimental mice with OVA in parallel with the fVIII treatments. When we test for anti-fVIII Abs, we will also test for OVA Abs. We will then be able to determine if the conjugate and saporin are specifically inhibiting the humoral immune response to fVIII. Finally, we will exploit our panel of MAbs to test the integrity of the B cell epitopes of our conjugate before testing them again in our *in vivo* systems. Once all of our tests are completed, we will repeat our *in vivo* experiments to see if we can reproduce our original experimental results.

Once we have completed this further testing, we can better examine whether a targeted toxin can consistently deplete naïve and memory B cells, and decrease or inhibit the formation of anti-fVIII antibodies. If we are successful at optimizing our conjugate, then fVIII-saporin potentially could be used in the treatment of congenital hemophilia A patients with inhibitors and with patients with acquired hemophilia A.

In addition, fVIII-saporin potentially could be used in previously untreated patients with hemophilia A to prevent inhibitor development.

In our research, we have aimed to directly decrease the immunogenicity of fVIII using fVIII-saporin. FVIII is an ideal model antigen for studying immune disorders, as the antigen is known. If successful, similar therapeutic strategies could be extended to other immune disorders. Thus, as antigens become better defined in other immune disorders, these studies may provide information about how to reduce antigenicity and immunogenicity and treat disease.

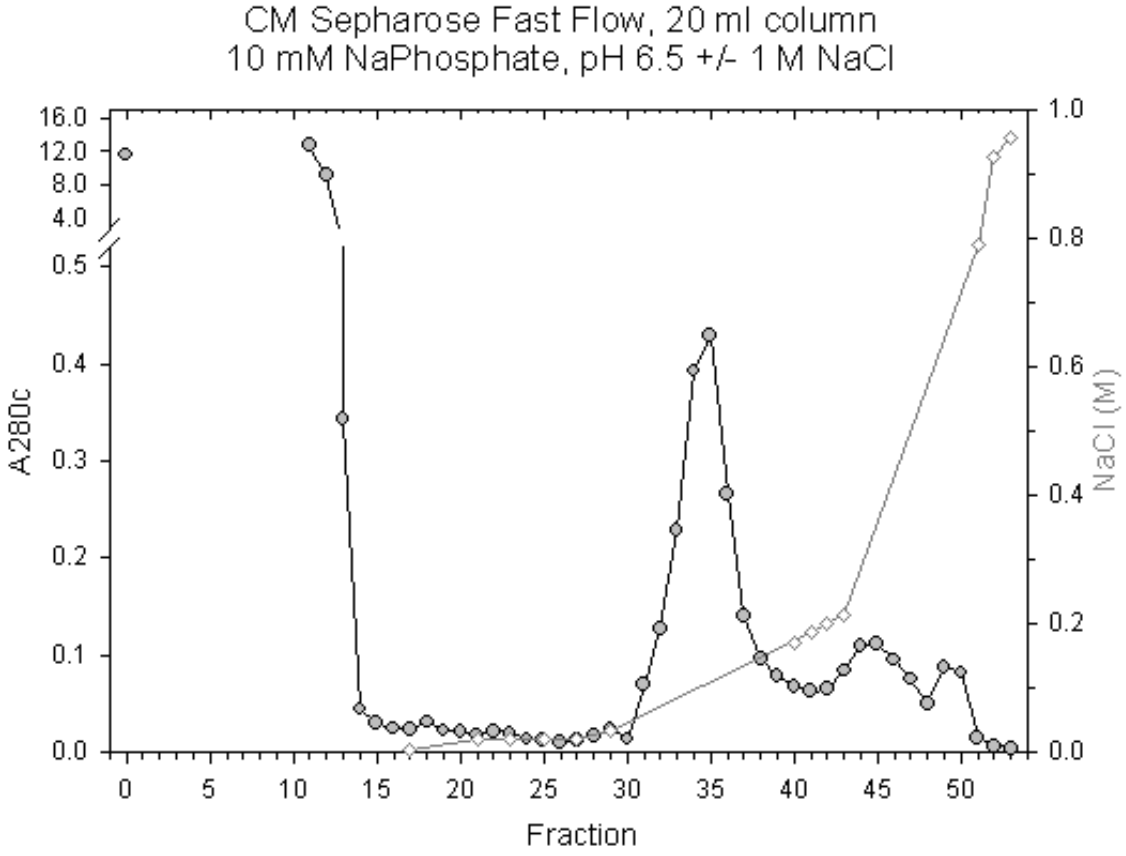
References

1. Prescott R, Nakai H, Saenko EL, Scharrer I, Nilsson IM, Humphries JE*, et al.* The inhibitor antibody response is more complex in hemophilia A patients than in most nonhemophiliacs with factor VIII autoantibodies. Recombinate and Kogenate Study Groups. *Blood* 1997,**89**:3663-3671.
2. Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Non-classical anti-factor VIII C2 domain antibodies are pathogenic in a murine in vivo bleeding model. *J Thromb Haemost* 2009,**7**:658-664.
3. Livnat T, Martinowitz U, Azar-Avivi S, Zivelin A, Brutman-Barazani T, Lubetsky A*, et al.* Combined administration of FVIII and rFVIIa improves haemostasis in haemophilia A patients with high-responding inhibitors--a thrombin generation-guided pilot study. *Haemophilia* 2013,**19**:782-789.
4. Doshi BS, Gangadharan B, Doering CB, Meeks SL. Potentiation of thrombin generation in hemophilia A plasma by coagulation factor VIII and characterization of antibody-specific inhibition. *PLoS One* 2012,**7**:e48172.
5. Dooriss KL, Denning G, Gangadharan B, Javazon EH, McCarty DA, Spencer HT*, et al.* Comparison of factor VIII transgenes bioengineered for improved expression in gene therapy of hemophilia A. *Hum Gene Ther* 2009,**20**:465-478.
6. Healey JF, Lubin IM, Nakai H, Saenko EL, Hoyer LW, Scandella D*, et al.* Residues 484-508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. *J Biol Chem* 1995,**270**:14505-14509.
7. Parker ET, Healey JF, Barrow RT, Craddock HN, Lollar P. Reduction of the inhibitory antibody response to human factor VIII in hemophilia A mice by mutagenesis of the A2 domain B-cell epitope. *Blood* 2004,**104**:704-710.
8. Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *J Thromb Haemost* 2004,**2**:1082-1095.
9. Collins PW, Hirsch S, Baglin TP, Dolan G, Hanley J, Makris M*, et al.* Acquired hemophilia A in the United Kingdom: a 2-year national surveillance study by the United Kingdom Haemophilia Centre Doctors' Organisation. *Blood* 2007,**109**:1870-1877.
10. Macauley MS, Pfrengle F, Rademacher C, Nycholat CM, Gale AJ, von Drygalski A*, et al.* Antigenic liposomes displaying CD22 ligands induce antigen-specific B cell apoptosis. *J Clin Invest* 2013,**123**:3074-3083.
11. de Virgilio M, Lombardi A, Caliandro R, Fabbrini MS. Ribosome-inactivating proteins: from plant defense to tumor attack. *Toxins (Basel)* 2010,**2**:2699-2737.
12. Mond JJ, Brunswick M. Proliferative assays for B cell function. *Curr Protoc Immunol* 2003,**Chapter 3**:Unit 3 10.
13. Allacher P, Baumgartner CK, Pordes AG, Ahmad RU, Schwarz HP, Reipert BM. Stimulation and inhibition of FVIII-specific memory B-cell responses by CpG-B (ODN 1826), a ligand for Toll-like receptor 9. *Blood* 2011,**117**:259-267.

14. Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK. Different B cell populations mediate early and late memory during an endogenous immune response. *Science* 2011,**331**:1203-1207.
15. Lenting PJ, Neels JG, van den Berg BM, Clijsters PP, Meijerman DW, Pannekoek H*, et al.* The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J Biol Chem* 1999,**274**:23734-23739.
16. Rastegarlari G, Pegon JN, Casari C, Odouard S, Navarrete AM, Saint-Lu N*, et al.* Macrophage LRP1 contributes to the clearance of von Willebrand factor. *Blood* 2012,**119**:2126-2134.
17. Saenko EL, Yakhyaev AV, Mikhailenko I, Strickland DK, Sarafanov AG. Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. *J Biol Chem* 1999,**274**:37685-37692.
18. Dasgupta S, Navarrete AM, Andre S, Wootla B, Delignat S, Repesse Y*, et al.* Factor VIII bypasses CD91/LRP for endocytosis by dendritic cells leading to T-cell activation. *Haematologica* 2008,**93**:83-89.
19. Herczenik E, van Haren SD, Wroblewska A, Kaijen P, van den Biggelaar M, Meijer AB*, et al.* Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain. *J Allergy Clin Immunol* 2012,**129**:501-509, 509 e501-505.

Appendix

1A. **Figure 1. Saporin expression and purification**

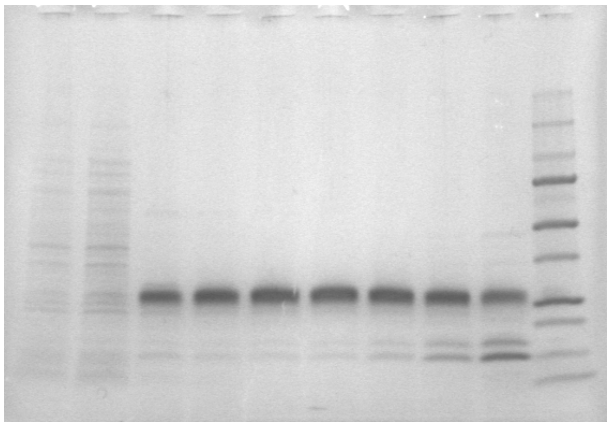


1B.

1C.

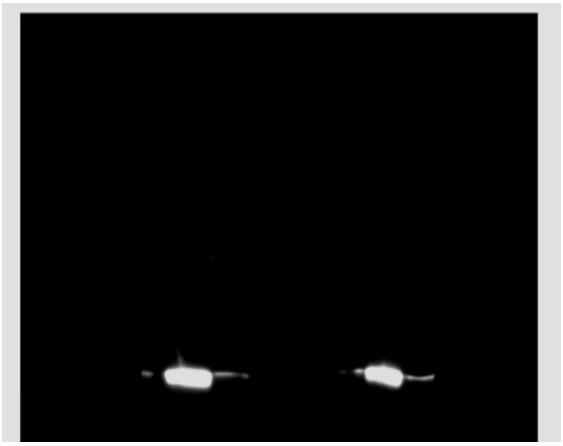
SDS-PAGE

Load FT 31 32 33 34 35 36 37 Stds



Coomassie Blue Staining

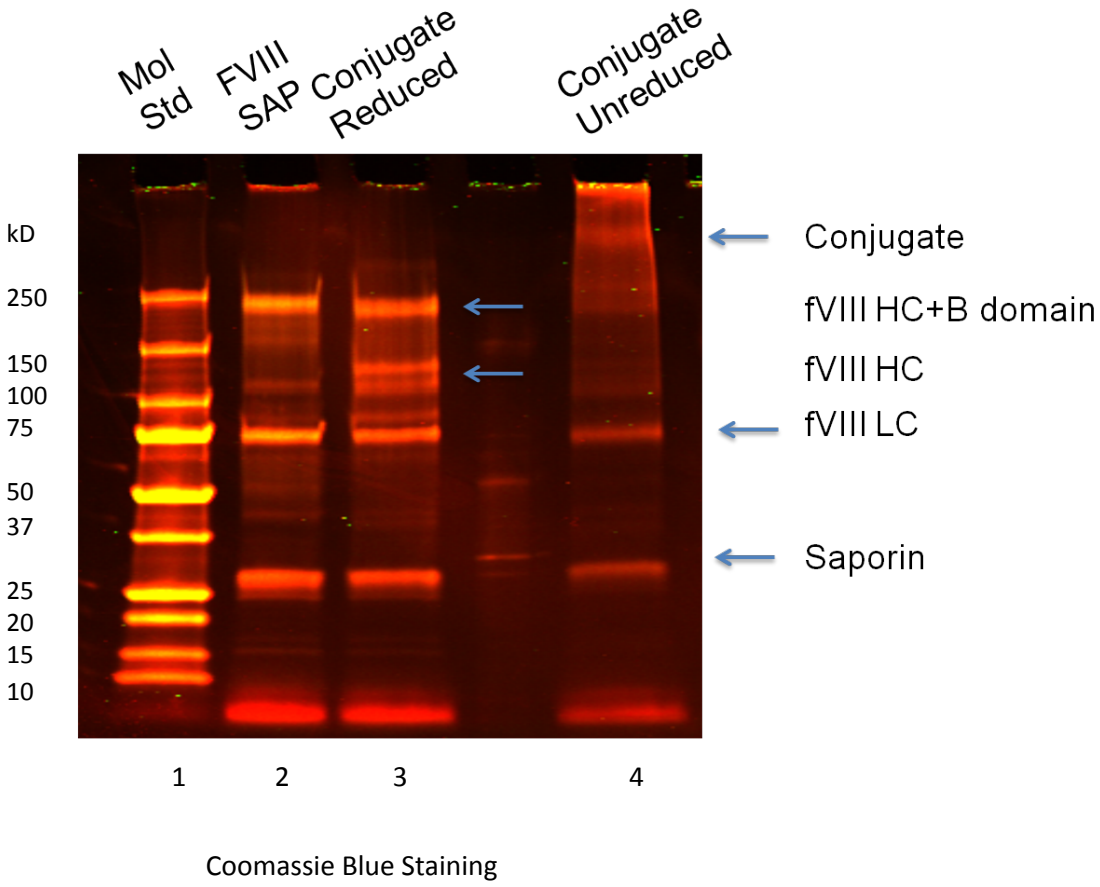
Western



SAP STD

Fraction 33

Figure 2. SDS-PAGE analysis of fVIII-saporin conjugate



Coomassie Blue Staining

Figure 3. FVIII specific ELISA to analyze the results of an adoptive transfer testing the fVIII-saporin conjugate

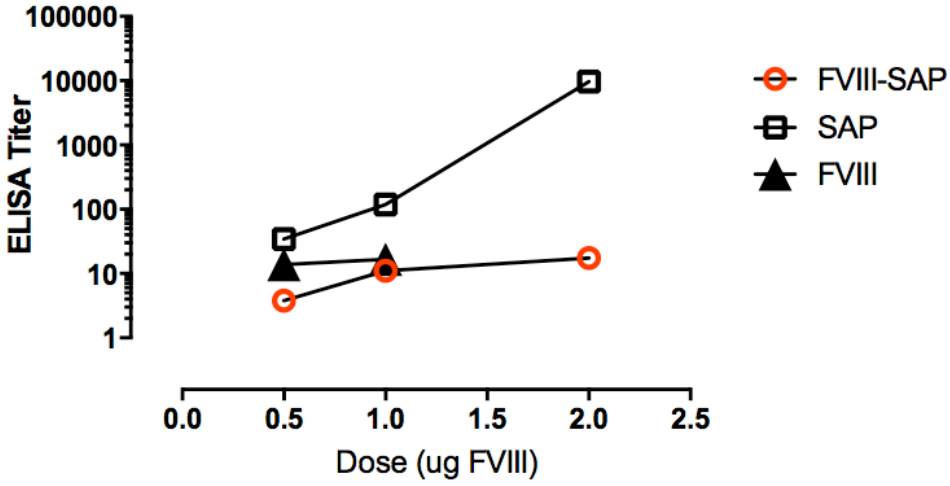
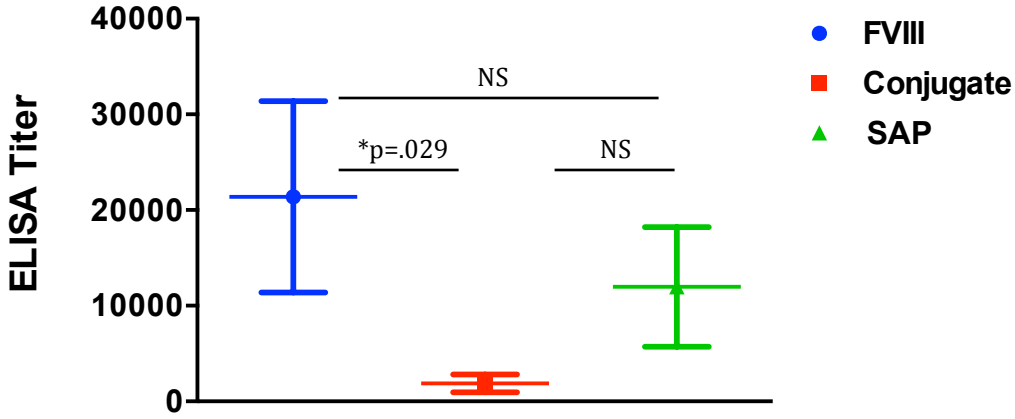
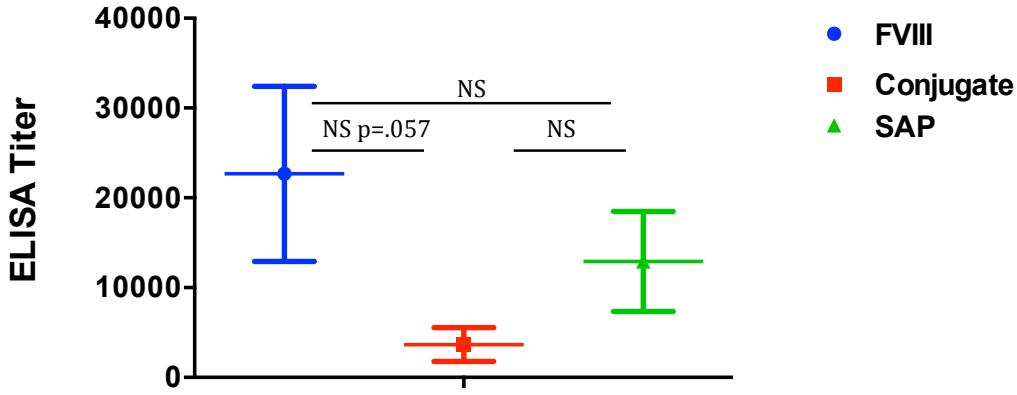


Figure 4. FVIII-saporin inhibits anti-fVIII Abs in naive mice

4A.

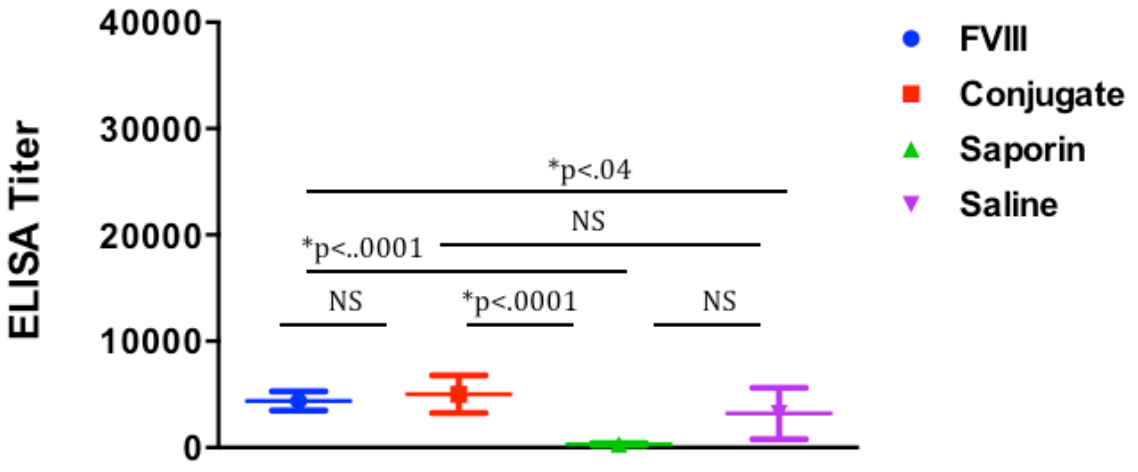


4B.

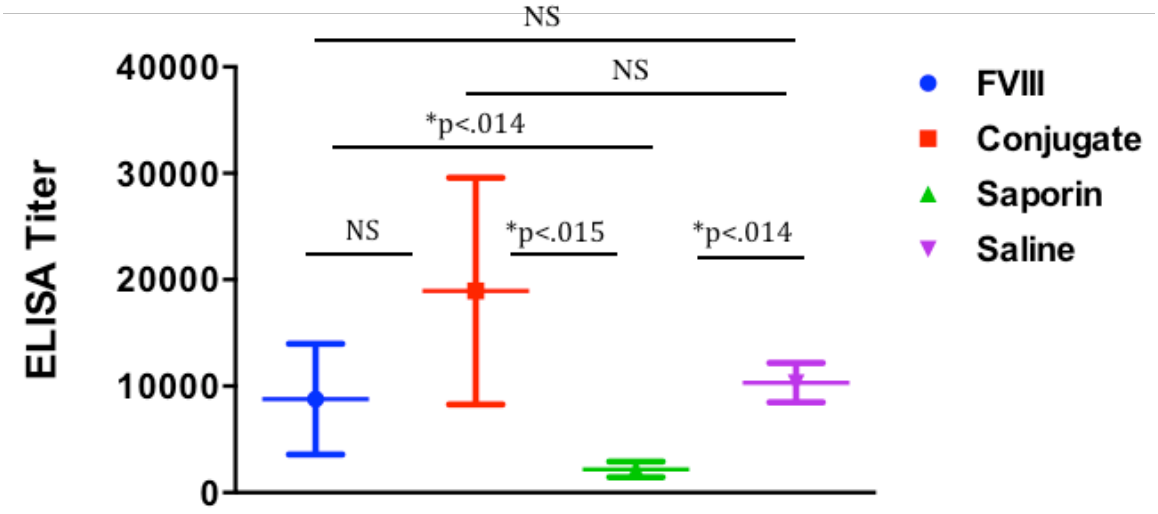


5A.

Figure 5. FVIII-saporin does not inhibit anti-fVIII Abs in naive mice



5B.



Figures

Figure 1. Saporin expression and purification. (A) After expressing saporin-3 using a pET system in *E. Coli* an ion exchange chromatography method was used for purification[1]. Saporin was diluted one to five in 10 mM sodium phosphate buffer, pH 6.5, and purified on a carboxymethyl-Sepharose column using a sodium chloride gradient at pH 6. (B) Pictured is SDS-PAGE and (C) a western blot demonstrating that saporin came off in a peak around fraction 33. The SDS-PAGE is stained with a non-specific dye, coomassie blue staining. The western was done with a saporin standard, fraction 33 of saporin, the flow through, a primary rabbit anti-saporin MAb, and polyclonal goat anti-rabbit horseradish peroxidase (HRP) for detection.

Figure 2. SDS-PAGE analysis of fVII-saporin conjugate. We incubated fVIII with Sulfo-LC-SPDP and after desalting the mixture quenched it with 0.1 M TRIS pH 7.0 pH and then incubated it with saporin. We then ran a SDS-PAGE gel and stained it with coomassie blue, a non-specific stain. Lane 1. Molecular weight standard, lane 2. fVIII and saporin control, Lane 3. fVIII-saporin conjugate reduced, Lane 4. fVIII-saporin conjugate unreduced.

Figure 3: FVIII-Specific ELISA to Analyze the Results of an Adoptive Transfer Testing the FVIII-saporin conjugate. Hemophilia A mice were immunized by intravenous injection with 2 μg of full-length fVIII every other week for eight weeks, followed by a final dose of 4 μg at ten weeks. Four weeks later, donor mice were

randomized into three treatment groups to receive equimolar doses saporin, fVIII, or fVIII-saporin, 2 μg of fVIII, 2.4 μg of the conjugate and 0.4 μg of saporin. Seven days after treatment, the mice were sacrificed and four million plasma cell ($CD138^+$)-depleted splenocytes were adoptively transferred as a source of fVIII-specific memory B cells into naïve recipient hemophilia A mice. Anti-fVIII IgG Abs in recipient mice were measured by a fVIII-specific high sensitivity ELISA developed for these experiments at 5 weeks after fVIII injection. Briefly, using a modification of a published protocol we coated 6 μg/ml of full-length fVIII onto ELISA plates, followed by an incubation with mouse plasma containing anti-fVIII Abs. We used goat-anti mouse streptavidin-alkaline-phosphatase conjugate for detection and doubled the volume used at every step[2].

Figure 4. FVIII-Saporin Inhibits Anti-FVIII Abs in Naïve Mice. 12 naïve E16 hemophilia A mice were divided into three treatment groups to receive equimolar doses of saporin, fVIII, or fVIII-saporin; 2 μg of fVIII, 2.4 μg of the conjugate and 0.4 μg of saporin. Starting seven days after the treatment, the mice were immunized with 2 μg of full-length fVIII every other week for twelve weeks. Anti-fVIII IgG Abs were measured after the (A)fourth and (B)sixth injections of fVIII. The Abs were measured using the high sensitivity ELISA described in Fig. 3. Comparisons between groups were made using the Mann-Whitney U test and Sigmaplot 12. A P value of less than 0.05 was considered statistically significant.

Figure 5. FVIII-Saporin Does Not Inhibit Anti-FVIII Ab Production in Naïve Mice. 32 naïve E16 hemophilia A mice were divided into four treatment groups to receive equimolar doses saporin, fVIII, or fVIII-saporin; 2 μg of fVIII, 2.4 μg of the conjugate and 0.4 μg of saporin. Starting seven days after the treatment, the mice were immunized with 2 μg of full length fVIII every other week for twelve weeks. Anti-fVIII IgG Abs were measured after the (A)fourth and (B)sixth injections of fVIII. The Abs were measured using the high sensitivity ELISA described in Fig. 3. Comparisons between groups were made using the Mann-Whitney U test and Sigmaplot 12. A P value of less than 0.05 was considered statistically significant.

RM designed experiments, analyzed data and made all the figures shown with the exception of figure 1A.

Appendix Results

We hypothesized that conjugation of fVIII to the toxin saporin, a Type I ribosome-inactivating protein, would target fVIII-specific cell surface immunoglobulin and selectively delete fVIII-specific naïve B cells and memory B cells. In order to test this hypothesis we planned to covalently link fVIII to saporin using a heterobifunctional crosslinker Sulfo-LC-SPDP[3]. To use this strategy we expressed and purified a mutant saporin, saporin-3, containing a single free cysteine. We purified saporin-3 using a two-step purification modified from a published protocol[1] after expressing saporin in *e. coli* using a pET system.

Next we covalently linked fVIII to saporin using Sulfo-LC-SPDP (Fig. 2.) SPDP is used for disulfide bond formation through amine to sulfhydryl conjugation. Sulfo-LC-SPDP initially was conjugated to free amines on fVIII. Saporin-3 then was coupled to the conjugate via a disulfide linkage using the in Sulfo-LC-SPDP. This form of conjugation has many advantages including stability in circulation[3, 4].

We tested our hypothesis that the fVIII-saporin conjugate could both prevent and eradicate fVIII inhibitors in an adoptive transfer model similar to models used by other groups[5, 6]. Hemophilia A mice were immunized with fVIII and four weeks after the last injection mice were treated with saporin, fVIII or the fVIII-saporin conjugate. A week later their spleens were harvested, depleted of plasma cells, and transferred into recipient hemophilia A mice. These recipient mice then received a single varying dose of fVIII. Naïve hemophilia A mice do not produce detectable anti-fVIII Abs following a single injection of fVIII. Therefore, any Abs detected after this

injection would be presumed to come from activated fVIII-specific B cells from the donor mice.

Anti-fVIII IgG Abs in recipient mice were measured by ELISA 5 weeks after the single fVIII injection. The mice that were treated with the fVIII-saporin conjugate after their initial five injections of fVIII had a blunted dose response to fVIII compared to the group treated with saporin, suggesting depletion of fVIII-specific naïve and memory B cells. This difference was not significant by a two-sample permutation test but the ratio of the linear regression lines of the slopes was 800. Therefore, this experiment should be repeated with the correct power to see a difference between the groups treated with saporin vs. fVIII-saporin.

To develop a model system to prevent fVIII inhibitor formation, naïve E16 hemophilia A mice were divided into three treatment groups to receive saporin, fVIII, or fVIII-saporin. Starting seven days after the treatment the mice were immunized with fVIII every other week for twelve weeks. Anti-fVIII IgG Abs were measured one week after the fourth and sixth injections of fVIII. Anti-fVIII IgG titers were significantly lower in the fVIII-saporin group compared to the fVIII group (1,900 vs. 21,400,$p=0.027$, $n=4$) after four injections of fVIII. After the sixth injections of fVIII the average titer of the fVIII group was 22,700 compared to 3,700 ($p=0.057$, $n=4$). The group that received saporin before their fVIII injections had a reduction in their anti-fVIII Abs of about half compared to the group that received only fVIII. This difference was not significant in the first naïve mouse experiment. A significant difference was seen in the second naïve mouse experiment when the group treated with saporin was compared to the group treated with fVIII.

In the second naïve mouse experiment (Fig. 5), there was no difference between the group treated with fVIII-saporin or the control groups, those treated with saline or fVIII. The same preparation of conjugate, shown in Fig. 2, was used for all three of these experiments. There was a difference between the groups treated with saline vs. fVIII that disappeared at week six. This suggests the difference was due to the extra injection of fVIII the "fVIII" group received at the beginning of the experiment.

References

1. Gunhan E, Swe M, Palazoglu M, Voss JC, Chalupa LM. Expression and purification of cysteine introduced recombinant saporin. *Protein Expr Purif* 2008,**58**:203-209.
2. Doering C, Parker ET, Healey JF, Craddock HN, Barrow RT, Lollar P. Expression and characterization of recombinant murine factor VIII. *Thromb Haemost* 2002,**88**:450-458.
3. Hermanson GT. *Bioconjugate techniques*. San Diego: Academic Press; 1996.
4. Polito L, Bortolotti M, Pedrazzi M, Bolognesi A. Immunotoxins and other conjugates containing saporin-s6 for cancer therapy. *Toxins (Basel)* 2011,**3**:697-720.
5. Ochsenbein AF, Pinschewer DD, Sierro S, Horvath E, Hengartner H, Zinkernagel RM. Protective long-term antibody memory by antigen-driven and T help-dependent differentiation of long-lived memory B cells to short-lived plasma cells independent of secondary lymphoid organs. *Proc Natl Acad Sci U S A* 2000,**97**:13263-13268.
6. Kafuye-Mlwilo MY, Mukherjee P, Chauhan VS. Kinetics of humoral and memory B cell response induced by the Plasmodium falciparum 19-kilodalton merozoite surface protein 1 in mice. *Infect Immun* 2012,**80**:633-642.