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Elizabeth S. York April 4, 2022

Investigating the Role of Immunoglobulin M in the Humoral Immune Response to Factor VIII

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Abstract

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Hemophilia A is an X-linked genetic disorder characterized by a deficiency in factor VIII (FVIII), an essential protein in the blood-clotting cascade. The most significant treatment complication for patients with hemophilia A is the humoral immune response to FVIII, which primarily consists of neutralizing polyclonal anti-FVIII antibodies (*i.e.*, inhibitors) that prevent successful replacement therapy. The mechanism of FVIII inhibitor formation and the role different immunoglobulin isotypes play in developing such an immunogenic response is not well understood. Previous laboratory data identified the presence of anti-C1 FVIII domain non-inhibitory and porcine cross-reactive immunoglobulin M (IgM) after immunization of a hemophilia A murine model with recombinant human FVIII. The persistence of these first responding IgM in the secondary humoral response to FVIII as well as the monoclonal binding to the C1 domain led to further investigation into their role in inhibitor formation. We set out to develop and characterize murine-derived anti-human FVIII IgM to evaluate their role in initiating and propagating the humoral response to FVIII. Hemophilia A mice lacking exon 16 in the *F8* gene were injected retro-orbitally with recombinant B domain deleted FVIII for a total of 5 weeks. Monoclonal anti-FVIII IgM antibodies were created using hybridoma technology fusing harvested splenocytes of the immunized hemophilia A mice with an immortal myeloma cell line. Enzyme-linked immunosorbent assays (ELISA) utilizing human recombinant FVIII protein constructs were performed after purification of anti-FVIII IgMs to assess FVIII-IgM binding interactions and FVIII domain-binding specificity. IgMs were determined to be porcine-cross reactive and non-inhibitory indicating similarities to the previously characterized IgM. However, IgM demonstrated weak binding affinity to FVIII which prevented definitive determination of domain-binding specificity. Weak binding interactions were hypothesized to be the result of alternative μ' variants of the IgM heavy chain leading to aggregation and decreased binding affinity. Further investigation of anti-FVIII IgMs is necessary to provide mechanistic insight into inhibitor development and pathogenicity.

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INTRODUCTION

Background

Hemophilia A is a congenital X-linked recessive bleeding disorder characterized by a deficiency in the blood coagulation protein Factor VIII (FVIII). Deficient FVIII levels result in the formation of inefficient blood clots during secondary hemostasis. The clinical phenotypes of hemophilia A are categorized by baseline plasma FVIII activity levels, where >50% is considered normal. Hemophilia A is classified as severe when FVIII activity levels are <1%, moderate if levels are between 1-5%, and mild for activity levels 6-49%.[1,2] FVIII deficiency is estimated to affect 1 in 5,000 live male births within the United States.[3] While symptoms vary depending on disease severity, characteristic bleeding symptoms include prolonged bleeding after injury, spontaneous joint bleeds (called hemarthrosis), muscle bleeds, and hematomas/bruising.[4] The same hemophilia A disease phenotype is caused by various mutations in the FVIII gene (*F8),* located at the terminal end of the long arm of the X chromosome.[5] The *F8* gene consists of 26 exons and codes for the FVIII glycoprotein consisting of 6 domains designated A1-A2-B-A3-C1-C2, with an additional activation peptide (ap) attached to the A3 domain (**Supplemental Figure S1**). The A1, A2 and B domains form the 200 kilodalton (kDa) heavy chain while the A3, C1, and C2 domains form the 80 kDa light chain. The B domain is the largest of these domains comprising 40% of FVIII mass.[5,6] Subsequently, the B domain has been identified as non-essential for the procoagulant function of FVIII in the clotting cascade.[5,7,8]

FVIII Procoagulant Function

The clotting cascade consists of two pathways (*i.e.,* the intrinsic and extrinsic pathways) each comprised of a series of enzymes in an amplified cascade that converges to one common

pathway which results in factor X (FX) activation. The main goal of the intrinsic pathway is to generate a "thrombin burst" in which thrombin uses feedback activation to rapidly amplify its response. The extrinsic pathway is initiated after damage to blood vessels causes abundant factor VII (FVII) to contact tissue factor (TF) expressed on stromal fibroblasts and various leukocytes.[9] Upon contact, TF and activated FVII (TF-FVIIa) form a complex known as the extrinsic tenase complex.[10] This complex activates coagulation factors IX (FIX) and FX. However, the process of FX activation to form FXa is rapidly inhibited, lasting just long enough to produce small amounts of FXa. FXa and activated factor V (FVa) form the prothrombinase complex, which proteolytically cleaves prothrombin into thrombin. Thrombin then activates FV and FVIII. FVIII plays an essential role in the convergence of the intrinsic pathway to the common pathway. Proteolytic cleavage of FVIII releases FVIII from its carrier protein von Willebrand Factor (VWF) and results in activated FVIII (FVIIIa). FVIIIa acts as a cofactor for activated FIX along with FX to form the intrinsic tenase complex. In the presence of calcium and a phospholipid surface, the intrinsic tenase complex continuously activates FX to FXa leading to the common pathway. In the common pathway, the prothrombinase complex (*i.e.*, FXa-FVa-prothrombin) converts large quantities of prothrombin to thrombin such that a critical quantity of fibrinogen is converted to fibrin monomers (**Supplemental Figure S2**). The fibrin monomers form the scaffolding from which a stable blood clot is formed. Thrombin also activates factor XIII which acts to cross-link fibrin monomers for further stabilization. FVIII therefore plays an essential role in the formation of stable fibrin clots via FX activation and assembly of the prothrombinase complex in large quantities.[9–12]

Treatment of Hemophilia A

The most widely used treatment for patients with hemophilia A is the intravenous infusion of FVIII concentrates for FVIII replacement. Therapeutic FVIII concentrates are either recombinant (rFVIII) or plasma derived (pdFVIII) products. In the clinical setting, FVIII replacement is administered on-demand for acute bleeding episodes or prophylactically for bleeding prevention.[13,14] FVIII replacement therapy has proven to be an effective treatment in hemophilia A. However, approximately 30% of patients with severe hemophilia A and 5% of patients with moderate to mild hemophilia A develop alloantibodies (*i.e.*, inhibitors) against the exogenous FVIII.[14–18] Inhibitors interfere with the procoagulant function of FVIII, thus preventing the efficacy of infused FVIII concentrates. Inhibitor formation results in significant morbidity as well as a higher rate of spontaneous and trauma-related bleeding, bleeding-associated complications, disability from chronic joint disease secondary to frequent bleeding episodes, and decreased quality of life.[19,20] Additionally, patients who develop inhibitors use significantly more health resources than patients who do not develop inhibitors as bleeding episodes become increasingly more difficult to treat. These patients have an increased clinical and financial burden of disease as they require more costly and less effective alternative hemostatic bypassing factor agents.[21,22] FVIII tolerance can be restored through immune tolerance induction (ITI): a treatment consisting of the infusion of frequent and high doses of FVIII concentrates over months to years. [23] ITI is the primary therapeutic approach for the elimination of existing inhibitors and has an estimated success rate of 70%.[24,25] This treatment, regardless of efficacy, imposes significant psychosocial and financial burdens on patients and their families. The formation of inhibitors consequently remains a significant clinical challenge in the management and treatment of hemophilia A.

Alternative therapies to FVIII replacement do currently exist, such as the non-factor therapy emicizumab, which significantly reduce the risk of bleeding when given prophylactically. Such therapies, however, are unable to treat active bleeds or eliminate existing inhibitors.[22,26] The inability to accurately predict or prevent inhibitor formation in patients with hemophilia A reflects the ongoing gaps in our understanding of important immune mechanisms underlying the humoral immune response to FVIII. The limitations of current therapies as well as the considerable psychosocial and financial burdens of ITI leave the formation of inhibitors a significant burden in the management of hemophilia A, which further emphasizes the need for improvements in our understanding of this humoral immune response.[22,25]

Immunoglobulins and FVIII Inhibitor Formation

Antibodies, also called immunoglobulins (Ig), are the secreted form of B cell receptors. The typical structure of an Ig consists of two constant and two variable regions in a 'Y' like structure linked via disulfide bonds. There are five different constant regions that define the five Ig isotypes and two different light chains. Although no functional differences have been found, the light chain of any Ig is described as kappa (κ) or lambda (λ).[27] Antibodies are major players in humoral immunity and have the ability to bind to any possible antigen. The diverse conformations of Ig isotypes result in varying functional roles such as opsonization, agglutination, activation of the complement cascade, and lymphocyte activation. The first antibody to appear in the immune response to an antigen has a μ constant region and is consequently termed immunoglobulin M (IgM). All other Ig isotypes are formed after IgM undergoes somatic hypermutation and class switching in their variable regions resulting in antigen specific adaptations.[27] For example, the immunoglobin G (IgG) antibody isotype, which

predominantly makes up the secondary immune response to an antigen, is the most abundant Ig in normal human serum and has a high binding affinity for antigens.

Following initial exposure to FVIII, the primary humoral immune response (similar to that of any antigen) consists of low binding-affinity IgM proceeded by high-affinity FVIII-specific IgG antibodies which constitute the more robust secondary immune response observed after subsequent FVIII exposures.[28,29] Most inhibitors which actively neutralize FVIII procoagulant function have been found to be polyclonal IgGs. IgG inhibitors have been found to target each FVIII domain with significant predominance to the A2 and C2 domains.[30–33] The binding of inhibitors to these domains is thought to result in steric hindrance of functionally critical epitopes on FVIII leading to inhibition of direct and indirect FVIII functions.[34] The formation of autoantibodies against endogenous FVIII can also occur in adults without a history of hemophilia A, termed acquired FVIII inhibitor disorder or acquired hemophilia A.[35] With a reported annual incidence of 1 case per million, acquired hemophilia A is extremely rare and most cases are idiopathic despite some association with underlying autoimmune diseases, pregnancies, and malignancies.[36] Natural antibodies to FVIII have also been described in healthy donors with normal FVIII activity and no bleeding symptoms.[32,33] There are two types of anti-FVIII inhibitors: type I inhibitors, which completely inhibit FVIII procoagulant activity, and type II inhibitors which incompletely inhibit such FVIII activity (**Supplemental Figure S3**).[37] Patients with mild, moderate, or acquired hemophilia A are more commonly found with type II inhibitors and patients with severe hemophilia A more commonly have both type I and type II inhibitors, although type II become increasingly more difficult to distinguish under the potent inhibition of type I antibodies.[38] In any case, both types of inhibitors leave patients more vulnerable to the characteristic bleeding symptoms of hemophilia A.[28] Of the recorded inhibitors

to FVIII IgM antibodies have been described in the majority of cases. However, neither the epitope nor the binding domain specificity of anti-FVIII IgMs have been determined.[29] Additionally, it is unknown whether anti-FVIII IgMs act to inhibit FVIII in a similar fashion as anti-FVIII IgG inhibitors or if IgMs have an alternative non-inhibitory role.[29,30]

Characteristics of IgM Antibodies

IgM is a highly glycosylated antibody isotype made of a ~25 kDa light chain consisting of both a single variable domain and single constant region, and a ~75 kDa heavy chain composed of a single variable domain and four constant regions referred to as Cμ(1-4).[27,39] IgM is the only class of antibody that exists in all vertebrates and show the highest evolutionary conservation of all Ig.[40,41] Such conservation results in low binding activities, an important characteristic of first responding IgM in their potential for affinity-maturation through multiple mutagenic processes.[42] IgM can exist in a monomeric form expressed on the surface of B cells as a B cell receptor or in a secreted pentameric form (sIgM) with 10 antigen binding sites. The pentameric form is linked via disulfide bonds to a long polypeptide 15 kDa joining (J) chain, which aids IgM in passage through mucosal barriers.[41,43,44] The 10 antigen binding sites of IgM allow for the highest binding avidity among all Ig isotypes, which assists in compensating for the weak binding affinity associated with IgM.

Secreted IgMs are produced by two different B cell populations: B1 cells produce natural antibodies, while B2 cells found in the spleen and lymph nodes secrete adaptive IgM (also termed “induced” IgM).[39,41] Conventional textbook views believe IgM to be planar, short-lived, and produced only during acute infection.[27,41] IgM-producing plasma cells are primarily found in the spleen and are unique in their ability to develop without germinal centers. They are therefore

not associated with somatic hypermutation/affinity maturation nor high binding affinity.[27] In concordance with this understanding, induced IgMs are not suspected to play significant roles in long-term immunity. However, this hypothesis may require further investigation with increasing evidence for long-term IgM in the adaptive immune response. There have been various studies demonstrating contrasting results for IgM in enhancing and suppressing adaptive immune response. For example, Bohannon et al. described a population of long-lived, antigen-specific IgM-secreting plasma cells that persisted 2 years in the spleen following mouse immunization with inactivated influenza virus.[45] These induced IgM plasma cells were found to be undergoing somatic hypermutation with the assistance of activation-induced cytidine deaminase (AID). High affinity B-cell clones are typically given survival signals through the process of antigen-selection which correlates to enriched AID mutations at complementary-determining regions (CDR).[27,46] In contrast, the mutations induced by AID in these IgM were widespread throughout the variable region without enhancement at CDRs, providing evidence that these long-lived, antigen-specific IgM did not undergo conventional antigen-selection for affinity maturation.[45] In a separate study, induced IgM alloantibodies showed immunosuppressive activity and lessened the severity of an autoimmune diseases associated with IgG autoantibodies.[47] Boes et al. induced a deleterious mutation into the sIgM gene in lupus-prone lymphoproliferative mice that halted sIgM production.[47] This autoimmune murine model with deleterious IgM showed IgG serum levels 3-10 times higher than wild type mice and had significantly shortened life spans.[47] The authors therefore concluded that sIgM are associated with the suppression of IgG autoantibody development.

Moreover, it has been well established that IgM have the potential to function as vigorous activators of the complement system.[27,39,42,48,49] Following binding to an antigen on an exposed

surface, sIgM is able to shift its conformation to a staple-like arrangement.[48,50] This conformational change enables complement protein C1q binding to the Fc tail portion of IgM, triggering complement activation.[48] Byproducts from the complement cascade, known as anaphylatoxins (i.e., C3a, C5a) are known to induce local inflammation and trigger pathways of lymphocyte activation.[51] Activation of the complement cascade can rapidly amplify the immune response to a specific antigen and its association with the Fc portion of IgM is evidence enough for the abilities of IgM to amplify local immune responses. The complement cascade has been shown to serve as an adjuvant in the primary immune response to FVIII.[52] Using a mouse model of hemophilia A, one study described the presence of the complement protein C3 to have an almost 2-fold increase in FVIII uptake by dendritic cells and presentation to CD4+ T cells. C3 depletion in hemophilia A mice resulted in a statistically significant decrease in anti-FVIII IgG production. The role of IgM activation in assisting this C3 adjuvant has yet to be confirmed.

In other expanding knowledge of IgM, it was found that IgM can serve as host protein carriers. Using cryo-atomic force microscopy, a new structural model shows sIgM in both mice and humans to appear as an asymmetric mushroom shaped “quasi-hexamer” missing one monomer.[50] Hiramoto et al. found these “gapped” pentameric IgM to carry circulating apoptosis inhibitor of macrophage (AIM) where the missing IgM monomer once was. AIM is a protein known to aid in the survival of macrophages and is associated with the prevention of various diseases.[53] The potential of IgM to carry proteins in this gapped form portrays another unique characteristic of these glycoproteins and provides evidence of the multi-faceted role IgM may play in various immune pathways. Taken together, these results suggest that IgM could play a role in either suppressing or amplifying the humoral immune response to FVIII, as well as play a more pertinent role in long-term immunity. Currently, most of the approved recombinant

antibody therapeutics are limited to the IgG subclass.[54] Novel discoveries on IgM function along with the presence of IgM in the early defense against antigens provides convincing evidence for the therapeutic potential of targeting these molecules with applications to a broad range of medical treatments.[22,54,55]

N-terminus Trimming of the IgM Heavy Chain Variable Region

A peculiar phenomenon that has been associated with decreased recombinant IgM activity is N-terminus trimming of the variable region of the heavy chain yielding a 55 kDa truncated μ' chain.[56–58] These μ' chains are missing stretches of the variable regions and can be unreactive in antigen/antibody binding interactions.[58] Various lengths of truncated μ' chains have been found and are hypothesized to result from alternative splicing or post-translational modifications.[57,59–61] The effects of such μ' IgM variants on IgM activity and immune responses is unknown. IgM aggregation was also observed in these alternatively processed IgM and concluded to be a prominent contributor to weak antigen binding by IgM.[57] Klaus et al. attempted to identify the factors of μ' generation and proposed IgM heavy chain cleavage to be a non-enzymatic process with physiological relevance. They isolated a site for non-enzymatic cleavage at asparagine (Asn) residue 209. Site-directed mutagenesis at Asn209 with any amino acid prevented the formation of μ'. Such cleavage produced both free antigen binding fragments (Fab) and a truncated heavy chain. The Fab fragments may bind to cognate epitopes and sterically block antigen availability for other Ig fragments. Therefore, the μ' heavy chain can act to suppress early immune responses.[57] This potential "auto-cleavage" of the variable chain at Asn209 and creation of alternative variable chains could be the missing link in understanding how IgM

undergo affinity maturation for long-term adaptive immunity without the need for germinal centers.[45]

Summary of the Importance of FVIII IgM Investigation

The development of anti-FVIII alloantibodies is recognized as the single most significant complication in the management of hemophilia A in the modern era.[62] Such a highly prevalent and robust humoral immune response to FVIII in both the treatment for hemophilia A as well as the spontaneous development of inhibitors in acquired hemophilia A necessitates an improved understanding of FVIII immunogenicity. The gaps in our knowledge of the humoral immune response to FVIII include mechanisms of initiation of the primary immune response of FVIII, inhibitor formation, and the role different antibody isotypes play in inhibitor development or propagation.[28]

Characterizing IgM's response to exogeneous FVIII could start to fill the gaps in our understanding of key initiation and propagation events. The motivation for identifying the role of IgM in the humoral immune response to FVIII arouse during previous laboratory experiments performed during the process of anti-FVIII IgG characterization. In these experiments, five anti-FVIII C1 domain IgM were identified following immunization with six injections of FVIII in hemophilia A mice (Ito J and Batsuli G, unpublished laboratory data). These five IgMs were non-inhibitory in the Bethesda clot-based assay and cross-reactive to the porcine FVIII C1 domain (referred to as porcine-cross reactive). Interestingly, the FVIII C1 domain is the most conserved region of all the FVIII domains across species with 86-91% sequence homology between human, porcine, and murine FVIII.[63] The predominant binding of these IgMs to the C1 domain suggested an immunodominant IgM-binding region in the initiation or propagation of the

humoral immune response to FVIII. Whether IgM enhance or suppress the inhibitor response to FVIII has not been evaluated which further prompted the execution of this study in evaluating the ambiguous role of IgM.

We therefore hypothesized that murine-derived anti-human FVIII IgM detected in the secondary phase of the humoral immune response to FVIII would primarily recognize the FVIII C1 domain and will be non-pathogenic in a murine model of hemophilia A. To address our main objective of characterizing anti-FVIII monoclonal IgM to determine whether they act to amplify or suppress the immune to FVIII given our hypothesis, this project was encompassed by three aims: (1) to purify and confirm IgM classification of murine-derived monoclonal FVIII IgM using hybridoma technology, (2) to characterize the effect of FVIII IgM on the biochemical properties and biological functions of FVIII, and (3) to evaluate the *in vivo* effects of FVIII/IgM complexes on IgG antibody formation in a severe hemophilia A murine model.

MATERIALS and METHODS

Materials

GlutaMAX was purchased from Invitrogen (Carlsbad, CA, USA). ORI-GEN Hybridoma Cloning Factor (HCF) was purchased from IGEN (Gaithersburg, MD, USA). A Roche IsoStrip™ Mouse Monoclonal Antibody Isotyping Kit, Hybri-Max, and OPI Media Supplement were purchased from Sigma-Aldrich (St Louis,MO, USA). Streptavidin alkaline phosphatase was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Goat anti-mouse isotype-specific antibodies were purchased from Southern Biotech (Birmingham, AL, USA). Recombinant full-length human FVIII (Advate, Takeda) was donated to the laboratory from the Pediatric Hemophilia Treatment Center at Emory University and the Aflac Cancer and Blood

Disorders Center at Children's Healthcare of Atlanta. Recombinant B domain deleted (BDD) human FVIII was expressed from a baby hamster kidney-derived cell line and purified as described in previous publications.[64–66] Hybrid FVIII constructs of porcine-sequence BDD FVIII containing a single human substituted A1, A2, ap, A3, C1 or C2 domain were previously developed in the lab.[66,67] Alkaline phosphatase-conjugated goat anti-mouse antibody was purchased from Bio-Rad (Hercules, CA). Citrated pooled normal plasma (FACT) and FVIII deficient plasma for the Bethesda assay were purchased from George King Biomedical (Overland Park, KS). All other materials were reagent grade or are described in the cited literature.

Hemophilia A Murine Model

A breeding colony of Hemophilia A mice has been established in the laboratory of Drs. Shannon Meeks and Pete Lollar (Emory University, Atlanta, GA). This murine model lacks exon 16 (designated E16) in the *F8* gene ($FVIII^{-/-}$) on a mixed C57BL/6 and 129S4 background at a C57BL/6:129S4 ratio of 70%:30% and were originally obtained from Leon Hoyer (American Red Cross, Holland Laboratory, Rockville, MD).[68,69] Experiments were performed in male and female $FVIII^{-/-}$ mice between 8-12 weeks of age.[37,66,68] Emory University Institutional Animal Care and Use Committee approved the study methods and use of animals. The Emory University Division of Animal Resources provided training for the proper handling and euthanasia of animals.

Production of IgM Secreting Hybridomas

$FVIII^{-/-}$ mice were weekly injected retro-orbitally with 1 μg recombinant human BDD FVIII for four weeks followed by a 2 μg "boost dose." On week six, spleens were harvested, and plasma

collected via cardiac puncture for detection of anti-FVIII IgG and IgM titers by ELISA. Mice were characterized as having "low titer" antibodies for anti-FVIII IgG ELISA titers <2,000 and "high titer" antibodies for anti-FVIII IgG ELISA titers ≥2,000. This designation was based on prior studies that suggested a higher frequency of IgM antibodies in immunized mice with anti-FVIII IgG ELISA titers <2,000 (*i.e.*, >40 IgM hybridoma colonies isolated) in comparison to mice with "high titer" antibodies that had a lower frequency of IgM-secreting hybridomas isolated (*i.e.*, 0-5 IgM secreting hybridomas).[30] Subsequent experiments utilized antibodies produced from spleens of one "high-titer" FVIII immunized mouse and one "low-titer" FVIII immunized mouse.

Harvested splenocytes were centrifuged for 10 minutes at 1325 rotations per minute (RPM) at 4°C and resuspended in 1 mL hybridoma growth medium containing: S10 HEPES, heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 50 U mL^{-1} penicillin, and 50 µg mL^{-1} streptomycin.[67] To obtain FVIII-specific IgM antibodies, immortal monoclonal antibody-secreting hybridoma cell lines utilizing the two harvested spleens were produced using Köhler-Milstein hybridoma technology.[70] Splenocytes were fused with a NS-1 myeloma cell line using polyethylene glycol (PEG-1500) diluted in S10 HEPES, 5% HCF, 100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine medium (HAT), 2 mM glutamine, 50 U mL^{-1} penicillin, and 50 µg mL^{-1} streptomycin. Splenocytes successfully fused with NS-1 myeloma cells were transferred to 96-well plates at a concentration of ~60,000 cells per well.[67] Hybridomas were maintained at 37°C in 5% CO_2 standard atmosphere and were screened for anti-FVIII IgM antibodies via ELISA of hybridoma cell supernatant on day 7 and 10 post spleen fusion **(Figure 1)**. Hybridoma colonies with an ELISA optical density >0.27 at an absorbance of 405 nm were categorized as positive for anti-FVIII IgM secretion when compared to background optical

densities of conditioned medium without hybridomas. IgM-secreting hybridoma colonies were then expanded in hybridoma growth medium. Stocks of hybridoma colonies were also stored in hybridoma growth medium containing 10% dimethyl sulfoxide (DMSO) at −80°C for future experimental analyses.

Anti-FVIII IgM Expression and Purification

Freshly expanded hybridoma colonies in hybridoma growth medium or recently thawed frozen hybridomas were utilized to obtain purified IgM antibodies. Frozen hybridoma colonies were thawed in a 37°C water bath, centrifuged at 1000 RPM for 5 minutes, then resuspended in hybridoma growth medium. Cells were transferred to T-75 cm^2 flasks at 37°C in 5% CO_2 atmosphere in incremental volumes of hybridoma growth medium up to a max volume of 50 mL for 10-14 days contingent upon achieving a cell density of 1 x 10^6 cells mL^{-1}. Cells were centrifuged at 1100 RPM for 10 minutes and resuspended into a separate T-75 cm^2 flask containing 50 mL of antibody expression medium (*i.e.*, CD Hybridoma medium, 4 mL 250X cholesterol, 2 mM L-glutamine, 50 U mL^{-1} penicillin, and 50 μg mL^{-1} streptomycin). Hybridoma cells were allowed to express for 6-7 days in antibody expression medium before supernatants were harvested by centrifugation at 3400 RPM for 10 minutes. To inhibit bacterial growth, 0.05% sodium azide (NaN_3) was added to harvested hybridoma supernatant and kept at 4°C until purification.

Monoclonal IgMs were purified from hybridoma supernatants within 4 weeks of harvest via affinity chromatography. A chromatography column was packed to a column volume (CV) of 2 mL with loose commercial IgM-specific resin (LigaTrap Technologies®, Raleigh, NC), before the chromatography apparatus was washed using 20 mL (10 CV) of equilibrium buffer

containing 10 mg/mL adipic acid and 800 mM NaCl. The hybridoma supernatant containing secreted anti-FVIII IgM was diluted at a ratio of 1:4 in 50 mg/mL adipic acid and 4 M NaCl (pH 5.8) before being loaded onto the column. The chromatography column was washed once again with equilibrium buffer and IgM eluted with 0.1 M sodium acetate in 2 mL aliquots. IgM-containing aliquots were concentrated using a 15 mL filter tube with a 100 kDa molecular weight cutoff and centrifuged 2-4 times at 3500 RPM for 25 minutes. IgM concentrations were adjusted to ~1 mg/mL and stored at -80°C. To confirm successful purification of IgM and absence of contaminants, purified IgM were analyzed by 4-15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under non-reduced and disulfide-reduced conditions with TCEP. Proteins were run at 200 V for 20-30 minutes and analyzed with Brilliant Blue R250 staining solution.

Nijmegen Bethesda Assay

A modified Bethesda assay was carried out to determine the inhibitory effect of IgM on FVIII clotting activity.[71] Inhibition in the Bethesda assay is defined as the amount of antibody which inhibits 50% of FVIII activity in pooled normal plasma.[72] This is compared to a standardized reference curve for residual FVIII activity between 40% and 60%.[49,73] Purified IgMs were diluted at multiple concentrations with 0.15 M NaCl/20 mM HEPES/0.05% Tween-80 (pH 7.4) and incubated for 2 hours at 37°C. Samples were subsequently clotted after a 4 minute incubation with FVIII deficient plasma and an activated partial thromboplastin time (aPTT)-based reagent. Clots were induced by the addition of 2 mM $CaCl_2$ and clotting times were measured. Citrated pooled normal human plasma (known FVIII activity >95%) incubated with FVIII deficient plasma was used as the control sample for comparison.[67] The IgM dilution

resulting in ~50% inhibitory activity was converted to a Bethesda titer presented as Bethesda units per milligram of IgM antibody (BU/mg IgM). Inhibitors were determined to be type I or type II based on the inhibition pattern visualized with the Bethesda curve. Type I inhibitors caused >98% inactivation of FVIII, while type II inhibitors incompletely inhibit FVIII activity (**Supplemental Figure S3**).[38]

Anti-FVIII IgM Binding ELISA

IgM binding to FVIII was confirmed via ELISA using a high binding 96-well polypropylene plate incubated with 1.5 μg/mL full-length FVIII or BDD FVIII in 20 mM Bicine and 2 mM $CaCl_2$ (pH 9) incubated overnight at 4°C. Plates were washed with buffer A containing 20 mM HEPES, 0.15 M NaCl, 0.05% Tween-20, and 0.05% NaN_3. The following day ELISA plates were blocked with 160 μl blocking buffer (buffer A with 2.0% bovine serum albumin (BSA)) in each well and incubated overnight at 4°C. Plates were washed before the addition of purified IgM at 10-50 μg/mL were incubated for one hour, followed by alkaline phosphatase-conjugated goat anti-mouse IgM secondary antibody at a 1:250 dilution in blocking buffer after plate washes with buffer A between each incubation. IgM binding to FVIII was detected using substrate p-nitrophenyl-phosphate (*pnpp*) and absorbance was measured at 405 nm. Wells incubated with blocking buffer without IgM were utilized as negative controls and a non-inhibitory monoclonal anti-C2 IgG2a antibody 2-117 served as the positive control.

Identification of the FVIII Binding Domain

An ELISA utilizing hybrid FVIII constructs of porcine-sequence BDD FVIII containing a single human substituted A1, A2, ap, A3, C1 or C2 domain was performed to identify FVIII domains

bound by purified IgMs **(Supplemental Figure S4)**.[66,67] A second ELISA was performed using recombinant isolated A2, C1, and C2 domains conjugated to human serum albumin (i.e., HSA-A2, HSA-C1, and HSA-C2).[16,74] Single domain A1 and A3 constructs were not available due to poor protein expression. For both ELISAs, polypropylene plates were incubated with the protein constructs in 20 mM Bicine and 2 mM $CaCl_2$ overnight at 4°C. The remainder of ELISA procedures including washes, blocking, IgM incubation, capturing antibodies, and detection methods were the same as described above in Anti-FVIII IgM Binding ELISA. Wells incubated with blocking buffer without IgM were utilized as negative controls and non-inhibitory monoclonal anti-C2 IgG2a antibody 2-117 served as the positive control.

IgM Excipient Stabilization Assay

Aliquots of purified IgMs were maintained in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline (HBS) containing 0.05% NaN_3 at 4°C for 15-18 weeks or stored at -80°C. Prior studies have shown that mouse IgM antibodies are relatively unstable and susceptible to enzymatic cleavage.[54,57] Recent findings from Mueller et al. lead to the conclusion that the presence of excipients could suppressed proteolytic degradation of IgM through preferential exclusion.[75] This paper specifically found excipients of sorbitol and glycine to have strong osmoprotective effects against enzymatic degradation of IgM. In attempt to resolve the instability of IgM using the results from Mueller et al., an ELISA was performed to determine whether the addition of sorbitol and glycine could aid IgM stability and facilitate improved IgM characterization in the *in-vitro* assays evaluating FVIII and IgM binding. Buffers of four purified anti-FVIII IgM (IgMs designated R155, R284, S1310, and R44) stored at 4°C and -80°C were exchanged into buffer solutions containing excipients. Buffer solutions consisted

of (1) HBS and 0.05% NaN_3, (2) HBS, 0.05% NaN_3, and 20% sorbitol, (3) HBS, 0.05% NaN_3, and 1M glycine, and (4) HBS, 0.05% NaN_3, 20% sorbitol, and 1M glycine. Buffer exchanges were performed using 4 mL filter conical tubes with a molecular weight cutoff of 100 kDa. Purified IgMs maintained at 4°C in different excipient solutions were compared by ELISA to IgM-FVIII binding by freshly thawed IgM stored at -80°C in the corresponding excipient solutions.

Statistical Analysis

Data are presented as means ± standard deviation (SD) unless specified otherwise. A t-test was used to compare purification quantities between IgM antibodies derived from the low-titer versus the high-titer spleen. One-factor Analysis of Variance (ANOVA) test was used to evaluate significant differences in optical densities at A_{405} of IgM-FVIII binding in the enzyme linked immunosorbent assays (ELISA). A significance value of $P < 0.05$ was considered statistically significant.[76]

RESULTS

Production of Anti-FVIII Monoclonal IgM

IgM secreting hybridomas were produced from two spleens harvested from mice differing anti-FVIII IgG plasma titers (*i.e.*, one low-titer and one high-titer spleen). A low-titer spleen with an anti-FVIII IgG ELISA titer of 1133 produced 10 (3.33%) anti-FVIII IgM-secreting hybridoma colonies out of 300 colonies. The high-titer spleen with an anti-IgG ELISA titer of 8501, 11 (3.82%) anti-FVIII IgM-secreting hybridoma colonies were identified out of 288 colonies. From the 588 total colonies selected between the two spleens, 21 colonies were positive for anti-FVIII

IgM secretion corresponding to 3.58% of all hybridomas **(Figure 2)**. There were no significant differences in the frequency of IgM production based on IgG ELISA titer. Under reduced conditions, 75 kDa and 25 kDa bands was observed on SDS-PAGE corresponding to the heavy and light chains of IgM, respectively, in 19 of the 21 IgM-secreting hybridoma supernatants. The SDS-PAGE showed that two of the 21 IgM hybridomas has cessation of IgM production following transfer of hybridoma cells to hybridoma growth medium. Purification of these two hybridoma supernatants was not attempted.

The Low-Binding Affinity of IgM Necessitates Alternative Purification Approaches

Initial attempts at IgM purification by affinity chromatography using a SP Sepharose Fast Flow resin resulted in detectable IgM in the flow-through buffer despite adjustments in the NaCl concentration and flow rates. Moreover, purified IgM was absent from the elution buffer for most IgM antibodies. To improve IgM purification quality and yield, an alternative commercially IgM-specific resin (LigaTrap Technologies®) was utilized. The new IgM-specific resin allowed for the successful purification of all 17 IgM antibody **(Table 1)**. Of 19 anti-FVIII IgM-producing hybridomas identified by ELISA screening and SDS-PAGE, 17 (89%) IgM antibodies were successfully purified. These 17 IgMs were purified from 9 hybridomas isolated from the high titer spleen and 8 hybridomas isolated from the low-titer spleen **(Table 1)**. The average quantity of IgM produced was 1.93 ± 1.73 mg per monoclonal antibody (MAb). There was no significant difference between the quantity of IgM harvested from hybridoma supernatants derived from the low titer versus high titer spleen (1.33 ± 0.77 mg vs. 2.53 ± 2.22 mg, respectively, $P = 0.12$).

Although most of the purified IgM demonstrated poor or absent antibody yields with the SP Sepharose Fast Flow resin, IgM MAb R155 was successfully purified using both the SP

Sepharose Fast Flow and commercial IgM-specific resins. There were no differences in the antibody purity or integrity of the disulfide bonds within IgM between the two resin approaches **(Figure 3)**. These findings suggest that the SP Sepharose Fast Flow resin approach itself did not result in proteolysis or other alterations to the IgM antibodies and that the low binding affinity of IgM likely contributed to poor binding interactions with the SP Sepharose Fast Flow resin. Subsequently, an SDS-PAGE was performed on all 17 purified antibodies to confirm their IgM isotype. Under the reduced condition, the 75 kDa heavy chain (μ) and 25 kDa light chain of IgM were detected **(Figure 4)**. Each IgM monomer has a molecular weight of 180 kDa, while its pentameric form has a mass of ~ 970 kDa. Under non-reduced conditions, the anti-FVIII IgMs showed a single band >250 kDa on SDS-PAGE as expected. Interestingly, some of the IgM antibodies demonstrated fragmentation with lighter bands detected at 55 and 25 kDa under non-reduced conditions **(Figure 4)**.

The absence of contaminating IgG in purified IgM samples was confirmed by ELISA and a mouse antibody isotyping kit. ELISAs using a two-fold limiting dilution of IgM antibodies between 0-10 μg/ml and a secondary anti-IgG capture antibody excluded the presence of any concomitant IgG antibodies **(Figure 5).** There was limited absorbance below the cutoff for IgM positivity for purified MAb R3412, which was considered to be non-specific binding **(Figure 5B)**. Use of the antibody isotyping kit further confirmed the IgM isotype with kappa light chain for all 17 IgMs without concomitant IgG detected **(Figure 6)**.

Anti-FVIII IgMs are Porcine Cross-Reactive and Demonstrate Weak-Binding Interactions

Next, ELISAs were performed to confirm FVIII-specificity of the purified IgMs. As anticipated, IgM demonstrated weak binding to B domain deleted (BDD) FVIII and full length (FL) FVIII as

demonstrated by a mean absorbance of 0.33 ± 0.43 and 0.29 ± 0.42, respectively **(Figure 7)**. The majority of IgM MAbs showed reduced binding to FL FVIII compared to a control monoclonal FVIII-specific IgG A2 domain inhibiting antibody (MAb 2-54) with a mean absorbance of 1.85 ± 0.02. The anti-FVIII IgMs did not inhibit FVIII activity and were classified as non-inhibitory **(Table 1).**

To identify domain-specificity of IgM binding to FVIII, an ELISA utilizing porcine FVIII with single-human substituted domains was performed in a subset of 7 IgM MAbs. Six of the 7 IgMs were identified as porcine cross-reactive, which prevented accurate identification of the FVIII domain specificity **(Figure 8)**. Anti-FVIII IgM designated R281 had a mean absorbance of 0.01 ± 0.0044, which was below the limit of detection to determine porcine-cross reactivity. A secondary A2, C1, and C2 domain-specific binding ELISA was carried out using isolated FVIII domains conjugated to human serum albumin (HSA). Similarly, to IgM binding to BDD FVIII or FL FVIII, IgM MAbs demonstrated weak binding to isolated A2, C1 and C2 domains with mean absorbance of 0.44 ± 0.52, 0.55 ± 0.54, and 0.49 ± 0.48, respectively **(Figure 9)**. Weak binding interactions between IgM and the various FVIII protein constructs prevented the identification of IgM binding site.

IgM Binding is Not Improved in Buffers with Excipients

Attempting to improve IgM binding in the ELISAs by reducing IgM fragmentation, BDD FVIII binding by IgM in different buffer solutions with excipients were evaluated by ELISA. No consistent improvement in IgM binding to FVIII with various excipients was seen and no one excipient showed significant stabilization **(Figure 10)**. Mean absorbances of the 4 IgMs tested

were 0.026 ± 0.04 for control, 0.022 ± 0.01 for sorbitol, 0.021 ± 0.008 for glycine, 0.040 ± 0.03 for glycine/sorbitol, and 0.060 ± 0.07 for the IgM stored at 4 °C.

DISCUSSION

This study demonstrated the presence of anti-FVIII IgM in the secondary immune response to FVIII along with anti-FVIII IgG. IgM has previously been detected in the immune response to FVIII in murine models of hemophilia A and in patients with hemophilia A who have developed inhibitors, as well as healthy individuals without hemophilia A.[32,33] The significance of persistent IgM in FVIII immunogenicity remains unclear and has not been extensively explored. We therefore set out to characterize properties of murine-derived anti-human FVIII IgM with the goal of providing insight into mechanisms of antibody initiation and propagation.

Seventeen monoclonal IgMs were produced, purified, and confirmed as FVIII-specific monoclonal IgMs with all MAbs having kappa light chains. The frequency of antigen-specific IgM production by hybridomas were similar to previously published reports.[77] Weak binding interactions between purified IgM and FVIII in the various ELISAs proved to be a recurring challenge that prevented the identification of FVIII domain-binding specificity or an immunodominant FVIII epitope recognized by IgM. We hypothesized that relatively unstable anti-FVIII IgM undergoing enzymatic degradation could be the cause of low IgM binding affinity to FVIII. Although IgM fragmentation was observed on SDS-PAGE, we did not identify a significant quantity of protein fragments of differing masses which would be expected from enzymatic cleavage alone. Furthermore, there were no significant increases in IgM binding to FVIII in the IgM stabilization assays when freshly thawed IgM were exchanged into buffers with various excipients. From these results we can infer that enzymatic degradation is not the sole

contributor to weak IgM binding to FVIII and that other preventative forces are also at play in inhibiting IgM binding interactions.[75]

Various studies describe similar difficulties evaluating IgM binding activity. IgM's large molecular weight, extensive glycosylation, and complex protein structure with numerous disulfide bonds make this antibody isotype very difficult to express, purify, and characterize.[54,55,57] We observed a 55 kDa band on SDS-PAGE and hypothesized that this may be due to IgM truncation or the formation of the alternative variable region in the heavy chain resulting in μ' (**Figure 4**). [56–58] Bovine serum albumin (BSA) has been shown to be associated with IgM μ' generation and decreased binding activity.[57] The use of BSA in the blocking buffers and incubation periods in the ELISAs is further evidence for a correlation between μ' formation and the weak binding interactions in the *in vitro* assays.

Despite challenges in the *in vitro* assays evaluating IgM binding to FVIII, we were able to identify that the purified IgMs produced were largely porcine-cross reactive and non-inhibitory like the five non-inhibitory C1 domain IgM antibodies previously identified in the lab (Ito J and Batsuli G, unpublished laboratory data). However, to complete characterization of the effect of anti-FVIII IgM on the biochemical properties and biological functions of FVIII, alternative methods of investigation independent of IgM binding interactions will be necessary. To determine IgM domain-binding specificity, we are collaborating with the Emory Genomics Core at Yerkes to perform RNA sequencing to reconstruct the heavy and light chains of the 17 purified IgMs. Using the RNA sequences, we plan to collaborate with a structural biologist to compare these sequences with the well-established 3-D crystal structure of FVIII to elucidate the IgM binding domains.[78] The C1 domain of FVIII is the most conserved region of FVIII between species with 90% homology between human and porcine C1 domain sequences.[63] We propose

that the IgM-binding region may align with multiple epitopes within the C1 FVIII domain based on similarities with prior laboratory data. Identification of a specific binding domain or epitope could help in the development of a recombinant FVIII protein through elimination or alteration of the immunogenic site to lessen the immunogenicity of FVIII.

As previously outlined, IgM have been shown to both amplify and suppress immune responses through unique biological and conformational characteristics with their subsequent functions. IgM have been shown to play immunoregulatory roles in suppressing certain pro-inflammatory pathways resulting in lower IgG responses as well as amplify local inflammatory responses through fixation and activation of the complement cascade.[41,45,47,49,50,79] Innovative research has provided new evidence that these molecules play a multi-faceted role in long-term adaptive immunity. This has made IgM of therapeutic relevance especially in autoimmune diseases.[41,45] In an attempt to mimic clinical scenarios of FVIII administration in patients with detectable anti-FVIII IgM antibodies and evaluate the role of IgM in the humoral immune response to FVIII *in-vivo,* future experiments will measure anti-FVIII IgG development following hemophilia A mice immunized with FVIII/IgM complexes. Antibody titers from these mice will be compared to hemophilia mice immunized with FVIII alone and FVIII/IgG immune complexes. Anti-FVIII IgG and IgM titers will be measured by ELISA, and the neutralizing effect of inhibitors present in the plasma of hemophilia A mice determined by Bethesda assay. Given the correlation of complement protein C3 and an enhanced immune response to FVIII, as well as the association of IgM with complement activation, biomarkers of complement activation will be measured in the FVIII/IgM immunization experiments to determine if IgM may amplify the immune response to FVIII through complement fixation.[52] Finally, we plan to assess the pathogenicity of FVIII/IgM complexes compared to FVIII in hemophilia mice using an *in vivo*

tail transection assay. These *in vivo* assays will provide further insight into the role of IgM in this humoral immune response by circumventing the difficulties of IgM weak binding affinity observed in the *in vitro* assays.

In conclusion, understanding the effects of IgM on inhibitor development could provide important mechanistic insight into the initiation and propagation of the humoral immune response to FVIII through IgM-specific mechanisms. Confirmation of an immunodominant C1 domain or identification of an immunodominant epitope would enable the potential development of FVIII concentrates with reduced immunogenicity. Even with new therapeutic advancements for the management of hemophilia A, FVIII replacement remains the therapeutic standard for the treatment and prevention of bleeding events and inhibitors continue to significantly challenge the management of hemophilia A.[22] Understanding the immunogenicity of FVIII in the context of the humoral immune response could provide the necessary therapeutics to maintain FVIII replacement therapy efficacy and prevent inhibitor formation, greatly increasing the quality of life for patients with hemophilia A.

TABLES

Table 1. Quantities of Purified IgM Using Affinity Chromatography Purification Technique.

No.	IgM MAb	Spleen Origin No.*	Purified IgM Quantity (mg)	Purification Method (Resin Type)	Inhibitor Titer (BU/mg IgM)
1	S1310	1	1.06	IgM-specific resin	<1
2	S266	1	2.89	IgM-specific resin	<1
3	S311	1	1.75	IgM-specific resin	<1
4	S319	1	2.14	IgM-specific resin	<1
5	S110	1	1.44	IgM-specific resin	<1
6	S373	1	0.86	IgM-specific resin	<1
7	S336	1	0.50	IgM-specific resin	<1
8	S1711	1	1.39	IgM-specific resin	<1
9	S236	1	0.86	IgM-specific resin	<1
10	R169	2	0.33	IgM-specific resin	<1
11	R281	2	1.84	IgM-specific resin	<1
12	R441	2	2.30	IgM-specific resin	<1
13	R234	2	1.00	IgM-specific resin	<1
14	R155	2	2.85	IgM-specific resin & SP Sepharose resin	<1
15	R3412	2	1.60	IgM-specific resin	<1
16	R154	2	1.36	SP Sepharose resin	<1
17	R284	2	2.91	SP Sepharose resin	<1

*Spleen no. 1 represents IgM MAbs derived from the high-titer spleen (designated initial of S) and spleen no. 2 represents IgM MAbs derived from the low-titer spleen (designated initial of R).

FIGURES

Figure 1

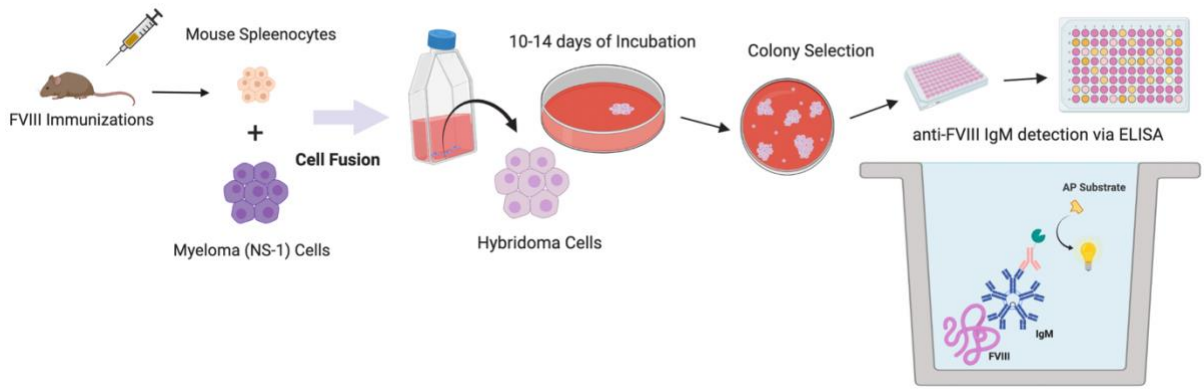

Figure 1. Schematic of Hybridoma Production Process. FVIII$^{-/-}$ mice were immunized with six injections of recombinant human B domain deleted (BDD) FVIII. Mouse splenocytes were fused with NS-1 myeloma cells to create immortal IgM-secreting hybridoma cell lines. Hybridomas were incubated in hybridoma growth medium for 10-14 days and monoclonal IgM-secreting colonies were selected for colony expansion. IgM binding to FVIII was determined by ELISA and read at an absorbance of 405 nm.

Figure 2

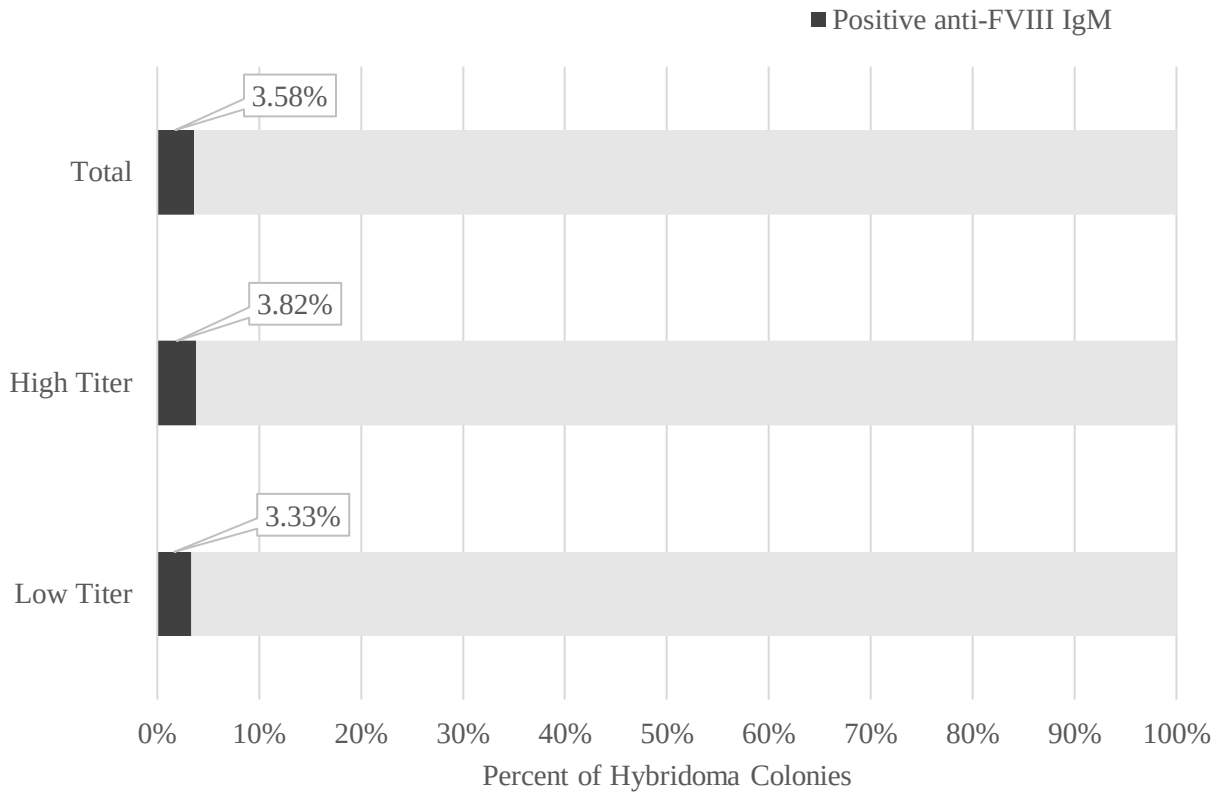

Figure 2. Frequency of Anti-FVIII IgM-Secreting Hybridomas. The frequency of positive anti-FVIII IgM-secreting hybridomas from 588 total hybridoma colonies screened by ELISA and derived from two spleens of immunized FVIII$^{-/-}$ mice are shown. Percentage of hybridomas derived from a high-titer spleen (*i.e.*, anti-FVIII IgG ELISA titer of ≥ 2,000) and a low-titer spleen (anti-FVIII IgG ELISA titer <2,000) are shown for comparison.

Figure 3

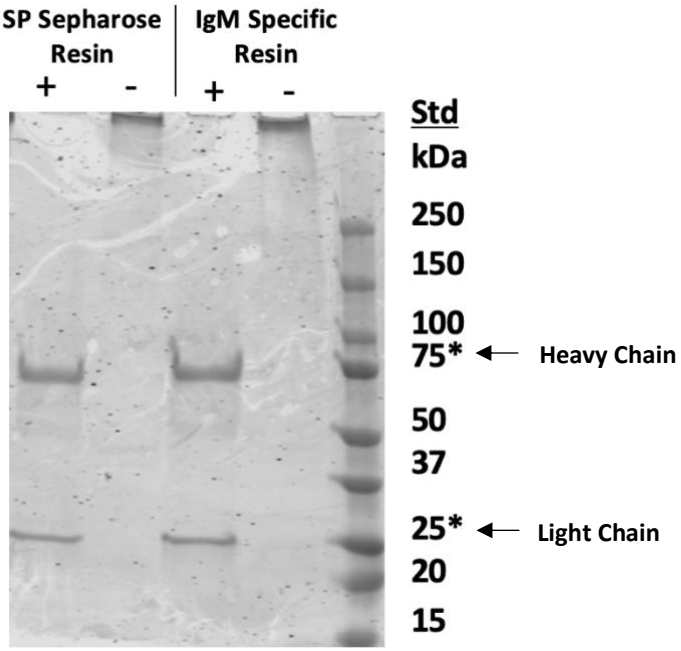

Figure 3. SDS-PAGE of IgM MAb R155 by Different Purification Approaches. SDS-PAGE of purified IgM MAb R155 derived from a low-titer spleen under reduced (+) and non-reduced (-) conditions. IgM was purified using affinity chromatography with a SP Sepharose Fast Flow resin and a commercially IgM-specific resin. In the reduced lane (+), a dark band at ~75 kDa shows the heavy chain of IgM monomer and a ~25 kDa band represents the light chain of IgM monomer. In the non-reduced lane (-), a thick band at the top of the gel indicates a pentameric IgM with a molecular weight >250 kDa.

Figure 4

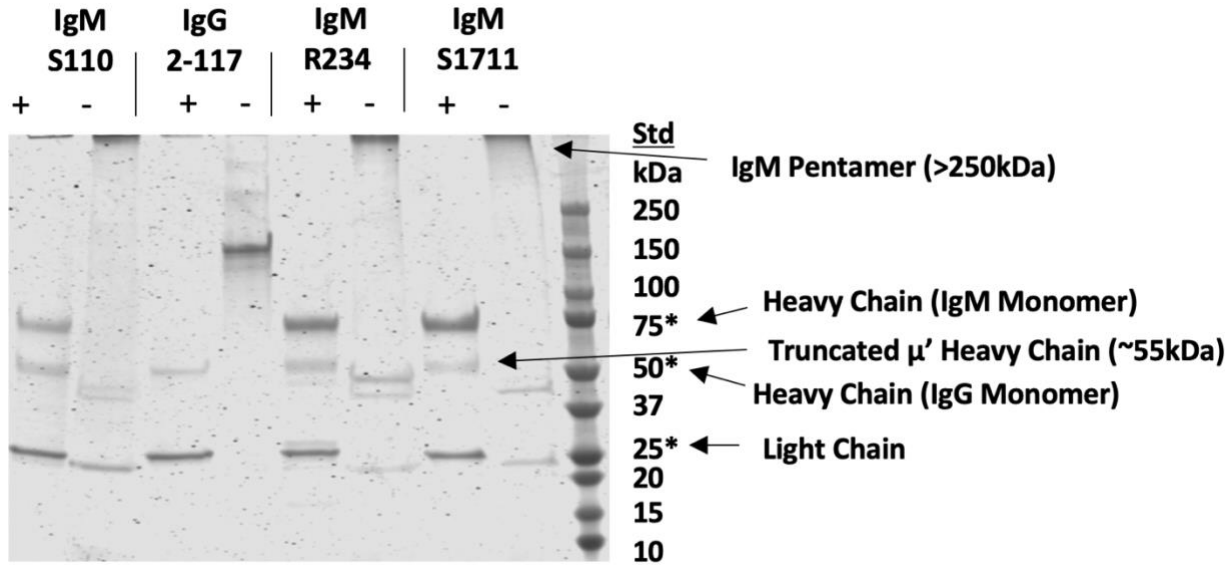

Figure 4. Purity of Purified IgM by SDS-PAGE. The SDS-PAGE show size separation of purified IgM under reduced (+) and non-reduced (-) conditions of 3 purified anti-FVIII IgM monoclonal antibodies (S110, R234, and S1710). The 'S' prefix indicates an anti-FVIII IgM derived from a high-titer spleen (anti-FVIII IgG ELISA titer of ≥ 2,000) and 'R' prefix indicates an anti-FVIII IgM derived from a low-titer spleen (anti-FVIII IgG ELISA titer <2,000). In the reduced lane (+) for the IgM antibodies, a dark band at ~75 kDa represents the heavy chain of the IgM monomer and a ~25 kDa band represents the light chain of the IgM monomer. The reduced lane for the IgM antibodies also shows a band at ~55 kDa for the truncated heavy chain known as mu prime (μ'). In the non-reduced lane (-) for the IgM antibodies, a thick band at the top of the gel represents a pentameric IgM with a molecular weight >250 kDa. Additional fragmentation can be seen in the non-reduced conditions for each of the 3 IgM antibodies. A previously characterized anti-FVIII C2 domain IgG MAb (designated 2-117) used for comparison. In the reduced lane (+) for the IgG antibody, a dark band at ~50 kDa represents the heavy chain of the IgG monomer and a ~25 kDa band represents the light chain of the IgG monomer. In the non-reduced lane (-) for the IgG antibody, a thick band at 150 kDa represents a monomeric IgG.

Figure 5

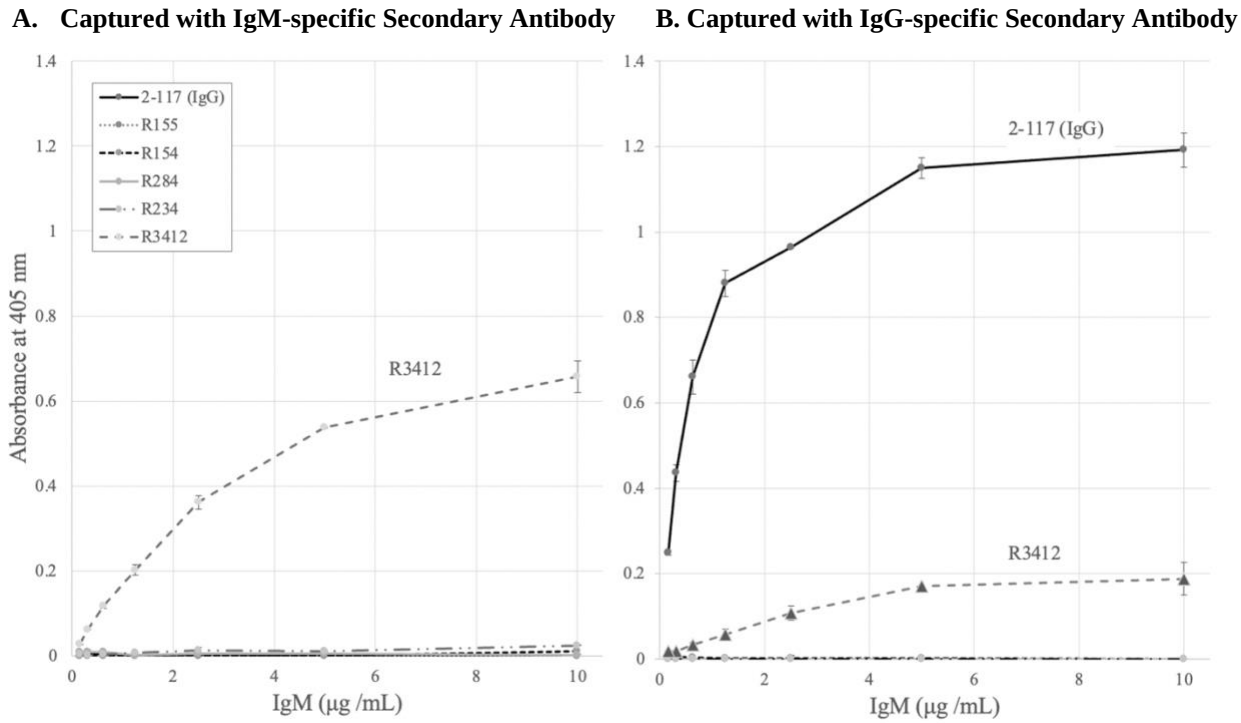

Figure 5. IgM Isotype Confirmation ELISA. Absorbances at 405 nm from an FVIII/IgM binding ELISA are shown. Binding of a two-fold limiting dilution of IgM between 0-10 µg/ml to 1.5 µg/ml full-length FVIII was measured. A previously characterized anti-FVIII C2 domain IgG MAb (designated 2-117) was used as a control. The 'S' prefix indicates anti-FVIII IgMs derived from a high-titer spleen (anti-FVIII IgG ELISA titer of ≥ 2,000) and the 'R' prefix indicates anti-FVIII IgMs derived from a low-titer spleen (anti-FVIII IgG ELISA titer <2,000). Optical densities were detected using (A) a goat anti-mouse IgM-specific secondary capture antibody or (B) a goat anti-mouse IgG-specific secondary capture antibody.

Figure 6

Figure 6. Confirmation of Purified IgM Isotype by Mouse Monoclonal Antibody Isotyping Kit. Confirmation of the IgM antibody isotype utilizing an antibody isotyping kit are shown. The blue bands indicate positivity and the absence of a blue band indicates negativity. The first (+) signs represent a positive control for protein presence. The boxes pictured (A) are for detection of an antibody of the IgA isotype and the (M) indicates detection of IgM isotype. Light chains are distinguished and pictured as (κ) for kappa and (λ) for lambda. The 'S' prefix indicates anti-FVIII IgMs derived from a high-titer spleen (anti-FVIII IgG ELISA titer of ≥ 2,000) and the 'R' prefix indicates anti-FVIII IgMs derived from a low-titer spleen (anti-FVIII IgG ELISA titer <2,000).

Figure 7

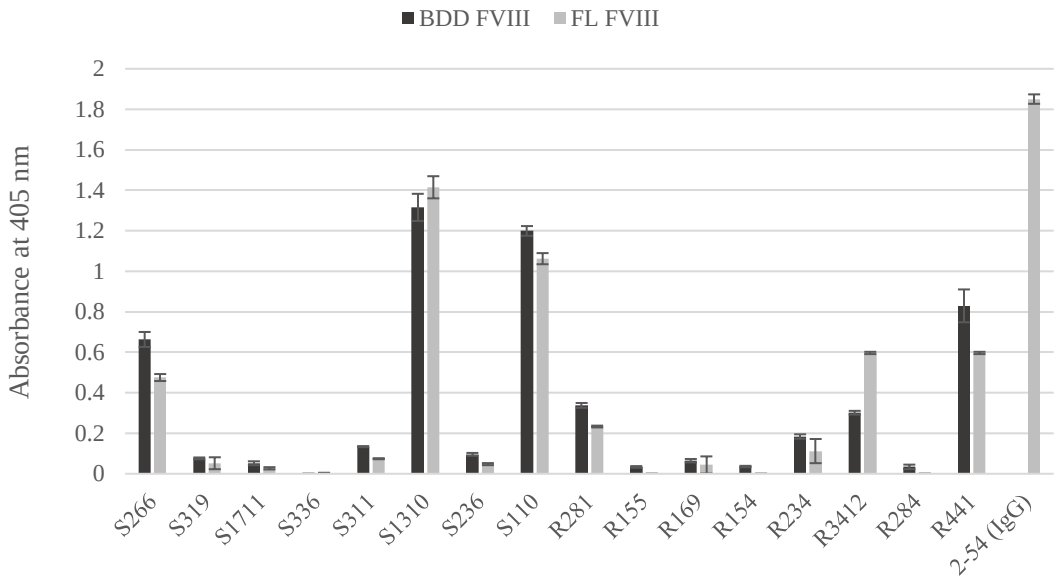

Figure 7. IgM Binding to FVIII. Absorbances at 405 nm 10 μg/ml of purified IgM binding to full length FVIII (FL FVIII) (pictured dark grey) and B domain deleted FVIII (BDD FVIII) (pictured lighter grey) using ELISA are shown. Binding of a previously purified anti-FVIII A2 domain IgG antibody (designated 2-54) to FL FVIII only was used as a positive control. Data is presented as means with standard deviation of anti-FVIII IgM binding measured in duplicates. The 'S' prefix indicates anti-FVIII IgMs derived from a high-titer spleen (anti-FVIII IgG ELISA titer of ≥ 2,000) and the 'R' prefix indicates anti-FVIII IgMs derived from a low-titer spleen (anti-FVIII IgG ELISA titer <2,000).

Figure 8

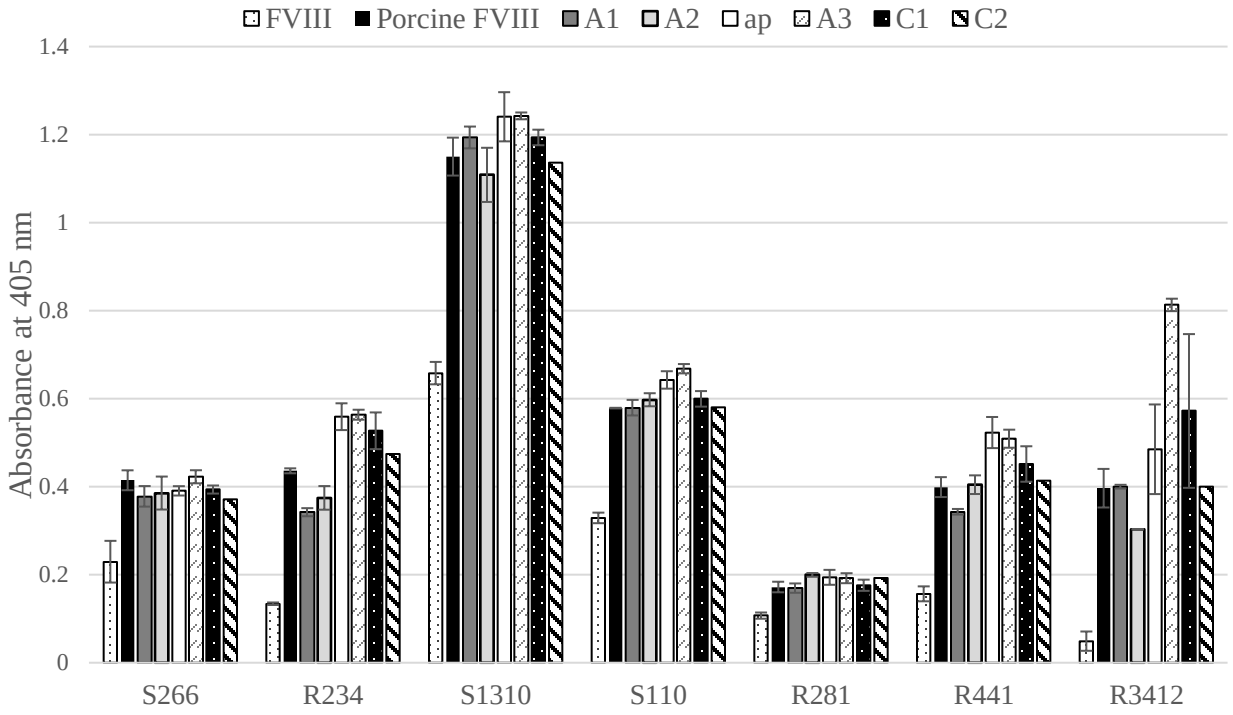

Figure 8. FVIII Domain-Specific Binding by Purified IgMs. IgM binding to human FVIII, porcine FVIII, or isolated human A1, A2, ap, A3, C1, or C2 FVIII domains evaluated by ELISA at an absorbance of 405 nm. FVIII constructs were coated on ELISA plates at 1.5 µg/ml and IgM incubated at 10 µg/ml. Data is presented as means with standard deviation of anti-FVIII IgM binding measured in duplicates. The 'S' prefix indicates anti-FVIII IgMs derived from a high-titer spleen (anti-FVIII IgG ELISA titer of ≥ 2,000) and the 'R' prefix indicates anti-FVIII IgMs derived from a low-titer spleen (anti-FVIII IgG ELISA titer <2,000).

Figure 9

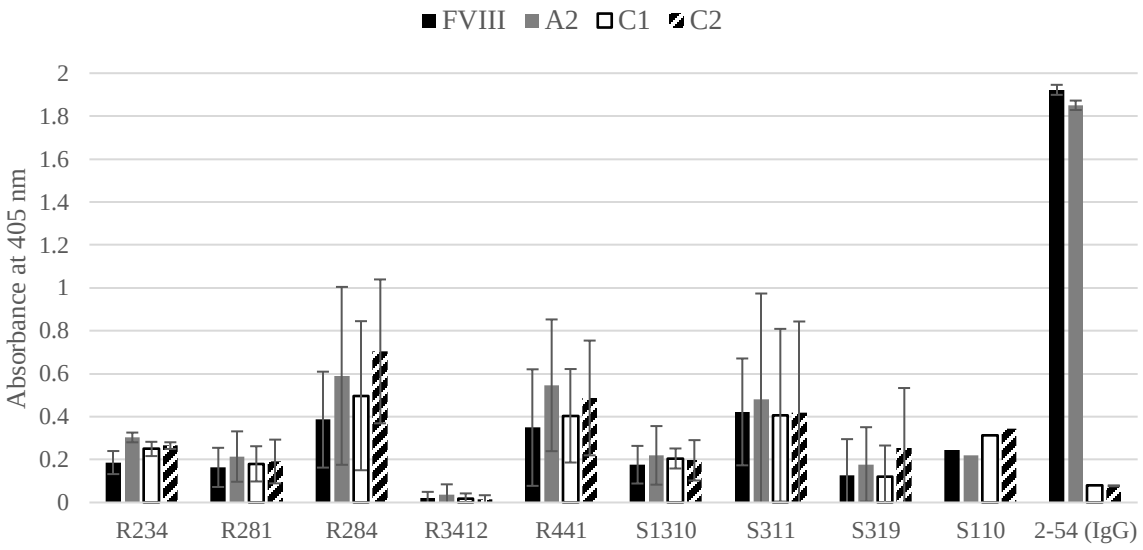

Figure 9. Domain-Specific Binding by Purified IgMs Using Single Human Domain A2, C1, and C2 Proteins. IgM binding to human FVIII domains A2, C1, or C2 was evaluated by ELISA. FVIII constructs were coated on ELISA plates at 1.5 µg/ml and IgM incubated at 10 µg/ml. Previously purified A2-domain IgG MAb 2-54 was used as a positive control. Data is presented as means ± standard deviation of IgM binding in duplicates. The 'S' prefix indicates anti-FVIII IgMs derived from a high-titer spleen (anti-FVIII IgG ELISA titer of ≥ 2,000) and the 'R' prefix indicates anti-FVIII IgMs derived from a low-titer spleen (anti-FVIII IgG ELISA titer <2,000).

Figure 10

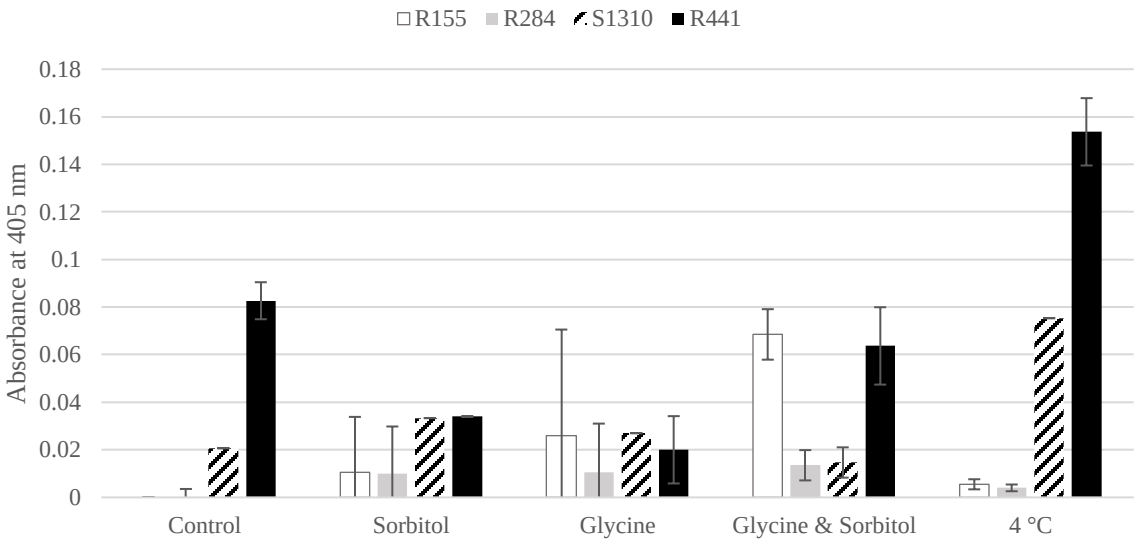

Figure 10. IgM Stabilization Binding Assay. IgM was exchanged into one of four different buffer solutions then evaluated for binding to B domain deleted FVIII (BDD FVIII) at 1.5 µg/ml. Conditions of excipient buffers were: Control represents thawed IgM from -80 °C (HBS, 0.05% NaN3), Sorbitol (HBS, 0.05% NaN3, 20% sorbitol), Glycine (HBS, 0.05% NaN3, 1M glycine), and Glycine & Sorbitol (HBS, 0.05% NaN3, 20% sorbitol, 1M glycine). Purified IgM at 4°C was maintained at 4 °C for 15-18 weeks. Data is presented as means with standard deviation anti-FVIII IgM binding measured in duplicates. The 'S' prefix indicates anti-FVIII IgMs derived from a high-titer spleen (anti-FVIII IgG ELISA titer of ≥ 2,000) and the 'R' prefix indicates anti-FVIII IgMs derived from a low-titer spleen (anti-FVIII IgG ELISA titer <2,000).

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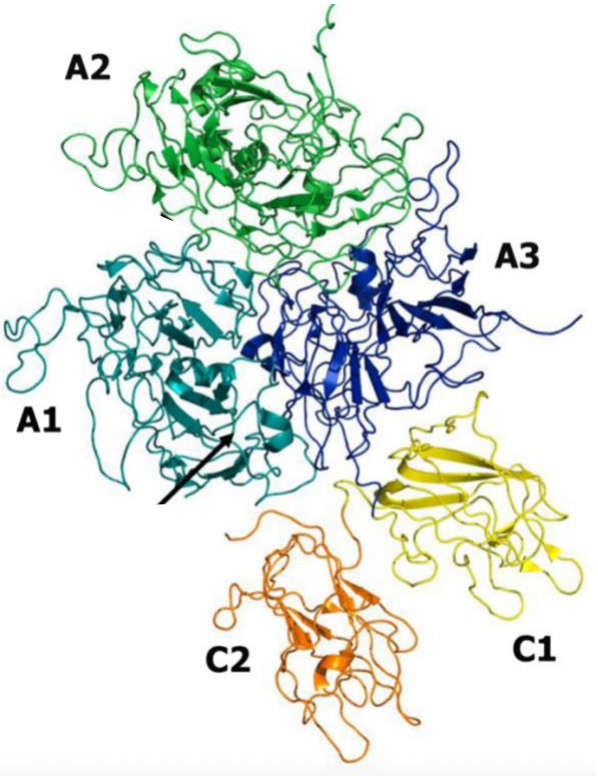
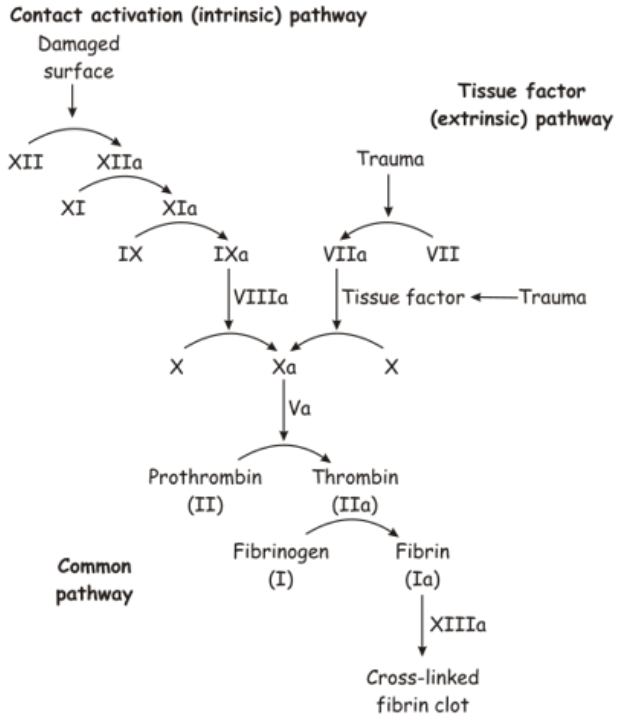
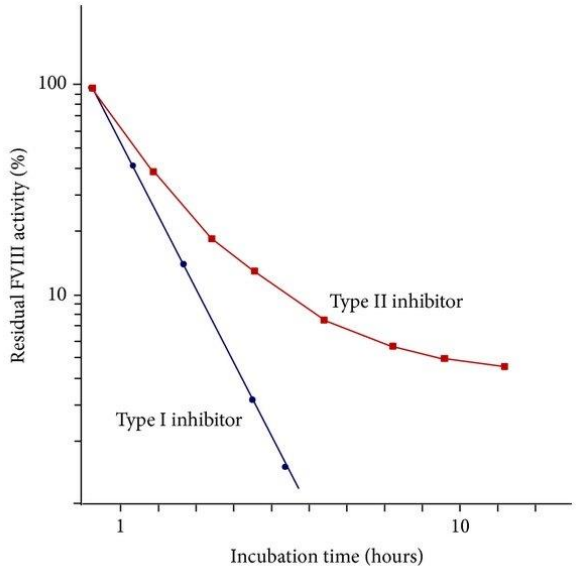
SUPPLEMENTAL FIGURES



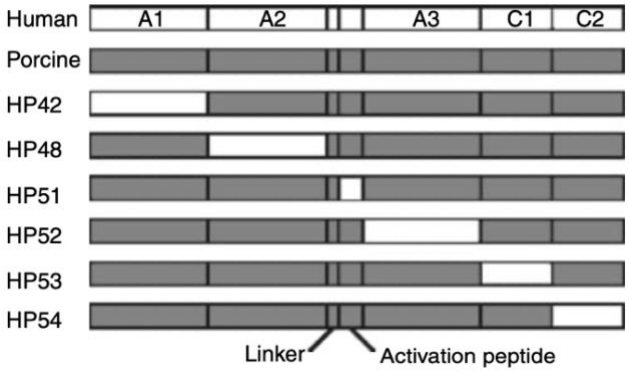
Supplemental Figure S1. The Crystalline 3-dimensional Structure of B Domain Deleted Factor VIII. Pictured in different colors are the five domains within the FVIII structure. This figure was altered from a figure by Elmahmoudi H et al *Diagn. Pathol.* 2012.[80]



Supplemental Figure S2. The coagulation cascade. Schematic diagram of the clotting factors within the tissue factor (extrinsic) and contact activation (intrinsic) pathways which converge to the common pathway to form a stable fibrin clot. This figure was adapted from Le Yaouanq S et al. *Adv. Artif. Life ECAL* 2011.[81]



Supplemental Figure S3. Kinetics of Type I vs Type II Inhibitors. The Y-axis shows the percent of residual FVIII activity while the X-axis shows the incubation time in hours. Type I inhibitors cause >98% inactivation of FVIII, while type II inhibitors incompletely inhibit FVIII activity. Figure was adapted from Sakurai et al. *J. Immunol. Res.* 2014.[82]



Supplemental Figure S4. Single Human Domain Hybrid Porcine FVIII Constructs. Pictorial of the B Domain Deleted (BDD) human FVIII construct, porcine FVIII construct, and porcine FVIII with single human domain hybrid constructs derived in the lab and utilized for IgM binding domain ELISAs. This figure was adapted from Healey JF et al. *J Thromb Haemost.* 2007.[67]