FVIII/anti-FVIII Immune Complex and prevention of anti-FVIII antibody production

The role of FVIII/Anti-FVIII antibody Immune Complexes in Preventing FVIII-Specific Antibody Production in a Hemophilia A Mouse Model

By Ghazaleh Dadashi Zadeh

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TITLE: The role of FVIII/Anti-FVIII antibody Immune Complexes in Preventing FVIII-Specific Antibody Production in a Hemophilia A Mouse Model

AUTHOR:

Ghazaleh Dadashi Zadeh (B.S.c., MSC)

Mashhad University of Medical Science

SUPERVISOR:

Davide Matino (MD, MSc)

SUPERVISORY COMMITTEE:

Anthony Chan (MD)

Peter Gross M **(**M.D., M.Sc., FRCPC (C)**)**

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Lay Abstract

Hemophilia A is an X-linked bleeding disorder caused by a deficiency or absence of a clotting protein called factor VIII (FVIII). Sometimes, when patients with Hemophilia A receive treatment, their immune systems produce antibodies that make it ineffective.

We found a way to block B Cells from producing anti-FVIII antibodies by targeting specific receptors expressed on the surface of B cells. These immune complexes are made by mixing FVIII with particular antibodies.

We used a Hemophilia A mouse model. We first produced anti-FVIII by immunizing other mice with FVIII. Then, we combined these antibodies with FVIII in varying ratios to formulate immune complexes. We injected these immune complexes into the mice according to the predefined timeline.

When we only gave the mice FVIII without the immune complexes, they developed a lot of antibodies. However, when we gave them a balanced mixture of FVIII and immune complexes in different ratios, some did not develop detectable levels of antibodies. This suggests that these immune complexes can stop the body from producing antibodies against FVIII.

Our study demonstrates that using these immune complexes may represent a promising strategy to manage Hemophilia A. It seems like the ICs can "switch off" the process that forms anti-FVIII antibodies.

Abstract

Background: Hemophilia A is an X-linked bleeding disorder characterized by a deficiency or absence of FVIII. Patients can develop anti-FVIII IgG, rendering FVIII therapy ineffective. Antibody production can be suppressed by targeting CD32b receptors in B cells using ICs. Binding rhFVIII/anti-FVIII immune complexes (rhFVIII-ICs) to FcγRIIB (CD32b) leads to phosphorylation of the immune receptor tyrosine-based inhibition motif (ITIM) tail of CD32b, resulting in the transduction of inhibitory signals to B cells, thereby inhibiting antibody production.

Aim: To evaluate the effect of rhFVIII-ICs administration on the suppression of antibody production by B cells in a mouse model.

Methods: A high-affinity anti-FVIII IgG pool was obtained by immunizing of FVIII KO mice with rhFVIII (Kovaltry). After immunization, blood was collected from immunized mice, and plasma was obtained. rhFVIII-ICs were prepared by incubating the mice plasma or anti-FVIII monoclonal antibody pool with Kovaltry at 1:1 and 1:10 ratios. FVIII KO mice were injected retro-orbitally with rhFVIII-ICs, PBS, or Kovaltry diluted in naïve plasma once weekly for four weeks (tolerization phase). Next, the mice were administered with FVIII retro-orbitally at a dose of 25 IU/kg once weekly for four weeks. Finally, blood samples were collected from all mice, and ELISA determined the concentration of anti-FVIII IgG.

Results: Mice that received the combination of FVIII and naive plasma developed anti-FVIII IgG (mean increase 6.1 μg/mL, p-value = 0.0001). In contrast, 69% of mice that received 1:1 rhFVIII-ICs did not develop antibodies (mean increase 1.3 μg/mL, p-value =

0.0723), while 50% of the mice that received 1:10 rhFVIII-ICs did not develop antibodies (mean increase 2.3 μg/mL, p-value = 0.1030).

Conclusion: In this study, we demonstrated that a 1:1 balanced ratio of rhFVIII-ICs injection suppresses antibody production. Thus, antibody feedback may play a role in the immune response to FVIII by suppressing FVIII-specific B cells. Future work will investigate the mechanism of this suppression, particularly the potential involvement of CD32b.

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To all those who have supported me through academic guidance, friendship, or family ties, I sincerely appreciate your contributions. This thesis is a culmination of collective effort and unwavering support, and I am profoundly grateful to each of you. With gratitude and humility, I present this work as a testament to our shared journey and the enduring pursuit of knowledge.

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List of Abbreviations

TXA2	Thromboxane A2
PF	Platelet Factor
PK	Prekallikrein
HMWK	High molecular weight kininogen
VWF	von Willebrand factor
EDTA	Ethylenediaminetetraacetic acid
ACE910	Emicizumab
APC	Antigen presenting cells
MHC	major histocompatibility complex
APCC	activated prothrombin complex concentrate
ITIM	Immunoreceptor tyrosine-based activation motif
TMB	3,3′,5,5′-Tetramethylbenzidine
SHIP	SH2-containing Inositol Phosphatase
PIP3	phosphatidylinositol 3,4,5-trisphosphate

Declaration of Academic Achievement

I, Ghazaleh Dadashi Zadeh, declare that this thesis titled "**The Role of FVIII/Anti-FVIII antibody Immune Complexes in Preventing FVIII-Specific Antibody Production in a Hemophilia A Mouse Model**" is a record of my independent research work under the supervision of Dr. Davide Matino. I am the sole author of this work. No part of this thesis has been submitted or published for the award of any other degree.

My supervisor, Dr. Davide Matino, and my committee members, Dr. Anthony Chan and Dr. Peter Gross, have provided support and guidance during this project.

Chapter I – Introduction

1.1 Overview of Hemostasis

The hemostatic system, responsible for maintaining the balance between bleeding and clotting, has two essential parts: the coagulation cascade and the fibrinolytic system (Stassen et al., 2004) (Figure 1). The coagulation component consists of two main systems: the primary and the secondary hemostasis systems (Stassen et al., 2004). The primary hemostatic system is a rapid response system that involves two processes: vascular contraction and platelet plug formation (Stassen et al., 2004). Vascular contraction occurs within a few seconds of injury and is followed by the formation of a platelet plug, which takes around fifteen to twenty seconds to develop (Stassen et al., 2004). In contrast, the secondary hemostatic system is a slower process that requires more than ten minutes to form a fibrin clot. The formation of the clot depends on coagulation factors. The primary hemostatic system plays a crucial role in developing the fibrin clot by providing platelet phospholipids that allow coagulation factors to bind and create a stable clot (Stassen et al., 2004).

Figure 1. Hemostasis. Collaboration of Vasoconstriction, Platelet Plug, and Coagulation Cascade. This figure visually presents how vasoconstriction, platelet plug formation, and the coagulation cascade work together in response to a vascular injury. Initially, vasoconstriction occurs, where the blood vessels narrow to reduce blood flow at the injury site, limiting blood loss (Stassen et al., 2004). Following this, platelet plug formation takes place. Platelets adhere to the exposed tissue (Stassen et al., 2004). As the process continues, the coagulation cascade is activated. This involves a series of steps where clotting factors are sequentially activated, forming a stable blood clot (Stassen et al., 2004). Figure created in Biorender.

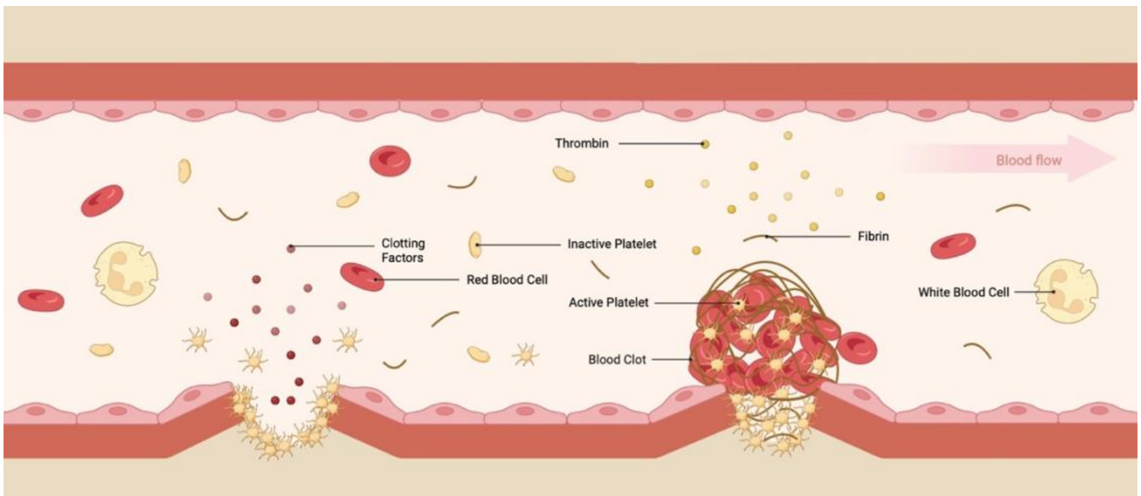

Figure 1. Hemostasis

(Med Lab Study Hall)

1.2 Coagulation cascade and coagulation factors

The coagulation cascade involves a complex cascade of biochemical reactions and the activation of multiple coagulation factors, forming a stable fibrin clot (Monagle & Massicotte, 2011).

The primary coagulation system provides the necessary phospholipid surface for the coagulation factors to interact and form a stable clot (Mertens et al., 1984). On the other hand, the secondary coagulation system uses fibrinogen as its main substrate, which is converted into fibrin polymers by various coagulation factors (Mertens et al., 1984). The fibrin fibers then form a network that traps erythrocytes and platelets, creating a stable clot that prevents further bleeding (Mertens et al., 1984).

Moreover, the fibrin network provides a surface for the migration of endothelial cells, which initiate the process of tissue repair and healing(Yakovlev & Medved, 2009). The fibrin clot also acts as a scaffold for other cells, including fibroblasts, that lay down new tissue to repair the damaged area (Yakovlev & Medved, 2009).

Coagulation factors, mostly glycoproteins, are synthesized primarily in the liver (Yakovlev & Medved, 2009). These coagulation factors circulate in the blood at regulated concentrations in their inactive proenzyme or zymogenic state(Yakovlev & Medved, 2009). Following an injury or damage to the blood vessel, platelets, VWF, and fibrinogen combine to form a white clot through the primary coagulation system(Yakovlev & Medved, 2009). This clot provides a surface on which coagulation factors can be activated and converted from their zymogenic to enzymatically active forms (Yakovlev & Medved, 2009).

The cascade of enzymatic reactions in the secondary hemostasis system is initiated by the exposure of tissue factor, a membrane-bound glycoprotein found on cells surrounding the blood vessel(Mackman et al., 2007). This interaction activates factor VII, which activates factor X, leading to thrombin formation(Mackman et al., 2007). Thrombin then cleaves fibrinogen to form fibrin, polymerized into a stable clot (Mackman et al., 2007). More precisely, the secondary coagulation system can be divided into three separate pathways based on how coagulation factors are activated: the intrinsic pathway, the extrinsic pathway, and the common pathway (Mackman et al., 2007).

The intrinsic pathway includes factors XII, XI, IX, and VIII, while the extrinsic pathway includes tissue factor (TF) and factor VII(Grover & Mackman, 2019). The common path includes factors X, V, II, and I. The intrinsic pathway is activated by negatively charged surfaces, such as kaolin, glass, silt, and alginic acid (Grover & Mackman, 2019). The extrinsic pathway is activated by exposure to tissue factors or thromboplastin, released from leukocytes and tissue cells (Grover & Mackman, 2019). Finally, the common pathway is activated by both the intrinsic and extrinsic pathways (Grover & Mackman, 2019). All these pathways lead to the production of thrombin, which leads to the formation of a fibrin polymer, which forms the basis of a stable clot (Figure 2).

Figure 2. Coagulation Cascade overview. The secondary hemostasis system involves a series of enzymatic reactions. The system comprises three pathways: intrinsic (factors XII, XI, IX, VIII), extrinsic (tissue factor and factor VII), and common (factors X, V, II, I). Intrinsic is activated by negatively charged surfaces, while extrinsic is triggered by tissue factors released from cells. Both pathways converge to produce thrombin, which is essential for forming stable clots (Grover & Mackman, 2019). Figure created in Biorender.

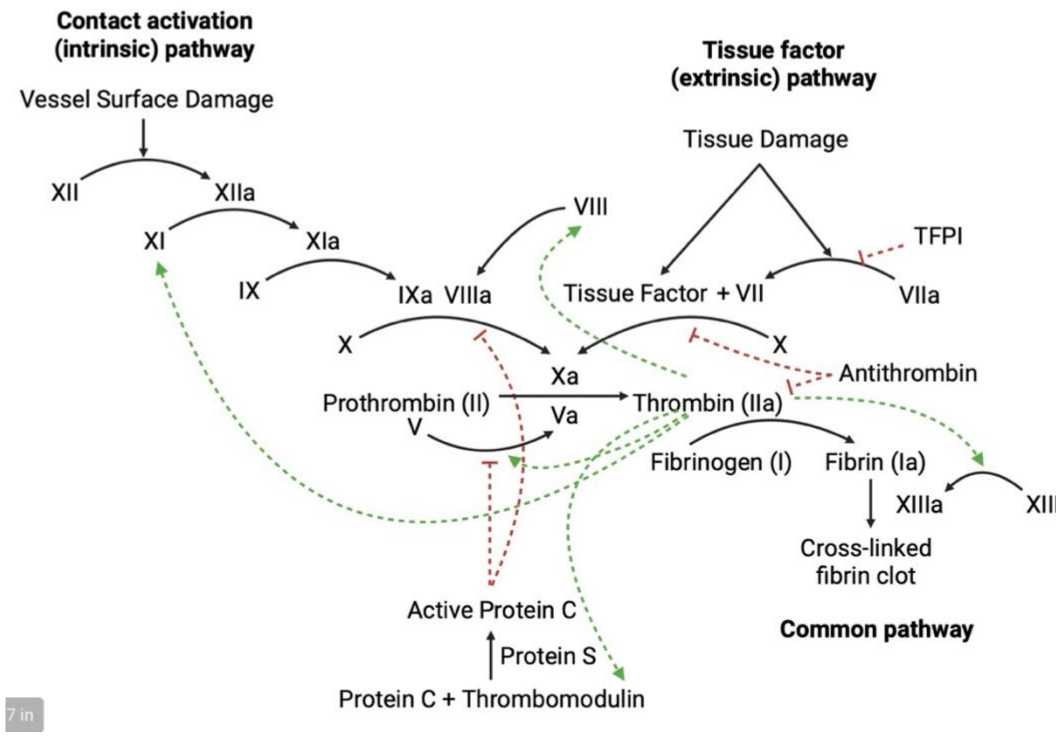

Figure 2. Coagulation Cascade Overview

https://step1.medbullets.com/hematology/111004/coagulation-cascade

1.3 Factor VIII

FVIII is a protein that plays a critical role in blood coagulation. The gene encoding FVIII (F8) is located in band Xq28 (Fay, 2006). Therefore, the inheritance of FVIII deficiencies follows an X-linked pattern and may affect males and females differently (Fay, 2006). The FVIII gene is relatively long, containing approximately 186 kilobases and 26 exons (Fay, 2006). Mutations in the FVIII gene can cause Hemophilia A, a bleeding disorder (Fay, 2006).

FVIII is a large plasma glycoprotein of 2332 amino acid residues organized in six domains: A1-A2-B-A3-C1-C2 (Fay, 2006). These domains are generated from three main regions: ceruloplasmin-like 3-domain, discoid-like domain, and glycosylated domain (Fay, 2006). The heavy chain comprises A1, A2, and domain B, while the light chain comprises domains A3, C1, and C2 (Fay, 2006). The heavy chain is 200 kDa, while the light chain is 80 kDa (Figure 3). Thrombin incisions can be found in three locations between the domains - a1, a2, and a3, which are located at Arg372, Arg740, and Arg1689, respectively, and are cleaved during activation by thrombin (Chavin, 1984; Fay, 2006).

Figure 3. FVIII is a plasma glycoprotein of 2332 amino acids organized into six domains: A1-A2-B-A3-C1-C2 (Fay, 2006). These domains stem from three main regions: ceruloplasmin-like 3-domain, discoid-like domain, and glycosylated domain (Fay, 2006). The heavy chain encompasses A1, A2, and domain B, while the light chain encompasses domains A3, C1, and C2. The heavy chain constitutes 200 kDa, and the light chain comprises 80 kDa (Fay, 2006). Figure created in Biorender.

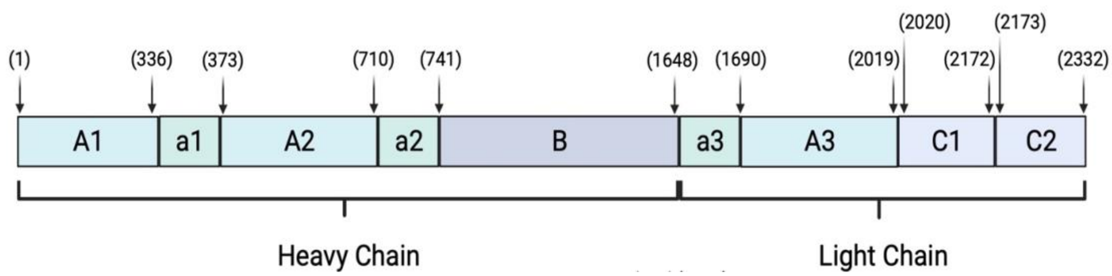

Figure 3. Coagulation FVIII structure (Graw et al., 2005)

FVIII is involved in the intrinsic coagulation pathway, activated when a blood vessel is damaged (Pittman et al., 1993). Upon activation, FVIII is converted to FVIIIa, which acts as a cofactor for factor IXa, activating factor X and subsequent thrombin generation (Pittman et al., 1993).

1.4 Hemophilia A

Hemophilia A is a bleeding disorder inherited in an X-linked pattern resulting from various FVIII gene mutations. It occurs in approximately 1 in 5,000 males (den Uijl et al., 2011). In Hemophilia A patients, the most common mutations in the F8 gene are intron 22 and 1 inversions affecting about 50% and 5% of patients, respectively (Abdulqader et al., 2020). About 5% of patients with severe Hemophilia A exhibit large gene deletions. The remaining severe, moderate, and mild cases result from various point mutations and small insertions/deletions (Castaldo et al., 2007). Hemophilia A exhibits varying clinical symptoms depending on the level of FVIII deficiency (den Uijl et al., 2011). The disease is classified to three categories based on the severity of symptoms: The mild form is characterized by having 6-49% residual FVIII and is detected only after significant surgeries or trauma (den Uijl et al., 2011). Spontaneous bleeding is rare in this form, and it is more common in adults, accounting for 30-40% of all Hemophilia A cases (den Uijl et al., 2011). The moderate form of Hemophilia A is characterized by having 1-5% residual factor VIII (den Uijl et al., 2011). These individuals do not usually bleed spontaneously (den Uijl et al., 2011). However, they may experience bleeding after minor injuries, tooth extractions, or surgeries. This form of Hemophilia A is primarily diagnosed in children and accounts for 10% of all cases (den Uijl et al., 2011). People with moderate Hemophilia A experience bleeding complications 4-6 times a year (den Uijl et al., 2011). The severe form of Hemophilia A is characterized by having 0-1% of factor VIII (den Uijl et al., 2011; van Dijk et al., 2005a). Patients with severe Hemophilia A bleed spontaneously and experience bleeding at the slightest injury if they are not receiving FVIII prophylaxis (den Uijl et al., 2011) (Figure 4). The number and frequency of bleeding episodes are high, and these patients suffer from bleeding complications 2-4 times a month (den Uijl et al., 2011). Bleeding is more common in soft tissues, with joint bleeding being the most common, leading to hemarthrosis (Peyvandi et al., 2016). Other symptoms of severe Hemophilia A include extensive ecchymosis and brain hemorrhage (den Uijl et al., 2011; van Dijk et al., 2005b).

Hemophilia A is typically diagnosed at an early age, with severe cases being diagnosed around nine months of age and moderate cases around 22 months of age (Peyvandi et al., 2016). The initial symptoms of Hemophilia A vary, with the most common being bleeding in soft tissues (41%), bleeding at injection sites, wounds, or after surgery (16%), oral bleeding (11%), and bleeding in muscles and joints, also known as hemarthrosis (Peyvandi et al., 2016). Hemarthrosis is the most specific sign of hemophilia, and it occurs frequently in people with severe hemophilia, causing painful swelling and limiting movement (Peyvandi et al., 2016). In addition, cerebral hemorrhage is the leading cause of death in hemophiliacs, as bleeding in the brain can be challenging to detect and can quickly become life-threatening (Marijke van den Berg, 2016).

Figure 4. A genetic mutation in Hemophilia A leads to a deficiency or dysfunction of clotting factor VIII, a critical component in the blood clotting process(Castaldo et al., 2007). This deficiency disrupts the formation of stable blood clots, resulting in prolonged bleeding after injury. Factor VIII is essential for activating factor IX, a critical step in the coagulation cascade. The cascade is disrupted without sufficient functional factor VIII, leading to unpaired hemostasis (Castaldo et al., 2007). This figure illustrates the mechanism underlying the clotting abnormalities observed in individuals with Hemophilia A (Castaldo et al., 2007). Figure created in Biorender.

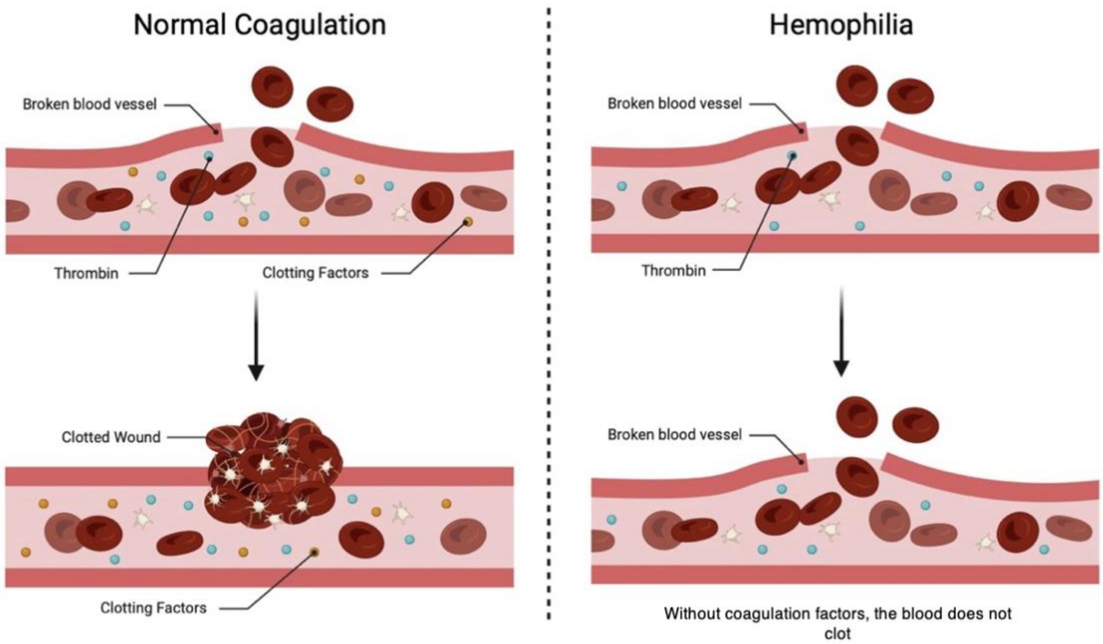

Figure 4. Hemophilia A

https://www.invitra.com/en/hemophilia-and-pregnancy/

1.5 Diagnosis of hemophilia

Coagulation screening tests diagnose Hemophilia A, which indicates a prolonged activated partial thromboplastin time and a normal prothrombin time (Kamal et al., 2007; Tiede et al., 2014). Additionally, diagnosis is confirmed by measuring FVIII clotting activity. Other methods for monitoring FVIII activity include thrombin generation assay and thromboelastography (Chalmers et al., 2011). Genetic analysis can also identify point mutations in the F8 gene, responsible for almost 40% of cases (Barrowcliffe, 2004). Nonsense mutations and large deletions are associated with the highest risk, while missense and splicing mutations carry the lowest risk (Barrowcliffe, 2004). Testing for the intron 22 and intron 1 inversions of the F8 gene is performed as they represent the most common genetic variations (Barrowcliffe, 2004). If these inversions are absent, full mutation screening of the gene is performed through direct Sanger sequencing, which covers all exons, intron-exon boundaries, and the promoter region (Antonarakis et al., 1995).

1.6 Treatment of Hemophilia A

Replacement therapy is the primary treatment approach for treating Hemophilia A, where the missing clotting factor is injected to achieve proper hemostasis (Franchini & Mannucci, 2014). The appropriate dose, frequency, and number of concentrate infusions depend on the type and severity of hemophilia (Franchini & Mannucci, 2014). Whole blood transfusions were the first treatment for hemophilia, followed by fresh frozen plasma in the 1950s and 1960s (Franchini & Mannucci, 2014). Modern medical approach to hemophilia A began in 1965 with the identification of cryoprecipitate from freshly frozen plasma (Franchini & Mannucci, 2014). Freeze-dried, lyophilized FVIII was then extracted from large pools of donor plasma, allowing for home therapy and improving patients' quality of life (Franchini & Mannucci, 2014). Recombinant protein products for Hemophilia A became commercially available in 1992, and numerous plasma-derived and recombinant products have been developed since (Franchini & Mannucci, 2014).

There are three distinct generations of rFVIII products. First-generation recombinant FVIII, like Recombinate®, were produced using the complete FVIII gene and contained animal-derived proteins in the cell culture medium and incorporated human serum albumin in the final formulation to stabilize FVIII (Ortel, 2019a). Second-generation products like Kogenate® use human-derived proteins in the culture medium but do not include albumin in the final formulation (Ortel, 2019b). In contrast, third-generation products are made without animal or human proteins, apart from FVIII itself, throughout the processing and final formulation stages (Santagostino, 2014).

Another treatment strategy for Hemophilia A is using a bispecific antibody called Emicizumab. Emicizumab is a recombinant, humanized, bispecific monoclonal antibody (Lippi & Favaloro, 2019). This antibody works by binding to both factors IXa and X, promoting the production (Lippi & Favaloro, 2019). Early research suggests that non-replacement products like Emicizumab could address some of the current challenges of prophylaxis, including frequent dosing and associated patient distress while reducing the risk of treatment-related immunogenicity (Oldenburg et al., 2017).

In addition, gene therapy is emerging as an approach for treating Hemophilia A. It involves introducing a functional copy of the F8 gene into the patient's cells, enabling the endogenous production of FVIII continuously (Perrin et al., 2019). This method can reduce the disease's severity from severe to moderate or mild after a single administration of a gene vector (Perrin et al., 2019).

1.7 Factor VIII inhibitor

The large and complex structure of FVIII makes it a potent immunogenic molecule that can activate the immune system. When FVIII is administered to individuals with severe Hemophilia A, it can trigger the production of allo-antibodies (Fay, 2006). Among the domains of Factor VIII, A2, C2, and A3 are predominantly immunogenic (Chavin, 1984). These antibodies can impair the function of FVIII and lead to a range of bleeding episodes in Hemophilia A patients (Grushin et al., 2014; Ronayne et al., 2021). Most FVIII inhibitors are polyclonal IgG1 and IgG4 antibodies that do not activate the complement system and are not associated with vascular and renal complications (Mammen et al., 2003).

The formation of FVIII inhibitors depends on the activation of specific helper T cells, or CD4+ T cells, against FVIII (Donald, 1969). To achieve an immune response against factor VIII, helper T cells need to be activated by professional antigen-presenting cells (APC) presenting epitopes through major histocompatibility complex class II (MHC II) molecules on their surface (Herzog et al., 2019). The process by which CD4+ T cells become activated involves endocytosis of FVIII by APCs, which then break down the protein into smaller peptides with intracellular proteolytic enzymes (Astermark et al., 2008). These activations are facilitated by various signals between the APC and the T cell, such as the CD28 and B7-1 (CD80) or B7-2 (CD86) signal and the CD40-CD40L signal (Astermark et al., 2008). T-cell stimulation is suppressed if these signals are not generated, leading to tolerance or anergy (Astermark et al., 2008). Once activated, helper

T cells can either differentiate into TH1 cells, which promote the production of IgG1 and IgG2 antibodies, or TH2 cells, which aid in producing of IgG and IgE antibodies by B cells (Astermark et al., 2008). TH1 secretes IL2 and interferon-gamma, while TH2 secretes IL4 and IL10, which help B cells in antibody production (Astermark et al., 2008).

When a patient with hemophilia A develops an inhibitor, the immune system quickly neutralizes the injected replacement clotting factor, rendering it ineffective; thus, higher doses or alternative treatments become necessary to manage bleeding (Dorgalaleh et al., 2016). About 10-15% of individuals with Hemophilia A have inhibitors (ZemaniFodil, 2014). Approximately 20-30% of children with severe Hemophilia A develop inhibitors against factor VIII, which is a higher prevalence than moderate and mild cases (ZemaniFodil, 2014). However, when inhibitors develop in these mild and moderate cases, inhibitors are primarily transient and low titer (Astermark et al., 2008). The management of bleeding in patients with inhibitors is a significant challenge as coagulation factors are quickly neutralized after injection, requiring higher amounts of FVIII or alternative treatments like activated prothrombin complex concentrate or rFVIIa, which can be very costly (Astermark et al., 2008).

To identify the presence of FVIII inhibitors, various techniques are used that involve measuring the levels of inactive FVIII in a mixture of test plasma that contains an FVIII inhibitor as well as an external source of factor VIII (Verbruggen et al., 2009). Currently, the Bethesda method, modified in Nijmegen, is the preferred method to measure the titer of an FVIII inhibitor (Halimeh et al., 2013; Verbruggen et al., 2009). One Bethesda unit is the amount of inhibitor that can inhibit 50% of coagulation factors in normal plasma after two hours of incubation at 37 °C (Verbruggen et al., 2009). If an inhibitory titer is less than

5 Bethesda units, it is considered transient, while an inhibitory titer equal to or greater than 5 Bethesda units is regarded as high responder (Duncan et al., 2013).

The Bethesda method has become the gold standard for inhibitor titer quantification due to its accuracy, reliability, and consistency in producing consistent results across different laboratories (Millner et al., 2016). Classifying inhibitors as high or low responders is essential because treating of Hemophilia A patients with inhibitors differs from those without inhibitors (Figure 5).

Figure 5. Bethesda assay. Standardize measurement of inhibitors in a factor VIII neutralization assay. The Bethesda assay is a laboratory test used to measure the concentration of inhibitors against factor VIII in patients with hemophilia A (Millner et al., 2016). It quantifies the inhibitory activity of antibodies that can neutralize the function of FVIII. Results are reported in Bethesda Units (BU), indicating the inhibitor activity level (Millner et al., 2016). One Bethesda Unit (BU) is a measure of the amount of inhibitor in a patient's blood that neutralizes the activity of one International Unit (IU) of FVIII (Millner et al., 2016). Figure created in Biorender.

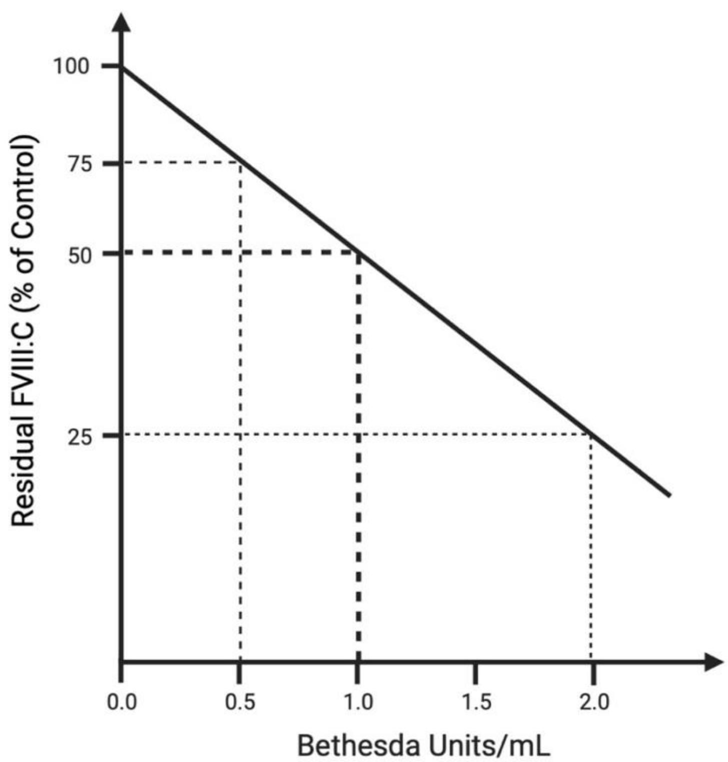

Figure 5. Bethesda assay

1.8 Factors affecting the prevalence of inhibitors

Current research has established that various genetic and environmental factors are crucial in developing FVIII inhibitors. Among these factors, the FVIII genotype is one of the most significant contributors to the development of inhibitors. Studies have shown that over 40% of patients with large deletions in the F8 gene are prone to developing anti-FVIII antibodies (Margaglione & Intrieri, 2018). On the other hand, 5% of patients with missense mutations and 15% with small deletions are also susceptible to inhibitor development (Mahmoud Abu Arra et al., 2020).

Studies have shown that polymorphisms in genes encoding molecules such as CTLA-4, TNFα, and IL10, as well as MHCII haplotypes, can affect antibody proliferation (Pavlova et al., 2009).

Table 1. Genetic and environmental factors in the formation of inhibitors

Genetic factors	Environmental factors
FVIII genotype	FVIII injection frequency
Family history	The injected FVIII
TNF, IL-10, CTLA-4, MHC II polymorphism	The age at the first injection
Race	

Apart from genetic factors, environmental factors also play a significant role in the development of inhibitors. Whether plasma-derived or recombinant, the type of FVIII used for treatment, can affect the risk of developing inhibitors (Peyvandi & Garagiola, 2018). Younger patients are also at an increased risk of developing inhibitors, as are patients who have been exposed to FVIII more than 20 times (Astermark et al., 2010). The intensity of FVIII treatment, inflammation caused by FVIII injection, and surgery can also increase the likelihood of developing inhibitors (Figure 6).

Moreover, it is worth noting that, a higher dose of FVIII is often required to be administered during surgical procedures (Astermark et al., 2010). Consequently, the combined effect of pro-inflammatory state and the increased dosage of FVIII during surgery may contribute to a break of tolerance to FVIII (Astermark et al., 2010). Like surgical situations, in cases where a patient experiences severe trauma with significant blood loss and tissue damage, the pre-inflammatory state is induced, necessitating large amounts of FVIII to manage bleeding and maintain adequate blood thickness (Astermark et al., 2010).

Figure 6. Environmental risk factors for development of inhibitors in severe Hemophilia A. The type of FVIII utilized for treatment (Peyvandi & Garagiola, 2018). Younger patients and those with over 20 exposures to FVIII face heightened susceptibility to inhibitor development (Astermark et al., 2010). Increased FVIII treatment intensity, inflammation resulting from FVIII administration, and surgical procedures further elevate this risk (Astermark et al., 2010). Figure created in Biorender.

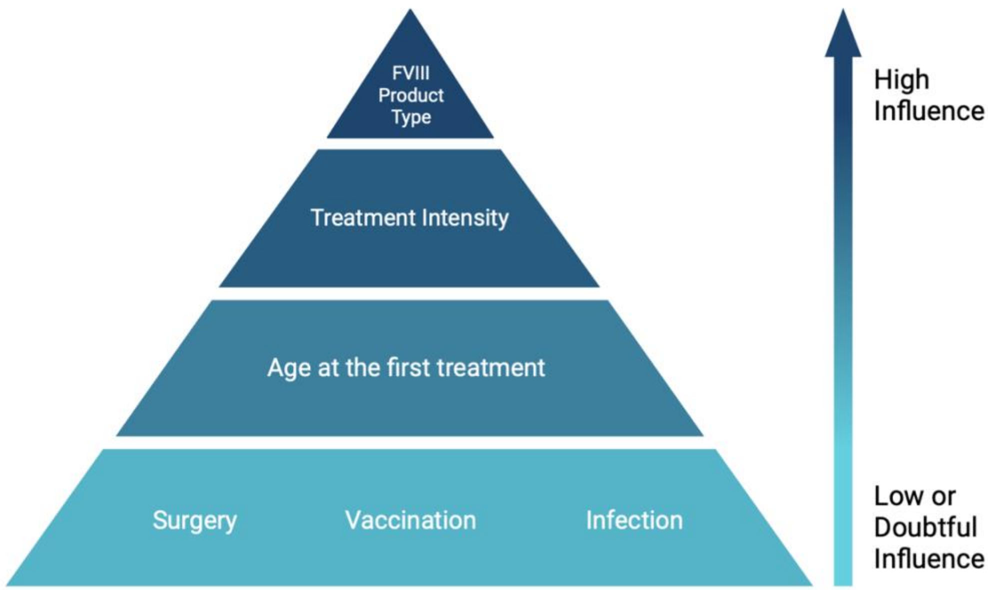

Figure 6. Environmental risk factors for the development of inhibitors in severe Hemophilia A

(Peyvandi & Garagiola, 2018)

1.9 Eradication of inhibitors (Immune Tolerance Induction (ITI) therapy)

Over the last three decades, immune tolerance induction (ITI) therapy has been the only treatment to decrease the immune response to FVIII (Schep et al., 2018). This therapy involves administering FVIII frequently at varying doses over time. While ITI has a success rate of 75%, it is both costly and time-consuming (Schep et al., 2018). In cases where patients do not respond to ITI treatment, options for controlling bleeding are limited. The titer of inhibitors affects the treatment of acute bleeding in patients with inhibitors. When the inhibitor titer is low, standard replacement therapy is administered by injecting a large amount of FVIII concentrate to neutralize the number of antibodies in the blood, allowing the rest of the FVIII to participate in the coagulation cascade. However, this method is unsuitable for patients with high inhibitor titers (Scott, 2022). For such patients, bypassing agents are the only effective therapies available. Bypassing agents bypass the need for factors VIII and IX by generating thrombin through other mechanisms (Schep et al., 2018). Activated prothrombin complex concentrate (APCC) and recombinant activated factor VII are the two bypassing agents currently available.

1.10 Fc Receptors

Fc receptors are proteins found on the surface of immune cells, such as leukocytes, which are essential in binding to the constant regions of antibodies (Figure 7) (Ravetch1 & Kinet, 1991). This binding promotes signal transduction pathways that modulate immune responses (Ravetch1 & Kinet, 1991). Various types of Fc receptors bind to different immunoglobin (Ig) heavy chain isotypes, each with distinct functions in the immune system. There exist receptors for immunoglobulin A (IgA), known as FcαR, for immunoglobulin G (IgG), referred to as FcγR and FcRn, and for immunoglobulin E (IgE), known as FcεR. Additionally, there are receptors for polymeric forms of immunoglobulin M and IgA called polyIgR (Mancardi & Daëron, 2014). Fcγ receptors are essential for the phagocytosis of particles coated with antibodies and are specific for the heavy chains of IgG antibodies (Mancardi & Daëron, 2014). These receptors play a vital role in the regulation of the immune response, and their dysregulation has been implicated in a variety of diseases, such as autoimmunity, cancer, and infectious diseases (Nimmerjahn & Ravetch, 2006; Raghavan & Bjorkman, 1996).

1.10.1 FcγRIIB

When the presence of an antigen activates B cells, they begin to produce antibodies (Kasahara et al., 2019). As the concentration of antibodies in the bloodstream increases, the antibodies themselves can signal to the B cells to slow down or stop their production (Kasahara et al., 2019). This is known as antibody feedback (Figure 7) (Kasahara et al., 2019).

B cells, a type of white blood cell, express a single type of Fc receptor known as CD32b. The CD32b receptor plays a crucial role in the process of antibody feedback. Antibody feedback occurs when the production of antibodies is decreased in response to the presence of secreted IgG antibodies (Kasahara et al., 2019). This process is achieved by binding of IgG antibodies to CD32b on B cells (Baerenwaldt et al., 2011). The cytoplasmic tail of CD32b contains an immune recepto0r tyrosine-based inhibition motif (ITIM), which plays a critical role in this process (Figure 10) (Kasahara et al., 2019). Moreover, the phosphorylation of SH2-containing Inositol Phosphatase (SHIP) also functions to suppress the Ras/ Mitogen-activated protein kinase (Ras-MAPK) signaling pathway involved in cell proliferation (Kasahara et al., 2019).

More precisely, the cytoplasmic tail of CD32b contains an ITIM that, when phosphorylated on tyrosine residues, forms a docking site for the phosphatase SHIP (Kasahara et al., 2019). SHIP then removes a phosphate on phosphatidylinositol 3,4,5-trisphosphate (PIP3), a signaling intermediate, and inactivates it (Kasahara et al., 2019). This process inhibits the B cell response to foreign antigens (Kasahara et al., 2019). When antigen-antibody complexes engage with CD32b and the antigen receptor simultaneously, inhibitory phosphatases are brought close to the antigen receptors, stopping their signaling (Kasahara et al., 2019). In other words, CD32b serves as an essential regulatory mechanism for controlling the B cell response to foreign antigens (Kasahara et al., 2019).

Figure 7. Antibody feedback. The immune complexes could neutralize B cell activation by co-engaging BCR and CD32b (Heyman, 2003). This negative feedback loop for antibody production relies on the interaction between the Fc domain of the antibody and FcγRIIb. The ITIM of CD32b is crucial for suppressing BCR-induced intracellular signals. Figure created in Biorender.

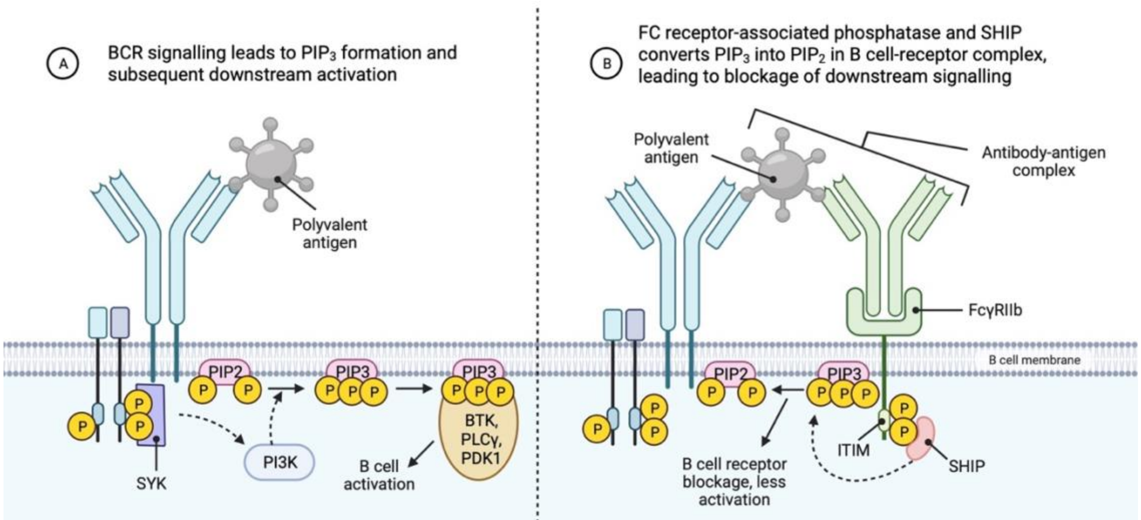

Figure 7. Antibody feedback

(Abbas, Abul K et al, 2010)

The equivalence zone in the context of immune complex (IC) formation refers to the point at which the antigen and antibody are present in equal amounts, forming stable ICs. ICs are formed when antibodies bind to antigens, and the immune system can clear the resulting complex from the body. In the equivalent zone, the concentration of free antigen and free antibody is minimal, and most of the antigen and antibody molecules are bound together in stable ICs. Researchers can improve their ability to target the CD32b on the surface of B cells by comprehending the dynamics of IC formation and the equivalent zone. This is because a proper ratio of antigen and antibody is required to activate the CD32b (Figure 8).

Figure 8. Equivalence Zone. In this zone, antigen and antibody molecules are present approximately equal amounts, allowing for efficient binding and formation of stable immune complexes. Figure created in Biorender.

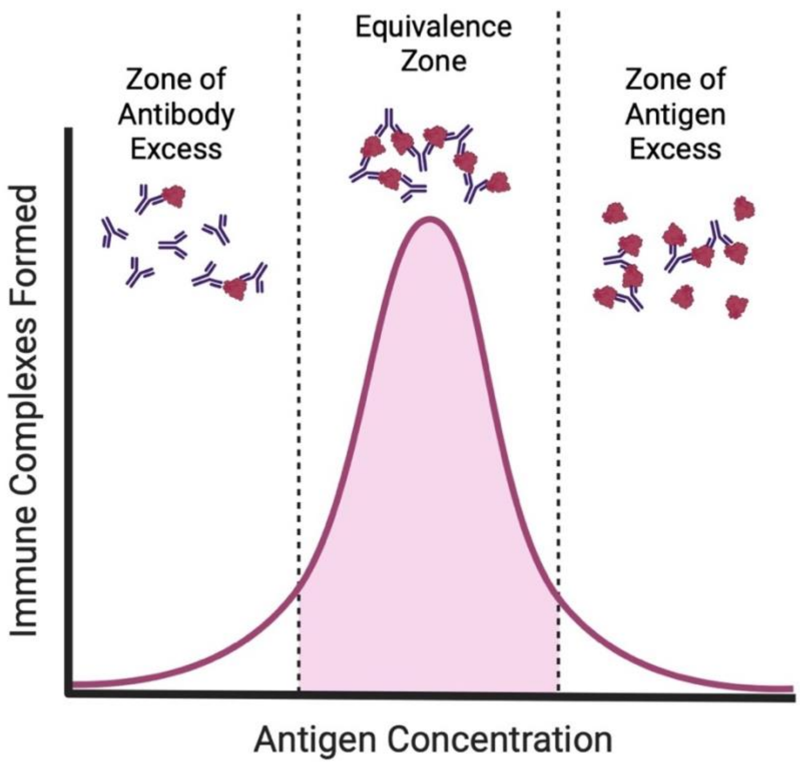

Figure 8. Equivalence Zone

2 Chapter II- Study Rational and Hypothesis

The development of inhibitors is a significant challenge in treating hemophilia A patients. The CD32b pathway plays a crucial role in regulating the activation of B cells. Targeting this pathway could potentially suppress the production of anti-FVIII antibodies by FVIII-specific B cells. Therefore, we suggested an experiment that involves inducing tolerance in Hemophilia A mice by engaging the CD32b on FVIII-specific B cells. This experiment could offer valuable insights into preventing inhibitor formation in patients with Hemophilia A and could lead to the developing of new therapeutic strategies for this condition.

Purpose of the Study:

This study investigated the impact of FVIII-Anti-FVIII immune complexes on FVIII-specific antibody formation in a Hemophilia A mouse model.

Hypothesis:

We hypothesize that administering a balanced amount of rhFVIII and anti-FVIII IgG will suppress FVIII-specific B cells, reducing inhibitors compared to the PBS control groups.

Objectives:

1. **IC Formation:** Determine the optimal rFVIII and anti-FVIII IgG concentration for preparing rh-FVIII-IC and identify the optimal ratio.
2. **Effect of F8-ICs Injection:** Investigate the effect of rFVIII-ICs injection in a mouse model.

3. Determine whether the induced tolerance is maintained upon rFVIII re-exposure.
4. **Impact of CD32b Blockade:** Explore the effect of CD32b blockade on developing of antibodies against FVIII.

3 Chapter III - Materials and Methods

3.1 Hemophilia A Mouse Model

FVIII knockout (KO) mice were used as a Hemophilia A mouse model. 12954/SvJae mice were inbred with C57BL6 background mice. Specifically, F8 exon 16 knockout results in Hemophilia A. The mouse colony is routinely genotyped to ensure the mice retain the gene knockout.

3.2 FVIII Source

Kovaltry (Bayer HealthCare LLC, Whippany, NJ, US) is a third-generation, unmodified, full-length recombinant human FVIII that is approved for both prophylactic use and to treat bleeding episodes on-demand in Hemophilia A patients (Figure 12).

3.3 Methods

3.4 FVIII/ Anti-FVIII Polyclonal Antibody Immune Complex Experiment

3.4.1 Immunization

All FVIII knockout (KO) mice were aged 9 ± 2 weeks at the beginning of experiments. To prepare the rhFVIII-ICs, FVIII KO mice were administered 25 IU/kg Kovaltry via retro-orbital injections twice a week for four weeks to develop anti-FVIII IgG (Figure 13). After this regimen, a terminal cardiac blood collection was used for blood collection. An ELISA was used to determine the concentration of anti-FVIII IgG, and then mice plasma was pooled together before the preparation of rhFVIII-ICs.

The total amount of FVIII to be injected in each mouse for these experiments was calculated based on its weight.

3.4.2 Retro-orbital blood collection

For retro-orbital blood collection, mice were put under anesthesia with Isoflurane. A capillary tube was used to collect blood from the medial canthus of the eye. Typically, 3 to 4 drops of blood were collected. Citrate 3.8% was used as a 1 to 9 ratio. After retro-orbital blood collection, mice were monitored for two days to ensure their well-being.

3.4.3 Cardiac blood collection

For cardiac blood collection, 50-70 µL of Sodium citrate 3.8% (258.07 g/mol) was loaded into the syringe. Mice were put under anesthesia with Isoflurane. Specifically, for 1 ml of blood collected, 100 µl of sodium citrate must be added to prevent blood clot formation. Finally, a cervical dislocation was performed to euthanize the mice (Figure 14).

3.5 Plasma Isolation

To isolate plasma from blood, whole blood was centrifuged for 10 minutes at 2000 RCF. Once the plasma supernatant was collected into a new Eppendorf tube using sterile conditions, the plasma sample was centrifuged for 15 minutes at 2000 RCF. Then, the supernatant plasma was again collected into a new Eppendorf tube using sterile conditions. Plasma samples were then pooled and aliquoted into smaller Eppendorf tubes to be stored at -80°C.

3.6 Indirect ELISA Determining Anti-FVIII IgG Concentration

An ELISA was used to quantify the concentration of anti-FVIII IgG present in plasma samples of mice. High affinity 8 Well Strips (Coaster, US) were assembled onto an ELISA frame. Kovaltry was diluted in Na2CO3 (0.1 M) as a coating buffer at a 1:100 ratio to have 10 ng/µL concentration, and 100 µL of this solution was added to every well in the plate. The plate was then incubated for 2 hours at room temperature. After the first incubation, the plate was washed thrice with 200 µL/well of PBS/T buffer solution (10 mM). Then 200 µl of blocking buffer (Milk 5%) was added to each well, and the plate was incubated for 2 hours at room temperature. After this second incubation, the plate was rewashed three times. The standard solution was prepared by diluting mouse anti-FVIII monoclonal antibody (1mg/mL) (Invitrogen, Carlsbad, California, US) in blocking buffer serially to concentrations of 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000, 1/64000, in addition to 1 blank tube containing only the blocking buffer. Each sample was serially diluted in blocking buffer to concentrations of 1:100, 1:200, and 1:400. The prepared standard and sample dilutions were added to the plate in duplicates of 100 µL/ well volume. The plate was incubated overnight at 4°C. The next step was washing the plate with 200 µL/well of PBS/T. The goat anti-mouse IgG (H&L) alkaline phosphatase-conjugated detecting antibody (1 mg/m) (Abcam, Waltham, Boston, US) was diluted in blocking buffer (1:1000 ratio), and 100 µL was added to each well. The plate was incubated for 2 hours at room temperature. The para-nitrophenylphosphate (p-NPP) (Sigma, Massachusetts, US) was diluted in diethanolamine (DEA) buffer (1M) to obtain 1 mg/mL concentration and was added to each well with a volume of 100 µL. The plate was allowed to develop color for

20 minutes and read with a spectrophotometer with an absorbance wavelength of 405 nm. The standard curve was plotted in SigmaPlot using the 4-parameter logistic regression model (Figure 9).

Figure 9. ELISA to quantify anti-FVIII IgG concentrations in mouse plasma. After coating the plate with Kovaltry (10 ng/μL). Subsequent steps included blocking with milk 5%, adding standard and sample solutions, and further incubation. The goat anti-mouse IgG (H&L) alkaline phosphatase-conjugated detecting antibody (1 mg/mL) was added, followed by the substrate p-NPP (1mg/ml) for color development. Spectrophotometry was used to measure absorbance at 405 nm. The standard curve was generated using the 4-parameter logistic regression model in SigmaPlot. Figure created in Biorender.

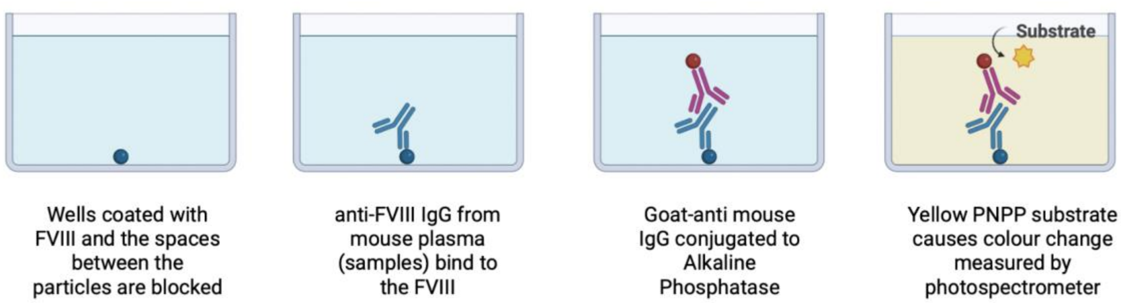

Figure 9. Indirect ELISA

3.7 FVIII antigen ELISA

The VisuLize™ FVIII Antigen Kit (Affinity Biological Inc, Ancaster, Ontario, Canada) was used to measure the FVIII concentration in each Kovaltry microliter. Strip wells were pre-coated with sheep polyclonal antibody to FVIII (Invitrogen, Carlsbad, California, US). The Kovaltry was diluted and added to the wells. Then, the plate was incubated for 1 hour at room temperature. After the first incubation, the plate was washed thrice with 200 µL/well of PBS/T buffer (10mM). After washing away unbound substances, a peroxidase-labeled detecting antibody was added and allowed to bind to the captured FVIII with 45 minutes of incubation at room temperature. The wells were rewashed, and the TMB (the peroxidase substrate tetramethylbenzidine) was used and allowed to react (Figure 10).

Figure 10. This sandwich ELISA involved coating wells with sheep polyclonal antibodies targeting FVIII. Subsequently, diluted Kovaltry was added into the wells and incubated for one hour. Following a thorough wash, a peroxidase-labeled detecting antibody was added and permitted to bind to the captured FVIII over a 45-minute incubation. Finally, a TMB solution was used to initiate a reaction. Figure created in Biorender.

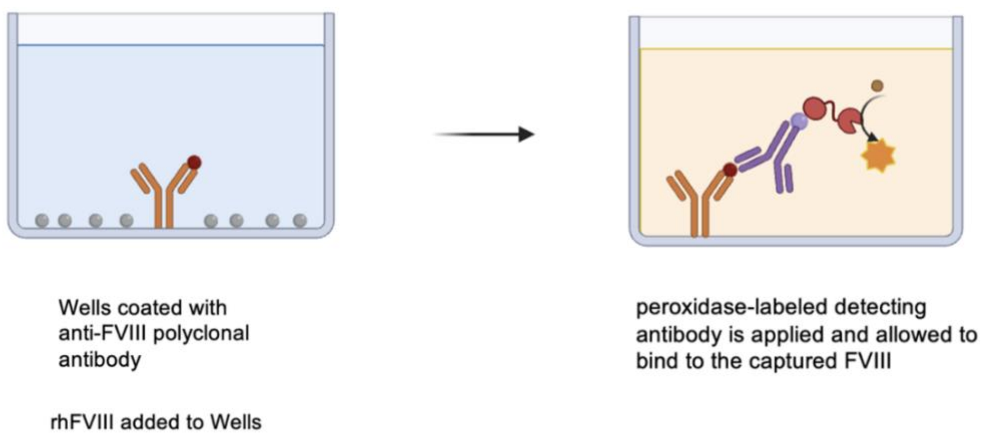

Figure 10. FVIII antigen sandwich ELISA

3.8 Preparation of rhFVIII-ICs

Sera of 10 immunized FVIII$^{-/-}$ mice were used to generate a pool of murine polyclonal anti-FVIII antibodies. The antibody pool was incubated with Kovaltry at 37 °C for 120 minutes to produce rhFVIII-ICs (Figure 11).

Figure 11. rhFVIII-ICs formation. The serum from 10 immunized FVIII-deficient mice, containing the anti-FVIII antibody, was mixed with Kovaltry at a temperature of 37 °C for 120 minutes, resulting in the formation of rhFVIII-ICs. Figure created in Biorender.

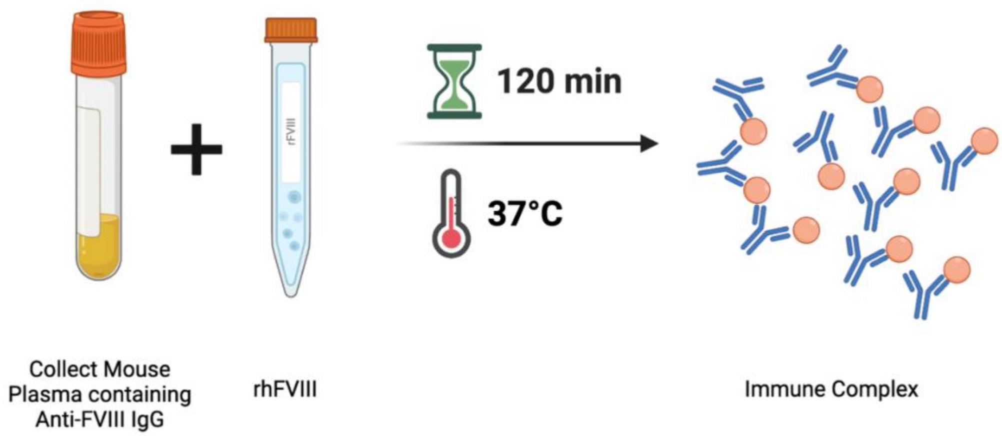

Figure 11. rhFVIII-ICs formation

3.9 rhFVIII/anti-FVIII antibody Immune Complex Sandwich ELISA

For the coating step, 96-well low-affinity polystyrene microplates (MaxiSorb, US) were coated for 18 hours at 4 °C with 50 μL/well of sheep anti-FVIII polyclonal antibody (40 μg/ml) (Invitrogen, Carlsbad, California, US) in 0.06 M carbonate-bicarbonate buffer (pH 9.6). Then, the plate was incubated at 4 °C overnight. After the first incubation, the plate was washed thrice with 200 μL/well of PBS/T buffer solution (10mM). The blocking buffer (5% BSA in PBS/T) was added to each well in the blocking step. The plate was incubated for 2 hours at room temperature. The plate was washed as before. A checkerboard ELISA evaluated the binding affinity of Kovaltry and anti-FVIII monoclonal antibody 1mg/mL (Invitrogen, Carlsbad, California, US) at varying concentrations. The experiment was carried out by adding different Kovaltry and anti-FVIII monoclonal antibody concentrations, ranging from 0-25 μg /mL of anti-FVIII IgG and 0-40 IU/mL of Kovaltry, respectively. Serial dilutions were used for the different ratios of ICs. The diluted Kovaltry and anti-FVIII antibody were added to each well in duplicate. The plate was incubated for 2 hours at room temperature. The next step was washing the plate with 200 μL/well of PBS/T. The detecting antibody, which was goat anti-mouse IgG (H&L) alkaline phosphatase-conjugated (1 mg/mL) (Abcam, Waltham, Boston, US), was diluted in blocking buffer at a ratio of 1:1000, and 100 μL was added to each well. The plate was incubated for 2 hours at room temperature. P-NPP (Sigma, Massachusetts, US) was dissolved in DEA buffer (1M) for having) 1 mg/mL concentration and was added to each well with a volume of 100 μL (Figure 12).

Figure 12. Microplates were coated with a sheep anti-FVIII polyclonal antibody, followed by overnight incubation at 4°C. After washing, blocking with 5% BSA in PBS/T occurred for 2 hours at room temperature. Different ratios of rhFVIII-ICs were tested via serial dilutions. Following further incubation and washing, a detecting antibody was applied, and p-NPP was added for color development. Figure created in Biorender.

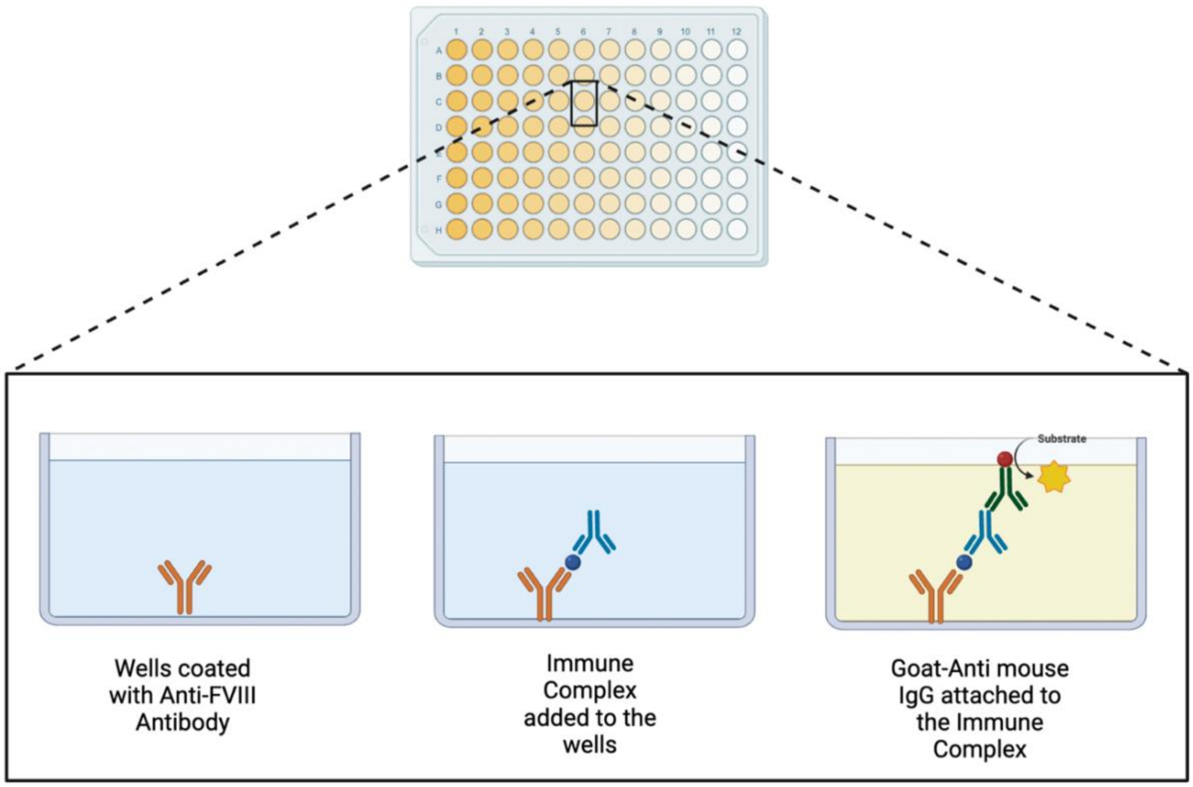

Figure 12. Sandwich ELISA for rhFVIII-ICs detection

3.10 rhFVIII-ICs Injection

During the experimental phase, Hemophilia A mice were administered once-a-week retro-orbital injections of either 1:1 or 1:10 ratios of rhFVIII-ICs, or positive control, which is naïve plasma mixed with FVIII or PBS as a negative control for four weeks. This phase aimed to induce tolerance to rhFVIII and prevent the production of anti-FVIII antibodies. Following the immunization phase, Kovaltry was administered retro-orbitally at a dose of 25 IU/kg once weekly for four weeks, while the mice in the PBS group received PBS for the next four weeks. The immunization phase aimed to stimulate an immune response. Finally, an ELISA was used to determine the concentration of anti-FVIII IgG. In this experiment, 11 mice were part of each group: the 1:1 ratio group, the 1:10 ratio group, and the control group. The positive control group had received a mixture of Kovaltry and naïve plasma, with a total amount of Kovaltry equal to what was used in the mice group that received the 1:1 and 1:10 ratio of our rhFVIII-ICs (Figure 13).

Figure 13. Our study involved four distinct groups: Control 1, consisting of mice administered PBS over the entire 8-week period; Control 2, involving mice receiving a combination of Kovaltry and Naive plasma for four weeks; a group of mice receiving rhFVIII-ICs in a 1:1 ratio; and a separate group receiving rhFVIII-ICs in a 1:10 ratio. Figure created in Biorender.

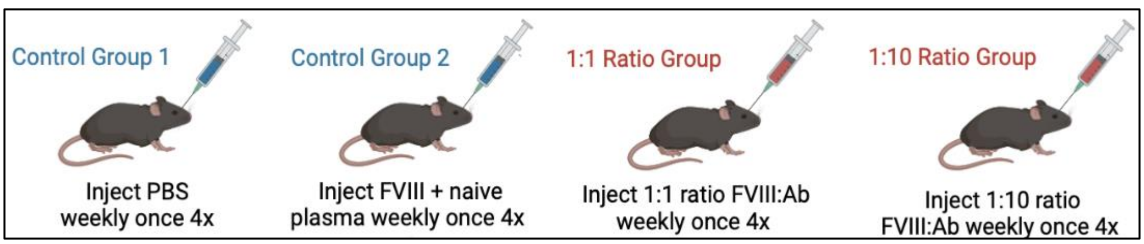

Figure 13. Experimental groups of mice

3.11 Booster Shot

After the final injection of the immunization phase, the mice were given a two-week rest period. Following the rest period, a booster injection of 25 IU/Kg Kovaltry was administered to all mice to stimulate the immune system further.

To evaluate the effectiveness of the tolerization phase, the mice were bled one week after receiving the booster injection on day 74 of the experiment. The aim was to determine if the tolerance was retained or diminished. Blood samples were collected and analyzed to measure the concentration of anti-FVIII IgG (Figure 14).

Figure 14. Experimental Plan. FVIII knockout mice received retro-orbital injections of rhFVIII-ICs, PBS, or Kovaltry mixed with naïve plasma once a week for four weeks during the tolerization phase. Subsequently, the blood was collected through retro-orbital blood collection. The mice were then administered Kovaltry retro-orbitally at 25 IU/kg once weekly for an additional four weeks. Following this, we conducted retro-orbital blood collection once again. Afterward, all the mice were given a two-week rest period, followed by a single dose of 25 IU/kg of Kovaltry. One week later, blood was collected via cardiac bleed. Finally, blood samples were obtained from all mice, and the levels of anti-FVIII IgG were assessed at all three different time points using ELISA. Figure created in Biorender.

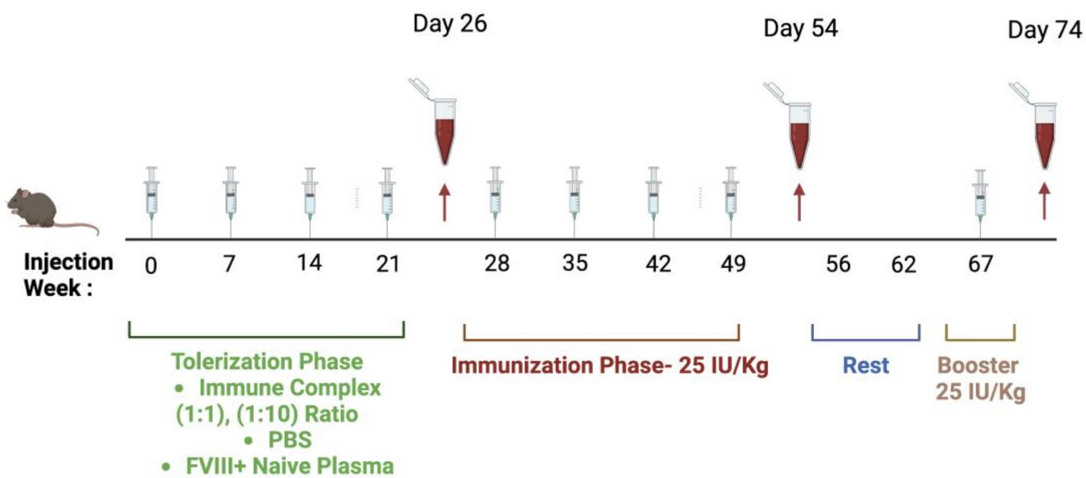

Figure 14. Experimental timeline

3.12 FVIII/ Anti-FVIII monoclonal antibody Immune Complex Experiment

In this study, an anti-FVIII monoclonal antibody was used to achieve a precise ratio of rhFVIII-ICs, as our previous rhFVIII-ICs generated with polyclonal anti-FVIII antibody has the potential to be close to a 1:1 or 1:10 ratio. To prepare the FVIII/Anti-FVIII monoclonal antibody IC, a mouse anti-FVIII monoclonal IgG (Invitrogen, Carlsbad, California, US) was used. To form the rhFVIII-ICs, the appropriate quantity of antibody was incubated with Kovaltry at 37 °C for 60 minutes, producing rhFVIII-ICs. The timeline of this experiment was comparable to the previous experiment, which involved a four-times-weekly injection of rhFVIII-ICs as a tolerization phase, followed by four injections of 25 IU/kg Kovaltry as an immunization step, and a single injection of a booster shot two weeks after. The negative control used in this experiment consisted of PBS. We also had an additional control, which was an anti-FVIII monoclonal antibody.

3.13 IgG subtype Elisa

The ELISA was used to determine the concentration of IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3) in the plasma samples collected from the mice one week after the booster shot.

A High-affinity EIA 8 Well Strip (Coaster, US) was assembled onto an ELISA frame. Kovaltry was diluted to a 10 ng/μL concentration as a coating buffer in Na2CO3 (0.1 M). Subsequently, 100 μl of this diluted solution was added to each well of the plate. The plate was then incubated at room temperature for 2 hours.

Following the initial incubation, the plate was washed using 200 μL of PBS/T buffer solution (10mM) per well. Subsequently, 200 μL of blocking buffer (Milk 5%) was added to each well. The plate was incubated for another 2 hours at room temperature. After this second incubation, the plate was subjected to three additional washes.

The mouse anti-FVIII monoclonal antibody (1mg/mL) (Invitrogen, Carlsbad, California, US) was mixed with a blocking buffer to prepare standard solutions. These solutions were serially diluted in separate Eppendorf tubes and a blank tube containing only a blocking buffer. In parallel, samples were prepared at an appropriate dilution in a blocking buffer.

The prepared standard and sample dilutions were added to the plate in duplicate, with each well receiving 100 μL of the respective dilution. The plate was incubated overnight at 4°C. The subsequent step involved washing it with 200 μL/well of PBS/T.

To detect the antibodies, the specific detecting antibody for each subclass ELISA was diluted in blocking buffer in a ratio of 1:2000, and then 100 μL of this mixture was added to each well. The plate was incubated for an additional 2 hours at room temperature.

100 μL TMB was added to each well to initiate the color development. The plate was stopped by 0.16 M sulfuric acid. Then, a spectrophotometer read the plate at an absorbance wavelength of 450 nm. The standard curve was plotted in SigmaPlot.

3.14 Blocking CD32b

We blocked CD32b by administering anti-mouse CD32b (Bio X Cell, Lebanon, New Hampshire, United States) through IP injection 24 hours before each 1:1 ratio of rhFVIII-IC injection. Additionally, we injected the Isotype control (Bio X Cell, Lebanon, New

Hampshire, United States) in the same manner as the control group. During the tolerization phase, FVIII-ICs were administered for four weeks, and blood samples were collected retro-orbitally to measure the levels of anti-FVIII IgG. Subsequently, the immunization phase proceeded by injecting 25 IU/kg Kovaltry, and blood samples were collected retro-orbitally to measure antibody levels. This experiment aimed to ascertain any differences in the levels of anti-FVIII IgG following the blocking of CD32b (Figure 15).

Figure 15. Timeline for blocking CD32b experiment. Anti-mouse CD32b and Isotype control were injected 24 hours before each 1:1 ratio of rhFVIII-IC injection once a week for four weeks during the tolerization phase. Subsequently, we collected blood through retro-orbital blood collection. The mice were then administered Kovaltry retro-orbitally at 25 IU/kg once weekly for an additional four weeks. Following this, we conducted retro-orbital blood collection once again. Afterward, all the mice were given a two-week rest period, followed by a single dose of 25 IU/kg of Kovaltry. One week later, blood was collected via cardiac bleed. Finally, blood samples were obtained from all mice, and the levels of anti-FVIII IgG were assessed at all three different time points using ELISA. Figure created in Biorender.

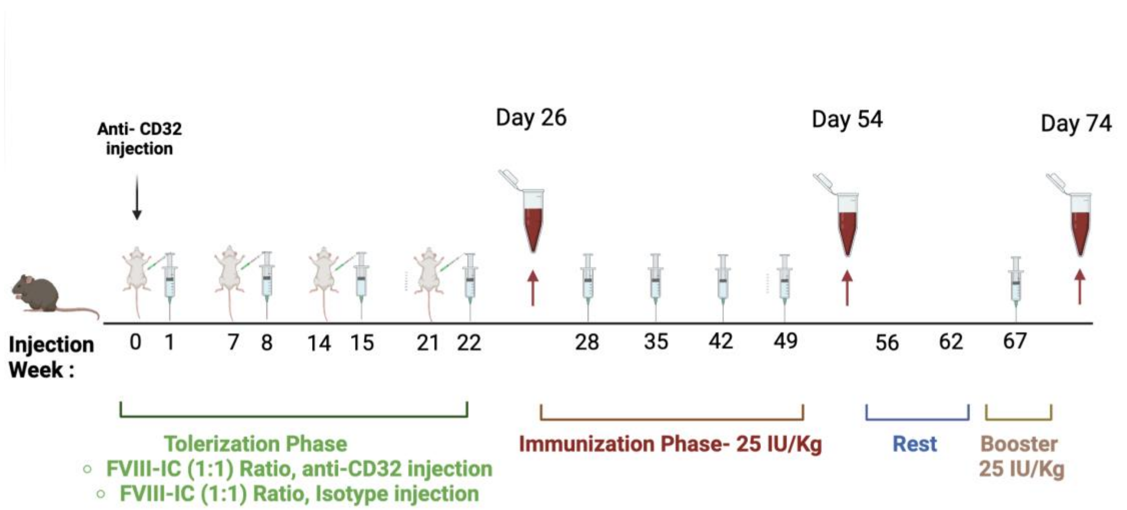

Figure 15. Timeline for Blocking CD32b Experiment

3.15 Bethesda Assay

To measure the titer of rFVIII-inhibitor, plasma was pooled from three mice in each mouse group: the PBS or negative control group, the non-tolerized mice group, and the tolerized mice group. Subsequently, the plasma samples were sent to Hematology McMaster University's core laboratory to quantify Bethesda units using the Bethesda assay.

4 Chapter IV – Results

4.1 rhFVIII-IC study using polyclonal anti-FVIII IgG antibody

4.1.1 FVIII antigen ELISA result

Our FVIII antigen ELISA kit utilized normal pooled human plasma as a calibrator to determine the exact concentration of FVIII antigen. The molecular weight of FVIII is 320 kDa, and its plasma concentration is 200 ng/mL, equivalent to 1 IU/mL. Based on our calibrator, the FVIII antigen concentration value was close to 200 ng/mL (1 IU/mL). However, based on the ELISA, we found that 1 IU/mL of Kovaltry consisted of approximately 190 ng/mL.

4.1.2 rhFVIII/anti-FVIII antibody Immune Complex ELISA result

Based on the ELISA results obtained in this study, the optimal concentration of Kovaltry for the formation of rhFVIII-ICs was determined to be between 10-20 IU/mL, while the optimal concentration for anti-FVIII IgG was determined to be 2-5 μg/mL. Additionally, the best ratio for the formation of rhFVIII-ICs was 1:1 for Kovaltry and anti-FVIII IgG (Figure 16).

Figure 16. The concentration of rhFVIII and anti-FVIII antibodies for FVIII-IC formation. The anti-FVIII IgG concentrations spanned from 0 to 25 μg/ml (B), while Kovaltry concentrations ranged from 0 to 40 IU/ml (A). The procedure was initiated by coating microplates with sheep anti-FVIII polyclonal IgG, followed by overnight incubation at 4°C. Subsequent steps involved blocking with 5% BSA in PBS/T for 2 hours at room temperature. We then explored different IC ratios through serial dilutions. After additional incubation and washing, a detecting antibody was applied, and the introduction of p-NPP facilitated color development.

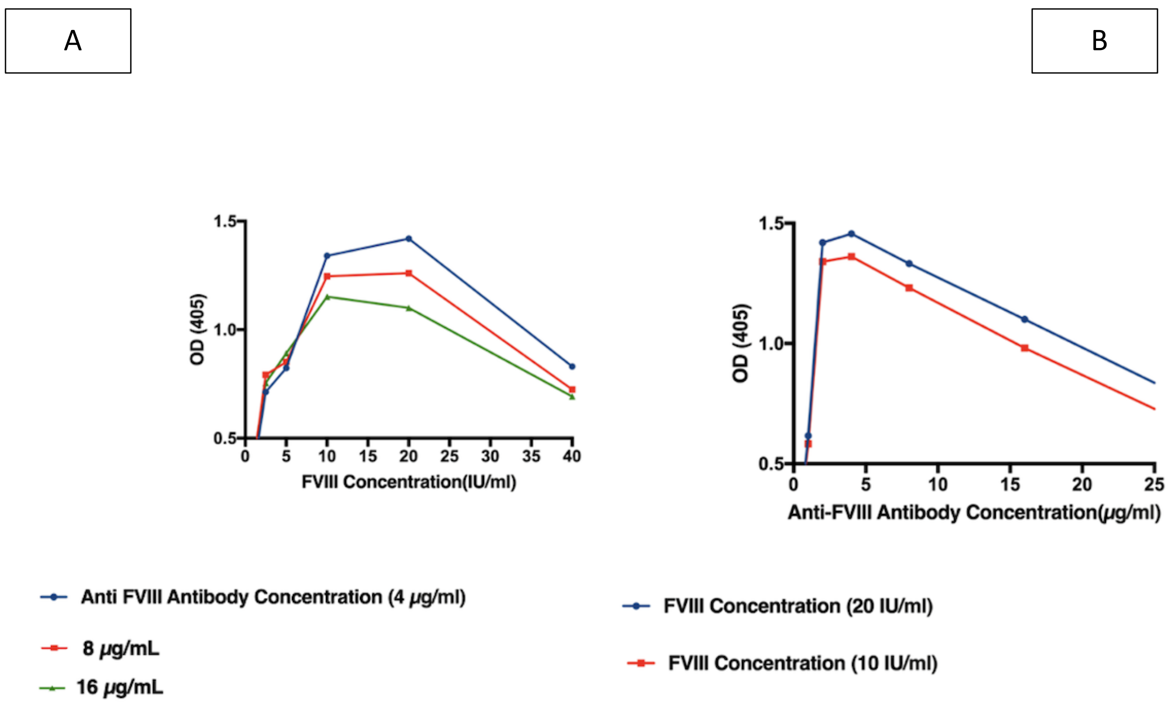

Figure 16. The concentration of Kovaltry and anti-FVIII IgG for FVIII-IC formation

4.1.3 Anti-FVIII IgG titer after tolerization and immunization phase

Hemophilia A mice underwent a 4-week tolerization phase with retro-orbital injections of rFVIII-ICs and controls, followed by a 4-week immunization phase of Kovaltry or PBS, and anti-FVIII IgG concentrations were assessed using ELISA. We used an unpaired t-test for our statistical analysis. The results of the study showed that the mice that received the combination of FVIII and naive plasma as a positive control (n=11) developed a significant increase in anti-FVIII IgG titer (mean increase of 6100 ng/mL, p-value = 0.0001).

Moreover, the results of this study demonstrate that the mice (n=11). Those who received only PBS during the tolerization and immunization phases did not develop detectable levels of anti-FVIII IgG (Figure 17).

On the other hand, when rhFVIII-ICs were administered to mice, the development of anti-FVIII IgG was significantly lower. Specifically, 65.3% of mice (n=11) that received rhFVIII-ICs at a 1:1 ratio did not develop detectable levels of IgG (mean increase of 1300 ng/mL, p-value = 0.0723), while 50% of mice (n=11) that received rhFVIII-ICs at a 1:10 ratio did not develop anti-FVIII IgG (mean increase of 2300 ng/mL, p-value = 0.1030).

Figure 17. Anti-FVIII IgG titer after the Tolerization phase and Immunization phase. The ELISA technique used for the measurement of anti-FVIII IgG concentrations in 4 groups of plasma mice samples (PBS, FVIII+ Naïve Plasma, rhFVIII-IC (1:1) ratio, rhFVIII-IC (1:10) ratio) in two-time point (Tolerization phase: after four times rhFVIII-ICs injection, and Immunization phase: after four times injection of 25 IU/kg of Kovaltry). The plate was coated with Kovaltry (10 ng/μL), followed by subsequent steps, including blocking with milk 5%, adding standard and sample solutions, and a further round of incubation. The goat anti-mouse IgG (H&L) alkaline phosphatase-conjugated detecting antibody (1mg/mL) was added, and p-NPP substrate (1mg/ml) was applied to initiate color development. Spectrophotometry was used to measure absorbance at 405 nm to determine concentrations. We established the standard curve by utilizing the 4-parameter logistic regression model in SigmaPlot. Results are presented as mean (SD) and analyzed using an unpaired t-test. A p-value of 0.05 was considered statistically significant.

(*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. non-significant. ns).

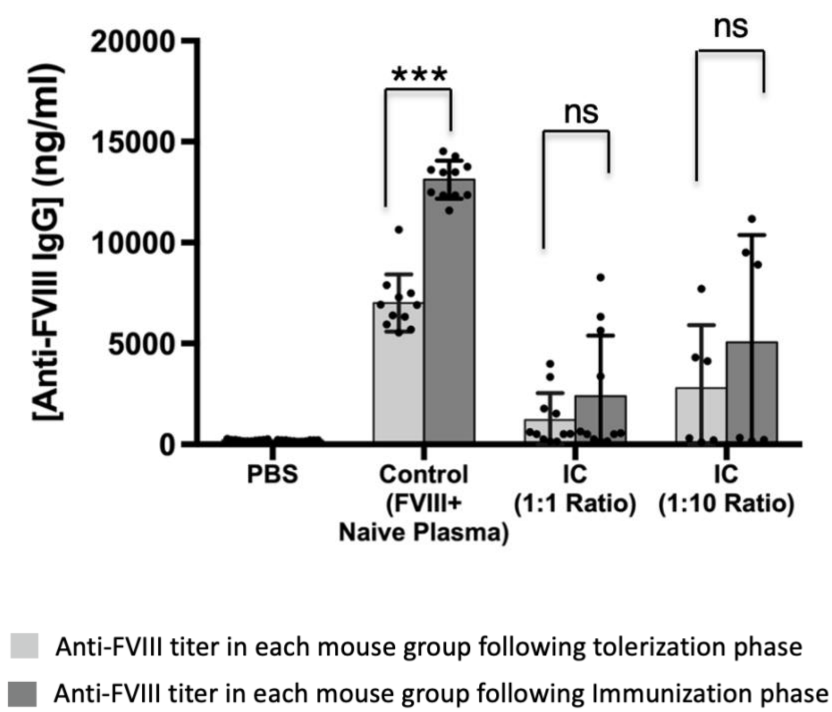

Figure 17. Anti-FVIII IgG titer after Tolerization phase and Immunization phase

4.1.4 Anti-FVIII IgG titer before and after the booster shot

A two-week resting period was implemented for all mice to evaluate the durability of the established tolerization during the tolerization phase, followed by administering a single 25 IU/kg dose of Kovaltry. The result of the booster shot experiment showed no significant difference in the levels of anti-FVIII IgG before and after the booster shot in any of the four groups ($p>0.05$). However, when comparing the levels of anti-FVIII antibodies between the groups after the booster shot, the positive control group showed a significantly greater increase in anti-FVIII IgG levels than the other two groups. This suggests that although the booster shot had a limited effect on anti-FVIII IgG levels overall, the positive control group had a more robust response to the booster shot than the other groups (Figure 18).

Figure 18. Anti-FVIII IgG titer before and after the Booster shot. The ELISA technique was run to quantify anti-FVIII IgG levels across four distinct groups of mouse plasma samples (PBS, FVIII+ Naïve Plasma, rhFVIII-IC (1:1) ratio, rhFVIII-IC (1:10) ratio). The plate was coated with Kovaltry (10 ng/µL) and underwent successive steps, adding 5% milk blocking the addition of standard and sample solutions. The goat anti-mouse IgG (H&L) alkaline phosphatase-conjugated detecting antibody (1mg/mL) was used, and the P-NPP substrate (1mg/ml) was added. The absorbance was measured by spectrophotometry at 405nm. The standard curve was constructed using the 4-parameter logistic regression model in SigmaPlot. Results are expressed as mean (SD) and were subjected to analysis via unpaired t-tests. Significance was designated at a p-value of 0.05 (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$, non-significant. ns).

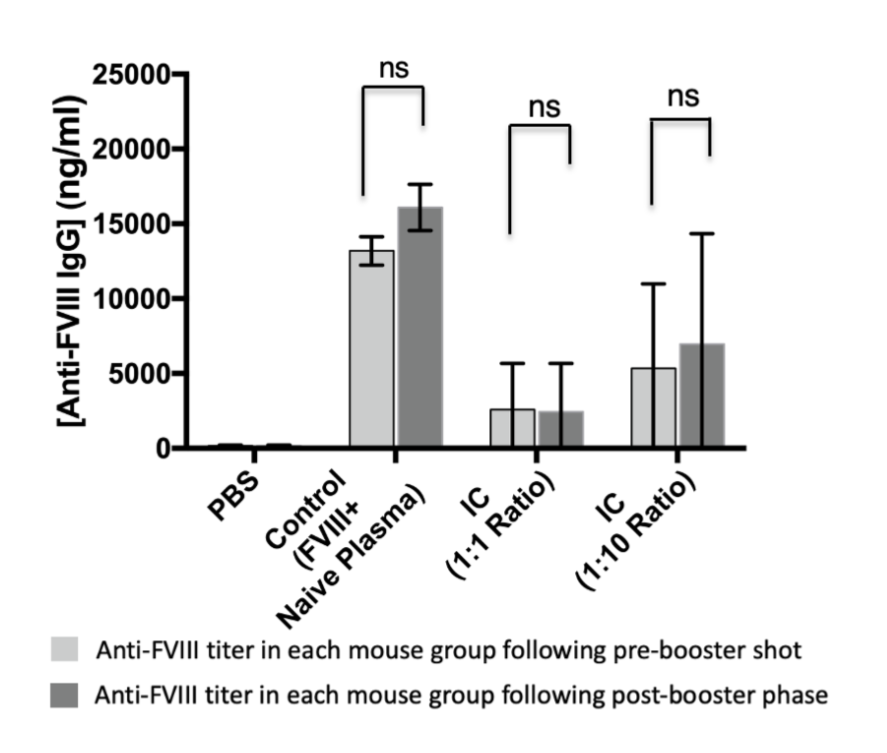

Figure 18. Anti-FVIII IgG titer before and after Booster shot

An interesting finding emerged from the study: the levels of anti-FVIII IgG were higher when FVIII was injected alone and not as a rhFVIII-ICs. After the tolerization phase, the mean antibody levels were as follows: Control group (FVIII + Naïve Plasma) at 7,004.5 ng/ml, IC (1:1) group at 1,208.66 ng/ml, and IC (1:10) group at 2,796.78 ng/ml. Furthermore, after the immunization phase, the mean antibody levels were Control (FVIII + Naïve Plasma) at 13,118 ng/ml, IC (1:1 Ratio) at 2,397.41 ng/ml, and IC (1:10 Ratio) at 5,050.23 ng/ml (Figure 19).

Figure 19. Comparison between the levels of ant-FVIII IgG in controls, IC (1:1) ratio, and IC (1:10) ratio groups after the Tolerization phase and Immunization phase. Significant differences were observed in the levels of anti-FVIII IgG between the control group (FVIII + naïve plasma) with both the IC (1:1) and (1:10) ratio groups during each phase of tolerization and immunization (p-value < 0.05). Significance was designated at a p-value of 0.05 (*: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001, non-significant. ns*)*

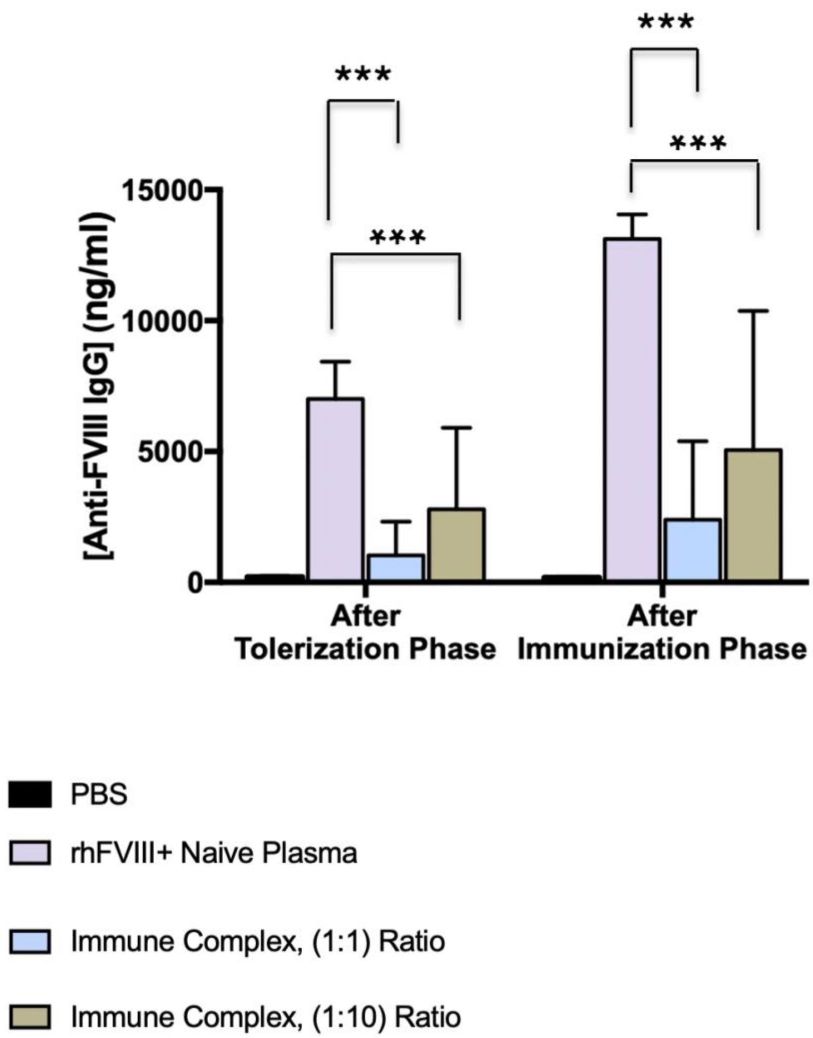

Figure 19. The comparison between the levels of ant-FVIII antibody after the Tolerization phase and Immunization phase

4.2 rhFVIII-IC study using monoclonal anti-FVIII IgG

4.2.1 Anti-FVIII IgG titer before and after immunization phase in Monoclonal Study

After a four-times-weekly injection of rhFVIII-ICs as a tolerization phase, followed by four injections of 25 IU/kg Kovaltry as an immunization step, blood samples were collected, and ELISA measured the levels of anti-FVIII antibodies. The ELISA results showed that the mice administered with solely PBS throughout the tolerization and immunization phases did not exhibit detectable levels of anti-FVIII IgG (mean increase of 1.55 ng/mL, p-value = 0.9488). Additionally, all mice in the Control group, which received the anti-FVIII monoclonal IgG, experienced a significant rise in anti-FVIII IgG (mean increase of 4890.4 ng/mL, p-value = 0.0001). Conversely, when mice were administered rhFVIII-ICs, 74.7% of mice receiving rhFVIII-ICs at a 1:1 ratio showed no significant anti-FVIII IgG development (mean increase of 768.5 ng/mL, p-value = 0.3312). In comparison, 63.3% of mice receiving rhFVIII-ICs at a 1:10 ratio did not develop anti-FVIII IgG (mean increase of 898.78 ng/mL, p-value = 0.4538) (Figure 20).

Figure 20. Anti-FVIII IgG titer after the Tolerization phase and Immunization phase. The ELISA was used to quantify anti-FVIII IgG in four groups of mouse plasma samples (control 1: PBS, control 2: Anti-FVIII IgG, rhFVIII-IC at 1:1 ratio, rhFVIII-IC at 1:10 ratio) at two-time points (after tolerization phase, four weekly injections of rhFVIII-ICs; after Immunization phase, four weekly injections of 25 IU/kg of Kovaltry). Kovaltry (10 ng/μL) was used to coat the plate, followed by blocking with 5% milk, adding standard and sample solutions, and further incubation. Detection was carried out with goat anti-mouse IgG (H&L) alkaline phosphatase-conjugated detecting antibody (1mg/mL), and color development was initiated with p\P-NPP substrate (1mg/ml). Concentrations were determined by measuring absorbance at 405 nm using spectrophotometry. The standard curve was established using the 4-parameter logistic regression model in SigmaPlot. Results are presented as mean (SD) and analyzed using an unpaired t-test. Statistical significance was set at $p < 0.05$. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$, non-significant. ns)

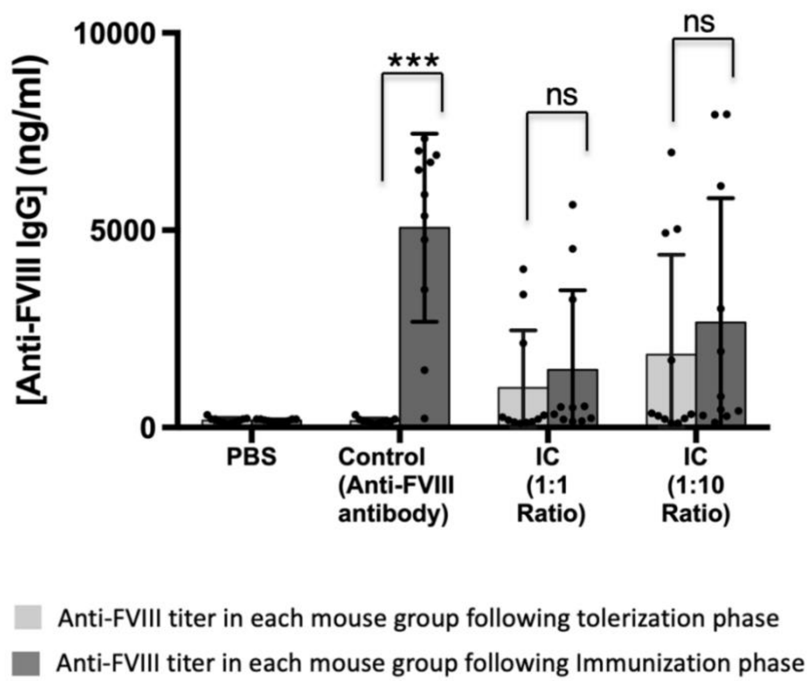

Figure 20. Anti-FVIII IgG titer after Tolerization phase and Immunization phase

4.2.2 Comparing Levels of Anti-FVIII IgG development between Monoclonal and Polyclonal FVIII-ICs groups

Compared to the polyclonal group, the monoclonal anti-FVIII IgG experiment demonstrated a higher incidence of mice that did not develop antibodies in the 1:1 and 1:10 ratios. In the polyclonal group, 65.3% of mice that received rhFVIII-ICs at a (1:1) ratio did not develop anti-FVIII IgG. In contrast, 50% of mice administered rhFVIII-ICs at a (1:10) ratio did not produce anti-FVIII IgG in this group. However, in the monoclonal group, 74.6% of mice that received rhFVIII-ICs at a (1:1) ratio and 63% of mice that received rhFVIII-ICs at a (1:10) ratio did not develop anti-FVIII IgG. This finding suggests that monoclonal antibody treatment may have an advantage in suppressing antibody production compared to its polyclonal counterpart (Figure 21).

Figure 21. Comparison of Monoclonal and polyclonal study groups regarding the percentage of mice without detectable anti-FVIII IgG. A significant difference in the levels of anti-FVIII IgG was shown in mice (both the IC (1:1) and (1:10) ratio groups) that received rhFVIII-ICs made by monoclonal antibodies, as opposed to those administered rhFVIII-ICs derived from polyclonal antibodies. n=15. Statistical significance was set at $p < 0.05$. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$, non-significant. ns).

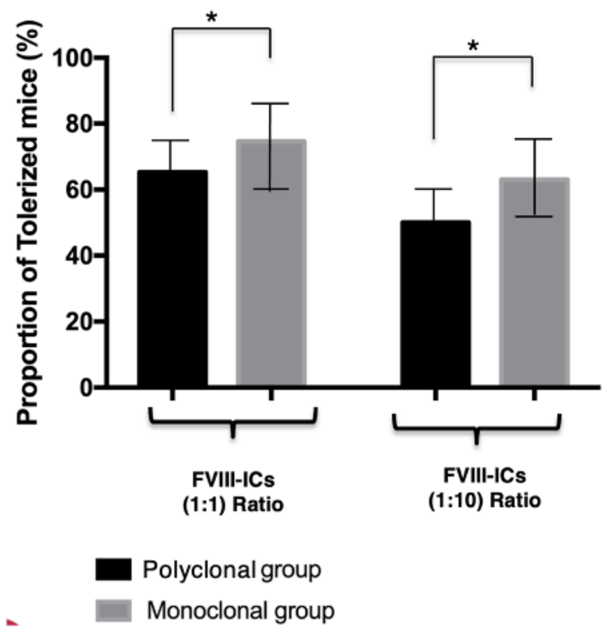

Figure 21. Comparison between Monoclonal and polyclonal study groups in terms of the percentage of mice without detectable amount of ant-FVIII antibody

4.3 IgG subtypes ELISA

Different types of ELISA were run to quantify the levels of IgG1, IgG2a, IgG2b, and IgG3 antibodies. Our findings revealed that IgG1 exhibited the highest antibody concentration among all subclasses (mean: 15,660 ng/ml), followed by IgG2a as the second most abundant (mean: 8,954.415 ng/ml). IgG2b (mean: 582.01 ng/ml) and IgG3 (mean: 172.17 ng/ml) were present in much lower amounts. We observed elevated levels of anti-FVIII antibodies and non-tolerized mice groups compared to the tolerize groups (Figure 22).

Figure 22. IgG subtypes ELISA. ELISA technique was used to determine IgG sub-class concentrations in mouse plasma samples. Kovaltry was diluted to 10 ng/μL. After a 2-hour incubation, the plate was washed and blocked with 5% milk. Both standards and samples were added to the plate in duplicate and incubated overnight. Detecting antibodies were added, followed by TMB for color development. Absorbance was measured at 450 nm, and the standard curve was generated using SigmaPlot.

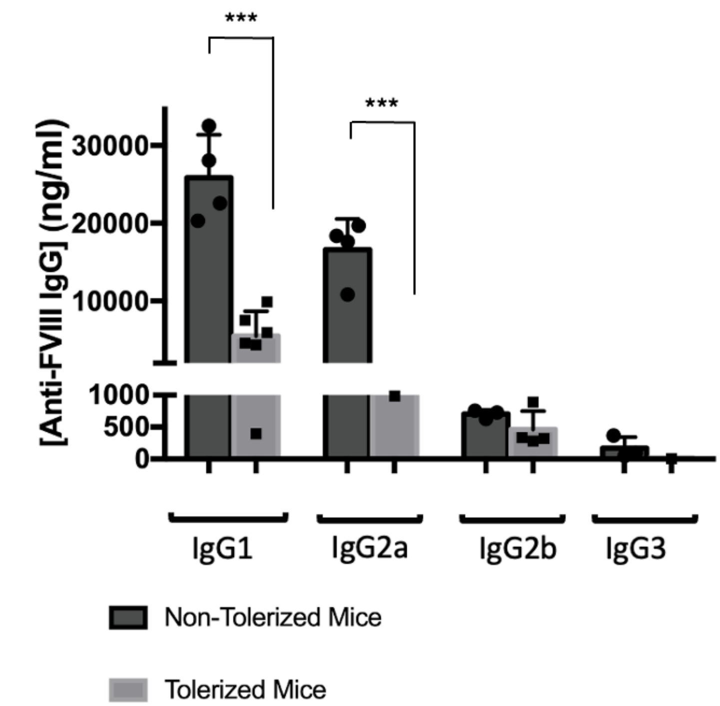

Figure 22. IgG subtypes ELISA

4.4 Blocking CD32b

Before each weekly 1:1 ratio injection of rhFVIII-IC during the tolerization phase, we injected Anti-mouse CD32b and Isotype control 24 hours in advance. Then, we collected blood. Then, the mice received 25 IU/kg of Kovaltry retro-orbitally once a week for an additional four weeks. Following this, we did another retro-orbital blood collection and measured the levels of anti-FVIII antibodies by ELISA. The result showed that 70% of mice in the Isotype group did not develop anti-FVIII IgG (mean increase of 781.3560 ng/mL, p-value = 0.3642). However, in the group in which CD32b was blocked before the injection of rhFVIII-IC, only 40% did not produce anti-FVIII IgG (mean increase of 3841.9550 ng/mL, p-value = 0.1194). These results suggest that CD32b plays a pivotal role in transmitting inhibitory signals to B cells, thereby regulating the production of anti-FVIII IgG (Figure 23).

Figure 23. Anti-FVIII IgG titer in Isotype and CD32b groups. Anti-mouse CD32b was administered via IP injection to block CD32b 24 hours before each 1:1 ratio of rhFVIII-IC injection. The control group was given an Isotype control in the same manner. Throughout the 4-week tolerization phase, FVIII-ICs were administered once a week. Retro-orbital blood samples were then collected to measure anti-FVIII IgG. Subsequently, during the immunization phase, we injected 25 IU/kg Kovaltry, followed by retro-orbital blood sample collection to measure antibody levels. The plate was coated with Kovaltry at a of 10 ng/µL concentration for the ELISA. This was followed by subsequent steps, including blocking with 5% milk, adding both standard and sample solutions, and an additional round of incubation. Next, the goat anti-mouse IgG (H&L) alkaline phosphatase-conjugated detecting antibody at a concentration of 1mg/mL was added, and the P-NPP substrate at a concentration of 1mg/ml was used to initiate color development. Concentrations were determined using spectrophotometry, precisely measuring absorbance at 405 nm. The standard curve was established using the 4-parameter logistic regression model in SigmaPlot. Results were presented as mean (SD) and analyzed using an unpaired t-test, where a p-value of 0.05 was deemed statistically significant (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. non-significant. ns).

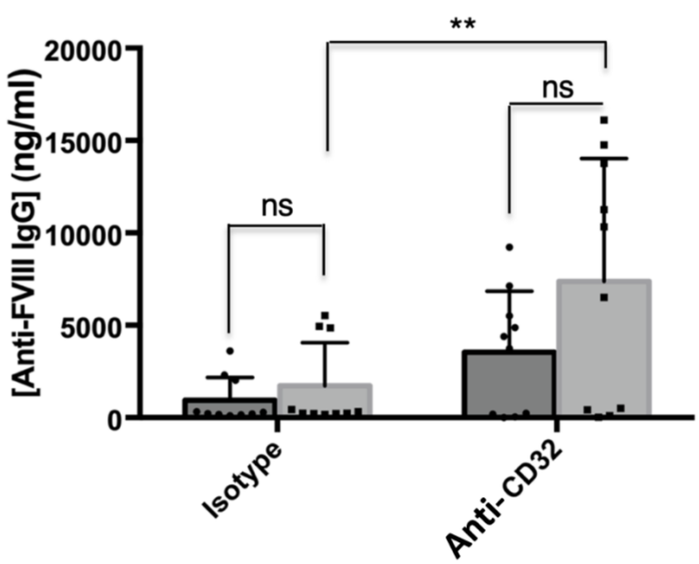

Figure 23. Anti-FVIII IgG titer in Isotype and anti-CD32b groups

Anti-FVIII titer in each mouse group following tolerization phase

Anti-FVIII titer in each mouse group following Immunization phase

4.5 Bethesda Assay Result

The Bethesda assay results indicated varying inhibitor levels across our three mouse groups: the PBS or negative control group, the tolerized mice group, and the non-tolerized mice group. Based on our findings, the PBS group exhibited an inhibitor titer below 0.5 BU/mL. In comparison, the tolerized mice displayed 1 BU/mL, and the non-tolerized group showed a significantly higher titer of 73.8 BU/mL (Figure 24).

Figure 24. Measurement of inhibitor titer by Bethesda Assay. Inhibitor titers were measured by Bethesda assay in the three groups of mice (PBS, Tolerized, and Non-Tolerized) after the booster shot. A single Bethesda Unit (Bu) is the amount of an inhibitor needed to neutralize half of one unit of FVIII:C in normal plasma after a 2-hour incubation at 37°C.

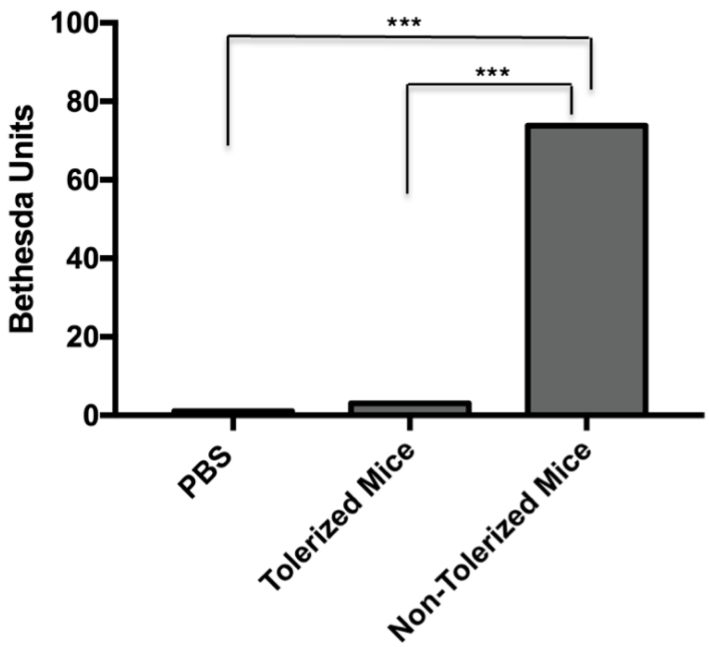

Figure 24. Measurement of inhibitor titer by Bethesda Assay

5 Chapter V – DISCUSSION

Specific immune complexes will form and increase in the bloodstream by interacting with a particular antibody and antigen (Heyman, 2003). These immune complexes could attach to the B cell and inhibitory CD32b receptors (Heyman, 2003). CD32b is the only Fc receptor found on B cells (Amigorena et al., 1992). The interaction between immune complexes and CD32b can lead to the suppression of specific B cells and the inhibition of antibody production (Heyman, 2003). This inhibitory effect is achieved through the phosphorylation of the ITIM on CD32b, which recruits SH2-containing inositol SHIP to counteract the intracellular calcium mobilization induced by ITAM (Kiener et al., 1997).

The role of CD32b in B cell suppression was demonstrated in studies with mice lacking CD32b. These mice were unable to control the immune response, which could lead to a higher risk of collagen-induced arthritis (Yuasa et al., 1999), manifesting conditions resembling lupus (Fukuyama et al., 2005), and Goodpasture’s syndrome (Akira Nakamura et al., 2000).

Several studies have shown the relationship between CD32b dysfunction and human autoimmune diseases. For instance, polymorphism in the promoter (Blank et al., 2005) and transmembrane domain (Chen et al., 2006) of CD32b have been associated with a higher risk of systemic lupus erythematosus (SLE). Moreover, Mackay’s study demonstrated that there is a decreased level of CD32b on the B cell surface in SLE patients, resulting in disrupted calcium signaling (Mackay et al., 2006).

In another study, Eckhardt and colleagues showed the association between the FcγRIIa R131H polymorphism and inhibitor development in severe Hemophilia A

(Eckhardt et al., 2014). They found that the R131H polymorphism is a significant risk factor for inhibitor development in Hemophilia A patients, particularly those with a family history of inhibitors (Eckhardt et al., 2014).

This study investigated the potential role of administering rhFVIII-ICs in suppressing antibody production in a Hemophilia A mouse model. The hypothesis is that the rhFVIII-ICs could prevent anti-FVIII IgG development when the mice are later challenged with Kovaltry.

The rhFVIII-IC ELISA results in this project showed the importance of anti-FVIII IgG and rFVIII concentrations and ratios required for rhFVIII-ICs formation. We measured the levels of anti-FVIII IgG concentrations in mouse plasma using an ELISA to investigate the effects of (1:1) and (1:10) ratios of rhFVIII-ICs injection on suppressing anti-FVIII IgG production. Anti-FVIII IgG concentrations were measured after the tolerization and immunization phases to compare anti-FVIII IgG titers between and within groups. This experiment was run to assess the impact of rhFVIII-ICs on developing anti-FVIII antibodies.

Among our experimental groups, all the mice in the positive control group, which received Kovaltry mixed with naïve plasma during the tolerization phase, had developed anti-FVIII IgG after the immunization phase. On the other hand, none of the mice in the negative control group, which had been injected with PBS during both the tolerization and immunization phases, produced anti-FVIII antibodies. The results from these two groups indicate that Kovaltry can stimulate the immune system, leading to the activation of FVIII-specific B cells that produce anti-FVIII IgG. In addition, our study showed that in the 1:1

and 1:10 ratio groups using rFVIII-Polyclonal anti-FVIII ICs, 68% of the mice in the 1:1 ratio group did not develop inhibitors. In comparison, 50% of the mice in the 1:10 ratio group did not produce inhibitors. These findings could suggest the effect of rhFVIII-ICs injection in preventing the immune response in the Hemophilia A mice.

Compared to the polyclonal study in which we used polyclonal anti-FVIII IgG, our study demonstrated lower levels of anti-FVIII IgG when we generated rFVIII-ICs using the anti-monoclonal anti-FVIII IgG.

Monoclonal antibodies can increase specificity and reduce cross-reactivity because they are derived from a single cell line, allowing them to target a particular antigen with high specificity. On the other hand, several B cell clones develop various types of polyclonal antibodies. These antibodies target different epitopes on a specific antigen, which can increase the risk of cross-reactivity with unintended antigens.

One significant factor to consider is that when using monoclonal anti-FVIII IgG for forming immune complexes, the rhFVIII-ICs are typically composed of a single antibody attached to an FVIII antigen, possibly with the inclusion of two FVIII molecules. In contrast, rFVIII-ICs humans using polyclonal anti-FVIII IgG can result in a variable binding pattern, where some rhFVIII antigens may be bound by multiple anti-FVIII antibodies, while others may be bound by only a few or even none. This distinction demonstrates the merit of monoclonal anti-FVIII IgG in generating more uniform and predictable rFVIII-ICs.

In the next phase of our study, we examined whether the mice stayed tolerant and were prevented from producing anti-FVIII antibodies after the injection of rFVIII-ICs. Thus, we allowed them a two-week rest before administering a single booster shot. Although our results demonstrated that there was no significant difference in the levels of anti-FVIII antibodies after the booster shot, indicating that the mice's immune system could suppress the production of anti-FVIII antibodies against the injected booster shot, our experiment needs to be continued for at least six months to obtain more reliable and long-lasting tolerization results in the mice following the administration of rFVIII-ICs.

The possible mechanism behind the role of rFVIII- ICs in inhibiting B cells is through CD32b on the surface of the FVIII-specific B cells. Thus, we decided to examine the influence of this receptor in the development of anti-FVIII IgG.

When we blocked CD32b using anti-CD32b, a significant contrast was found between the isotype group (control) and the group where CD32b was blocked. More precisely, 70% of the mice in the isotype group showed no development of anti-FVIII IgG. In contrast, when CD32b was blocked before the injection of rhFVIII-IC, only 40% of the mice did not show a detectable amount of anti-FVIII IgG. These findings emphasized the role of CD32b in transmitting inhibitory signals to B cells, which can lead to the regulation of anti-FVIII IgG production.

Chu and colleagues found that the CD32b and BCR co-engagement using a singular antibody can suppress the immune complexes's function in modulating B cell activation. According to their result, this antibody demonstrated a suppressive effect on BCR-induced calcium mobilization and the proliferative activity of B cells (Chu et al., 2008). They have shown that this antibody activated the SHIP signaling pathway while neutralizing BCR-mediated survival signals, ultimately inducing apoptosis in activated B cells (Chu et al., 2008).

Our study showed results similar to those of Chu et al. However, the difference was that we used the produced polyclonal ant-FVIII IgG or monoclonal Anti-FVIII IgG for immune complex production and triggered the CD32b on the surface of the B cells.

In another study, Whelan et al. found that IgG4 and IgG1 are the most common subclasses of anti-FVIII IgG antibodies in patients with FVIII inhibitors (Whelan et al., 2013).

Furthermore, additional studies have demonstrated murine IgG1 exhibiting functional affinities like human IgG4 (Lilienthal et al., 2018) and the mouse IgG2a antibody, often regarded as functionally comparable to human IgG1 antibodies (Shekhar et al., 2019).

In the next step, we were curious to observe the ratio of different anti-FVIII IgG subtypes in our mouse model. According to our results, IgG1 and IgG2a were the dominant subtype antibodies, which aligns with the findings of the Whelan study.

Our results reveal notable differences in inhibitor titers across the experimental groups. Specifically, the PBS-treated group demonstrated an inhibitor titer of less than 0.5 BU/mL, whereas the tolerized mice exhibited 1 BU/mL titer. Strikingly, the non-tolerized group displayed a substantially higher titer, measuring 73.8 BU/mL.

In conclusion, our study revealed evidence supporting the effect of a 1:1 balanced ratio of rhFVIII-ICs in suppressing anti-FVIII antibody production. It is a significant breakthrough in our understanding of the immune response to FVIII, as it showed antibody feedback as a critical player in this process, actively suppressing FVIII-specific B cell activity.

Furthermore, our investigation indicated the pivotal role of CD32b in modulating anti-FVIII IgG production. These findings highlight the significant role of CD32b in transmitting inhibitory signals to B cells, thereby regulating the production of anti-FVIII IgG.

Our study shows that using rhFVIII-ICs to target CD32b presents a promising avenue for future therapeutic interventions to mitigate the immune response to FVIII.

6 Future Direction

As a future direction, we suggest using CD32b knock-out mice and comparing the results with the present study to observe any difference in blocking CD32b with an antibody or using CD32b knock-out mice.

It would also be worthwhile to assess the cytokine profile of B cells in both tolerized and non-tolerized mice, with a particular focus on key inflammatory cytokines like TGF-beta and non-inflammatory cytokines like IL-10. Investigating how these cytokines modulate B cell responses can provide vital insights into immune tolerance mechanisms.

We used plasma to obtain polyclonal anti-FVIII IgG. Since plasma contains other antibodies, it might influence the results, particularly regarding potential cross-reactivity with CD32b. Therefore, purifying the anti-FVIII IgG and utilizing this antibody for producing rh-FVIII would be prudent.

7 Limitations

While invaluable for advancing scientific understanding, mice studies have several inherent limitations.

Firstly, there can be a need for more direct translatability to humans due to differences in biology, genetics, and physiology between the two species.

Additionally, ethical considerations and practical constraints can limit the scope and duration of experiments in mice. For instance, certain long-term studies or interventions may not be feasible or ethical in mice.

We need to extend the experiment beyond 12 weeks to confirm if tolerance is established and keep the mice alive for at least six months. This might be challenging for Hemophilia A mice. Moreover, because of the limitation in AUP, we were not allowed to do more than 12 injections.

Another limitation is the relatively short half-life of anti-CD32b, which ranges from 4 to 6 days. This means it rapidly diminishes in the bloodstream, making it impractical to effectively block CD32b and administer immune complexes a week or more later. This aspect can present a challenge, especially when working with Hemophilia A mice and necessitating swift injections.

Another limitation to consider is the occurrence of fatal internal bleeding due to inter-mouse aggression. To mitigate this issue, a potential strategy could involve housing the mice in smaller, compatible groups within each cage.

8 Chapter VI- References

FAbdulqader, A. M. R., Mohammed, A. I., Rachid, S., Ghoraishizadeh, P., & Mahmood, S. N. (2020). Identification of the Intron 22 and Intron 1 Inversions of the Factor VIII Gene in Iraqi Kurdish Patients With Hemophilia A. *Clinical and Applied Thrombosis/Hemostasis*, *26*. https://doi.org/10.1177/1076029619888293

Akira Nakamura, B., Yuasa, T., Ujike, A., Ono, M., Nukiwa, T., Ravetch, J. V, & Takai, T. (2000). Fc Receptor IIB-deficient Mice Develop Goodpasture's Syndrome upon Immunization with Type IV Collagen: A Novel Murine Model for Autoimmune Glomerular Basement Membrane Disease. In *J. Exp. Med*, 191(5). http://www.jem.org/cgi/current/full/191/5/899

Amigorena, S., Bonnerot, C., Drake, J. R., Choquet, D., Hunziker, W., & Guillet, J.-G. (1992). Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. In *From: Science*, 256.

Antonarakis, S. E., Rossiter, J. P., Young, M., Horst, J., De Moerloose, P., Sommer, S. S., Ketterling, R. P., Kazazian, H. H., Negrier, C., Vinciguerra, C., Gitschier, J., Goossens, M., Girodon, E., Ghanem, N., Plassa, F., Lavergne, J. M., Vidaud, M., Costa, J. M., Laurian, Y., … Lnaba, H. (1995). Factor VI11 Gene Inversions in Severe Hemophilia A: Results of an International Consortium Study. *Blood, 86(6), 2206-12.*

Astermark, J., Altisent, C., Batorova, A., Diniz, M. J., Gringeri, A., Holme, P. A., Karafoulidou, A., Lopez-Fernández, M. F., Reipert, B. M., Rocino, A., Schiavoni, M., von Depka, M., Windyga, J., & Fijnvandraat, K. (2010). Non-genetic risk factors and the development of inhibitors in haemophilia: A comprehensive review and consensus report. *Haemophilia*, *16*(5), 747–766. https://doi.org/10.1111/j.1365-2516.2010.02231.x

Astermark, J., Lacroix-Desmazes, S., & Redingà, M. T. (2008). Inhibitor Development. *Hemophilia,* 3, 36-42. http://hapmap.org/

Baerenwaldt, A., Lux, A., Danzer, H., Spriewald, B. M., Ullrich, E., Heidkamp, G., Dudziak, D., & Nimmerjahn, F. (2011). Fcγ receptor IIB (FcγRIIB) maintains humoral tolerance in the human immune system in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(46), 18772–18777. https://doi.org/10.1073/pnas.1111810108

Barrowcliffe, T. W. (2004). Monitoring haemophilia severity and treatment: new or old laboratory tests?. Hemophilia, 4, 109-14.

Blank, M. C., Stefanescu, R. N., Masuda, E., Marti, F., King, P. D., Redecha, P. B., Wurzburger, R. J., Peterson, M. G. E., Tanaka, S., & Pricop, L. (2005). Decreased transcription of the human FCGR2B gene mediated by the -343 G/C promoter polymorphism and association with systemic lupus erythematosus. *Human Genetics*, *117*(2–3), 220–227. https://doi.org/10.1007/s00439-005-1302-3

Castaldo, G., D'Argenio, V., Nardiello, P., Zarrilli, F., Sanna, V., Rocino, A., Coppola, A., Di Minno, G., & Salvatore, F. (2007). Haemophilia A: Molecular insights. In *Clinical Chemistry and Laboratory Medicine*, 45(4), 450–461. https://doi.org/10.1515/CCLM.2007.093

Chalmers, E. A., Williams, M., Brennand, J., Liesner, R., Collins, P., & Richards, M. (2011). Guideline on the management of haemophilia in the fetus and neonate. *British Journal of Haematology*, *154*(2), 208–215. https://doi.org/10.1111/j.1365-2141.2010.08545.x

Chavin, S. I. (1984). Factor VIII: Structure and in Blood Clotting Function. In *American Journal of Hematology*, 16(3), 297-306.

Chen, J. Y., Wang, C. M., Ma, C. C., Luo, S. F., Edberg, J. C., Kimberly, R. P., & Wu, J. (2006). Association of a transmembrane polymorphism of Fcγ receptor IIb (FCGR2B) with systemic lupus erythematosus in Taiwanese patients. *Arthritis and Rheumatism*, *54*(12), 3908–3917. https://doi.org/10.1002/art.22220

Chu, S. Y., Vostiar, I., Karki, S., Moore, G. L., Lazar, G. A., Pong, E., Joyce, P. F., Szymkowski, D. E., & Desjarlais, J. R. (2008). Inhibition of B cell receptor-mediated activation of primary human B cells by coengagement of CD19 and FcγRIIb with Fc-engineered antibodies. *Molecular Immunology*, *45*(15), 3926–3933. https://doi.org/10.1016/j.molimm.2008.06.027

den Uijl, I. E. M., Mauser Bunschoten, E. P., Roosendaal, G., Schutgens, R. E. G., Biesma, D. H., Grobbee, D. E., & Fischer, K. (2011). Clinical severity of haemophilia A: Does the classification of the 1950s still stand? *Haemophilia*, *17*(6), 849–853. https://doi.org/10.1111/j.1365-2516.2011.02539.x

Donald, B. (1969). Immunologic Characterization of 12 Factor VIII Inhibitors. *Blood, 34(1), 85-90.*

Dorgalaleh, A., Dadashizadeh, G., & Bamedi, T. (2016). Hemophilia in Iran. *Hematology*, *21*(5), 300–310. https://doi.org/10.1080/10245332.2015.1125080

Duncan, E., Collecutt, M., & Street, A. (2013). Nijmegen-bethesda assay to measure factor VIII inhibitors. *Methods in Molecular Biology*, *992*, 321–333. https://doi.org/10.1007/978-1-62703-339-8_24

Eckhardt, C. L., Astermark, J., Nagelkerke, S. Q., Geissler, J., Tanck, M. W. T., Peters, M., Fijnvandraat, K., & Kuijpers, T. W. (2014). The Fc gamma receptor IIa R131H polymorphism is associated with inhibitor development in severe hemophilia A. *Journal of Thrombosis and Haemostasis*, *12*(8), 1294–1301. https://doi.org/10.1111/jth.12631

Fay, P. J. (2006). Factor VIII structure and function. In *International Journal of Hematology*, 83(2), 103–108. https://doi.org/10.1532/IJH97.05113

Franchini, M., & Mannucci, P. M. (2014). The history of hemophilia. In *Seminars in Thrombosis and Hemostasis*, 40(5), 571–576. https://doi.org/10.1055/s-0034-1381232

Fukuyama, H., Nimmerjahn, F., & Ravetch, J. V. (2005). The inhibitory Fcγ receptor modulates autoimmunity by limiting the accumulation of immunoglobulin G+ anti-DNA plasma cells. *Nature Immunology*, *6*(1), 99–106. https://doi.org/10.1038/ni1151

Graw, J., Brackmann, H. H., Oldenburg, J., Schneppenheim, R., Spannagl, M., & Schwaab, R. (2005). Haemophilia A: From mutation analysis to new therapies. In *Nature Reviews Genetics*, 6(6), 488–501. https://doi.org/10.1038/nrg1617

Grover, S. P., & Mackman, N. (2019). Intrinsic pathway of coagulation and thrombosis: Insights from animal models. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *39*(3), 331–338. https://doi.org/10.1161/ATVBAHA.118.312130

Grushin, K., Miller, J., Dalm, D., Parker, E. T., Healey, J. F., Lollar, P., & Stoilova-McPhie, S. (2014). Lack of recombinant factor VIII B-domain induces phospholipid vesicle aggregation: implications for the immunogenicity of factor VIII. *Haemophilia : The Official Journal of the World Federation of Hemophilia*, *20*(5), 723–731. https://doi.org/10.1111/hae.12421

Halimeh, S., Bidlingmaier, C., Heller, C., Gutsche, S., Holzhauer, S., Kenet, G., Kurnik, K., Manner, D., Iorio, A., & Nowak-Göttl, U. (2013). Risk factors for high-titer inhibitor development in children with hemophilia A: Results of a cohort study. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/901975

Herzog, R. W., Kuteyeva, V., Saboungi, R., Terhorst, C., & Biswas, M. (2019). Reprogrammed CD4+ T cells that express Foxp3+ control inhibitory antibody formation in hemophilia A mice. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.00274

Heyman, B. (2003). Feedback regulation by IgG antibodies. *Immunology Letters*, *88*(2), 157–161. https://doi.org/10.1016/S0165-2478(03)00078-6

Kamal, A. H., Tefferi, A., & Pruthi, R. K. (2007). How to interpret and pursue an abnormal prothrombin time, activated partial thromboplastin time, and bleeding time in adults. In *Mayo Clinic Proceedings*, 82(7), 864–873. Elsevier Ltd. https://doi.org/10.4065/82.7.864

Kasahara, Y., Shirota, H., Umegaki, S., & Ishioka, C. (2019). Contribution of Fcγ receptor IIB to creating a suppressive tumor microenvironment in a mouse model. *Cancer Immunology, Immunotherapy*, *68*(11), 1769–1778. https://doi.org/10.1007/s00262-019-02413-w

Kempton, C. L., Soucie, J. M., Miller, C. H., Hooper, C., Escobar, M. A., Cohen, A. J., Key, N. S., Thompson, A. R., & Abshire, T. C. (2010). In non-severe hemophilia A the risk of inhibitor after intensive factor treatment is greater in older patients: A case-control study. *Journal of Thrombosis and Haemostasis*, *8*(10), 2224–2231. https://doi.org/10.1111/j.1538-7836.2010.04013.x

Kiener, P. A., Lioubin, M. N., Rohrschneider, L. R., Ledbetter, J. A., Nadler, S. G., & Diegel, M. L. (1997). Co-ligation of the antigen and Fc receptors gives rise to the selective modulation of intracellular signaling in B cells: Regulation of the association of phosphatidylinositol 3-kinase and inositol 5-phosphatase with the antigen receptor complex. *Journal of Biological Chemistry*, *272*(6), 3838–3844. https://doi.org/10.1074/jbc.272.6.3838

Lilienthal, G. M., Rahmöller, J., Petry, J., Bartsch, Y. C., Leliavski, A., & Ehlers, M. (2018). Potential of murine IgG1 and Human IgG4 to inhibit the classical complement and Fcγ receptor activation pathways. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.00958

Lippi, G., & Favaloro, E. J. (2019). Emicizumab (ACE910): Clinical background and laboratory assessment of hemophilia A. In *Advances in Clinical Chemistry*, 88, 151–167. Academic Press Inc. https://doi.org/10.1016/bs.acc.2018.10.003

Mackay, M., Stanevsky, A., Wang, T., Aranow, C., Li, M., Koenig, S., Ravetch, J. V., & Diamond, B. (2006). Selective dysregulation of the FcγIIB receptor on memory B cells in SLE. *Journal of Experimental Medicine*, *203*(9), 2157–2164. https://doi.org/10.1084/jem.20051503

Mackman, N., Tilley, R. E., & Key, N. S. (2007). Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. In *Arteriosclerosis, Thrombosis, and Vascular Biology*, 27(8), 1687–1693. https://doi.org/10.1161/ATVBAHA.107.141911

Mahmoud Abu Arra, C., Samarah, F., & Sudqi Abu Hasan, N. (2020). Factor VIII Intron 22 Inversion in Severe Hemophilia A Patients in Palestine. *Scientifica*, *2020*. https://doi.org/10.1155/2020/3428648

Mammen, E. F., Editor, G., Berntorp, E., & Thompson, A. R. (2003). Modern Management of Hemophilia A to Prevent Bleeding and Arthropathy; Editor in Chief Structure and Function of the Factor VIII Gene and Protein. In *Seminars in Thrombosis and Hemostasis*, 29(1).

Mancardi, D., & Daëron, M. (2014). Fc Receptors in Immune Responses. In *Reference Module in Biomedical Sciences*. Elsevier. https://doi.org/10.1016/b978-0-12-801238-3.00119-7

Margaglione, M., & Intrieri, M. (2018). Genetic Risk Factors and Inhibitor Development in Hemophilia: What Is Known and Searching for the Unknown. *Seminars in Thrombosis and Hemostasis*, *44*(6), 509–516. https://doi.org/10.1055/s-0038-1660816

Marijke van den Berg, H. (2016). Preventing bleeds by treatment: new era for haemophilia changing the paradigm. *Haemophilia*, *22*, 9–13. https://doi.org/10.1111/hae.12993

Mertens, K., Cupers, R., & Bertinat, R. M. (1984). Binding of human blood-coagulation Factors IXa and X to phospholipid membranes. In *Biochem. J*, 223.

Millner, A. H., Tiefenbacher, S., Robinson, M., & Boesen, H. T. (2016). A variation of the Nijmegen-Bethesda assay using heat or a novel heat/cold pretreatment for the detection of FIX inhibitors in the presence of residual FIX activity. *International Journal of Laboratory Hematology*, *38*(6), 639–647. https://doi.org/10.1111/ijlh.12552

Monagle, P., & Massicotte, P. (2011). Developmental haemostasis: Secondary haemostasis. In *Seminars in Fetal and Neonatal Medicine*, 16(6), 294–300. https://doi.org/10.1016/j.siny.2011.07.007

Nimmerjahn, F., & Ravetch, J. V. (2006). Fcγ receptors: Old friends and new family members. In *Immunity*, 24(1), 19–28. https://doi.org/10.1016/j.immuni.2005.11.010

Oldenburg, J., Mahlangu, J. N., Kim, B., Schmitt, C., Callaghan, M. U., Young, G., Santagostino, E., Kruse-Jarres, R., Negrier, C., Kessler, C., Valente, N., Asikanius, E., Levy, G. G., Windyga, J., & Shima, M. (2017). Emicizumab Prophylaxis in Hemophilia A with Inhibitors. *New England Journal of Medicine*, *377*(9), 809–818. https://doi.org/10.1056/nejmoa1703068

Ortel, T. L. (2019a). Introduction to a review series on new therapeutics for inherited and acquired bleeding conditions. In *Blood*, 133(5), 383–384. https://doi.org/10.1182/blood-2018-12-877779

Ortel, T. L. (2019b). Introduction to a review series on new therapeutics for inherited and acquired bleeding conditions. In *Blood*, 133(5), 383–384. https://doi.org/10.1182/blood-2018-12-877779

Páramo, J. A. (2021). Treatment of haemophilia: From replacement to gene therapy. In *Medicina Clinica*, 157(12), 583–587. https://doi.org/10.1016/j.medcli.2021.04.031

Pavlova, A., Delev, D., Lacroix-Desmazes, S., Schwaab, R., Mende, M., Fimmers, R., Astermark, J., & Oldenburg, J. (2009). Impact of polymorphisms of the major histocompatibility complex class II, interleukin-10, tumor necrosis factor-α and cytotoxic T-lymphocyte antigen-4 genes on inhibitor development in severe hemophilia A. *Journal of Thrombosis and Haemostasis*, *7*(12), 2006–2015. https://doi.org/10.1111/j.1538-7836.2009.03636.x

Perrin, G. Q., Herzog, R. W., & Markusic, D. M. (2019). *Review Series NEW THERAPEUTICS FOR INHERITED AND ACQUIRED BLEEDING CONDITIONS Update on clinical gene therapy for hemophilia*.

Peyvandi, F., & Garagiola, I. (2018). Product type and other environmental risk factors for inhibitor development in severe hemophilia A. *Research and Practice in Thrombosis and Haemostasis*, *2*(2), 220–227. https://doi.org/10.1002/rth2.12094

Peyvandi, F., Garagiola, I., & Young, G. (2016). The past and future of haemophilia: diagnosis, treatments, and its complications. In *The Lancet*, 388, 187–197. https://doi.org/10.1016/S0140-6736(15)01123-X

Pittman, D. D., Alderman, E. M., Tomkinson, K. N., Wang, J. H., Giles, A. R., & Kaufman, R. J. (1993). Biochemical, Immunological, and In Vivo Functional Characterization of B-Domain-Deleted Factor VI11. In *Blood*, 81(1). https://ashpublications.org/blood/article-pdf/81/11/2925/609734/2925.pdf

Raghavan, M., & Bjorkman, P. J. (1996). Fc RECEPTORS AND THEIR INTERACTIONS WITH IMMUNOGLOBULINS. In *Annual Reviews RAGACHPT.DUN*, 1. www.annualreviews.org

Ravetch1, V., & Kinet, P. (1991). *Fc RECEPTORS*. www.annualreviews.org

Ref47. (n.d.).

Ronayne, E. K., Peters, S. C., Gish, J. S., Wilson, C., Spencer, H. T., Doering, C. B., Lollar, P., Spiegel, P. C., & Childers, K. C. (2021). Structure of Blood Coagulation

Factor VIII in Complex With an Anti-C2 Domain Non-Classical, Pathogenic Antibody Inhibitor. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.697602

Santagostino, E. (2014). A new recombinant factor VIII: From genetics to clinical use. In *Drug Design, Development and Therapy*, 8, 2507–2515. https://doi.org/10.2147/DDDT.S73241

Schep, S. J., Schutgens, R. E. G., Fischer, K., & Boes, M. L. (2018). Review of immune tolerance induction in hemophilia A. In *Blood Reviews*, 32(4), 326–338. https://doi.org/10.1016/j.blre.2018.02.003

Scott, D. W. (2022). Until programmed death do us tolerant. *Journal of Clinical Investigation*, *132*(22). https://doi.org/10.1172/JCI164858

Shekhar, S., Khan, R., Khan, A. U. R., & Petersen, F. C. (2019). Mouse IgG2a Antibodies Specific for the Commensal Streptococcus mitis Show Stronger Cross-Reactivity with Streptococcus pneumoniae than IgG1 Antibodies. *Journal of Immunology Research*, *2019*. https://doi.org/10.1155/2019/7906724

Stassen, J. M., Arnout, J., & Deckmyn, H. (2004). The Hemostatic System. In *Current Medicinal Chemistry*, 11.

Tiede, A., Werwitzke, S., & Scharf, R. E. (2014). Laboratory Diagnosis of Acquired Hemophilia A: Limitations, Consequences, and Challenges. In *Seminars in Thrombosis and Hemostasis*, 40(7), 803–811. https://doi.org/10.1055/s-0034-1390004

van Dijk, K., Fischer, K., van der Bom, J. G., Grobbee, D. E., & van den Berg, H. M. (2005a). Variability in clinical phenotype of severe haemophilia: The role of the first joint bleed. *Haemophilia*, *11*(5), 438–443. https://doi.org/10.1111/j.1365-2516.2005.01124.x

van Dijk, K., Fischer, K., van der Bom, J. G., Grobbee, D. E., & van den Berg, H. M. (2005b). Variability in clinical phenotype of severe haemophilia: The role of the first joint bleed. *Haemophilia*, *11*(5), 438–443. https://doi.org/10.1111/j.1365-2516.2005.01124.x

Verbruggen, B., Van Heerde, W. L., & Laros-Van Gorkom, B. A. P. (2009). Improvements in factor VIII inhibitor detection: From Bethesda to Nijmegen. In *Seminars in Thrombosis and Hemostasis*, 35(8), 752–759. https://doi.org/10.1055/s-0029-1245107

Wang, W., Wang, Y. J., & Kelner, D. N. (2003). Coagulation factor VIII: Structure and stability. In *International Journal of Pharmaceutics*, 259, 1–15. https://doi.org/10.1016/S0378-5173(03)00227-8

Whelan, S. F. J., Hofbauer, C. J., Horling, F. M., Allacher, P., Wolfsegger, M. J., Oldenburg, J., Male, C., Windyga, J., Tiede, A., Schwarz, H. P., Scheiflinger, F., & Reipert, B. M. (2013). Distinct characteristics of antibody responses against factor VIII in healthy individuals and in different cohorts of hemophili A patients. *Blood*, *121*(6), 1039–1048. https://doi.org/10.1182/blood-2012-07-444877

Yakovlev, S., & Medved, L. (2009). Interaction of fibrin(ogen) with the endothelial cell receptor VE-cadherin: Localization of the fibrin-binding site within the third extracellular VE-cadherin domain. *Biochemistry*, *48*(23), 5171–5179. https://doi.org/10.1021/bi900487d

Yuasa, T., Kubo, S., Yoshino, T., Ujike, A., Matsumura, K., Ono, M., Ravetch, J. V, & Takai, T. (1999). Deletion of Fc Receptor IIB Renders H-2 b Mice Susceptible to Collagen-induced Arthritis. In *J. Exp. Med* , 189(1). http://www.jem.org

ZemaniFodil, F. (2014a). Factor 8 Gene Mutations and Risk of Inhibitor Development in Hemophilia A Algerian Patients. *Journal of Pharmacogenomics & Pharmacoproteomics*, *05*(01). https://doi.org/10.4172/2153-0645.1000124

ZemaniFodil, F. (2014b). Factor 8 Gene Mutations and Risk of Inhibitor Development in Hemophilia A Algerian Patients. *Journal of Pharmacogenomics & Pharmacoproteomics*, *05*(01). https://doi.org/10.4172/2153-0645.1000124