LOW ZONE TOLERANCE INDUCTION TO COAGULATION FACTOR VIII IN A HEMOPHILIA A MOUSE MODEL

LOW ZONE TOLERANCE INDUCTION TO COAGULATION FACTOR VIII IN A HEMOPHILIA A MOUSE MODEL

BY: WAJEEHA ZAHEER, B.Sc. (Honours)

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AUTHOR: Wajeeha Zaheer, B.Sc. (Honours)

SUPERVISOR: Dr. Anthony Chan

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ABSTRACT

Hemophilia A (HA) is a hemorrhagic disorder caused by a decrease/absence of coagulation Factor VIII (FVIII) in circulation. Management involves administration of FVIII to prevent bleeding episodes. The most serious complication of this replacement therapy is the development of inhibitory anti-FVIII antibodies which neutralize the infused FVIII. Low zone tolerance (LZT) is a state in which the immune system is unresponsive to an antigen induced by repeated prior exposure to low doses of said antigen. Previous animal studies exploring LZT demonstrated successful T-cell tolerance induction by this mechanism. This study investigated whether the administration of low-dose FVIII could induce immune tolerance to FVIII in a HA mouse model.

HA mice received intravenous FVIII at doses ranging from 0.01 IU/kg - 5 IU/kg to determine the most promising doses (0.25 IU/kg, 2.5 IU/Kg) to further investigate. Naïve mice were treated with 0.25 IU/kg or 2.5 IU/kg weekly for 6 weeks, then immunized with 25 IU/kg FVIII weekly for 4 weeks. Following a two-week rest period (week 12), all mice received a 25 IU/Kg booster shot. Blood was collected on weeks 7, 11 and 13 and anti-FVIII antibody concentrations were measured by ELISA. Control mice received phosphate buffered saline (PBS) during weeks 1-6 of the experiment, followed by identical immunizations as stated above.

Within the 2.5 IU/Kg FVIII treatment group, 11% of the mice developed tolerance to the treatment, indicated by undetectable anti-FVIII IgG titer by ELISA. The remaining 89% of these mice developed high titer antibodies, therefore they were not tolerized to FVIII. In the PBS treatment group, 62% of the mice developed high anti-FVIII antibodies. Conversely, 50% of the mice treated with 0.25 IU/kg were tolerized to FVIII and the remaining mice had significantly

reduced antibody titers when compared to the controls. Moreover, upon booster dose injection, 100% of 0.25 IU/kg and 2.5 IU/Kg treated mice that were previously tolerized retained tolerance, suggesting that tolerance through low-dose injections is maintained upon FVIII re-exposure.

The LZT experiment conducted here shines light on a new approach to preventing FVIII inhibitors. This study suggests that frequently administering low doses of FVIII effectively induces tolerance to FVIII in HA mice. Moreover, treatment through LZT induction may confer long lasting protection against inhibitor development as indicated by the retention of tolerance in mice subjected to a rest period and post-treatment booster shot.

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
APC	Antigen presenting cell
aPTT	Activated partial thromboplastin time
ATP	Adenosine triphosphate
BDD	B-domain deleted
BHK	Baby hamster kidney
BSA	Bovine serum albumin
BU	Bethesda unit
CHO	Chinese hamster ovary
DC	Dendritic cell
ED	Exposure day
EHL	Extended half-life
ELISA	Enzyme linked immunosorbent assay
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FII	Factor II, prothrombin
FIIa	Activated factor II, thrombin
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIIIa	Activated factor VIII
FX	Factor X
FXa	Activated factor X
FXI	Factor XI
FXIa	Activated factor XI

FXII	Factor XII
FXIIa	Activated factor XII
FXIII	Factor XIII
FXIIIa	Activated factor XIII
g	Gram
GC	Germinal centre
HA	Hemophilia A
HrFVIII	Human recombinant factor VIII
HRP	Horse radish peroxidase
HSA	Human serum albumin
IgG	Immunoglobulin G
ITI	Immune tolerance induction
IU	International unit
Kb	Kilobase
Kg	Kilogram
KO	Knock out
L	Litre
LZT	Low zone tolerance
M	Molar
mL	Millilitre
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline – tween
pdFVIII	Plasma derived factor VIII
PNPP	Para-Nitrophenyl phosphate
PUP	Previously untreated patient
rFVII	Recombinant factor VII
rFVIIa	Activated recombinant factor VII
TF	Tissue factor
TMB	Tetramethylbenzidine
VWF	Von Willebrand Factor

1 INTRODUCTION

1.1 HEMOSTASIS

Upon injury to the lining of a blood vessel, a complex multistep process known as hemostasis begins, the ultimate goal of which is the cessation of bleeding.[1,2] This makes it the most important protective mechanism for mammalian survival upon internal or external trauma.[1] The mechanism behind hemostasis follows four steps: vasoconstriction, platelet plug formation, activation of the proteolytic coagulation cascade and fibrin plug formation.[2]

There are two stages of hemostasis, primary and secondary. In primary hemostasis, a weak platelet plug is formed. This starts when endothelial damage occurs. At the site of vessel injury, the potent vasoconstrictor endothelin-1 is produced which mediates the narrowing of the blood vessels, thus slowing bleeding.[2] At this time, inflammatory molecules, sub-endothelial collagen, von Willebrand factor (VWF) and adenosine triphosphate (ATP) are also released, leading to platelet adhesion.[2] As circulating platelets flow past the vessel injury, they attach to the exposed sub-endothelial collagen and VWF. The first bond between platelets and VWF is mediated by the Glycoprotein-Ib-IX receptor that is expressed on the phospholipid bilayer of platelets.[3] Once the platelets have bound to VWF, they must undergo thrombin-mediated activation. The two hallmarks of successful platelet activation are a change in the cell's morphology, from smooth discs to elongated pseudopodal plugs, and secondly, secretion of their cytoplasmic granules.[3,4] As platelets activate, they aggregate to form a weak platelet plug stabilized by fibrinogen. [2]

The process of primary hemostasis temporarily protects from further hemorrhaging; however, the plug must undergo further stabilization with fibrin via thrombin generation. This

occurs during secondary hemostasis via the coagulation cascade.[2] This cascade consists of two pathways, the extrinsic and intrinsic pathways, which unite at the activation of factor X (FX to FXa) and continue downstream as the common pathway, ultimately forming a stable cross-linked fibrin clot.[4] The extrinsic pathway is activated by external bodily trauma, which exposes tissue factor (TF) leading to the activation of Factor VII (FVII to FVIIa).[5] The resulting TF-FVIIa complex goes on to activate FX, thereby commencing the common pathway.[5] The intrinsic pathway initiates when factor XII is activated (FXII to FXIIa) upon exposure to sub-endothelial collagen at the surface of intravascular injury. FXIIa then goes on to activate factor XI (FXI to FXIa), which then activates Factor IX (FIX to FIXa).[4] FIXa and activated factor VIII (FVIIIa) form the "tenase" complex which synergistically activates FX, at which point the cascade continues down the common pathway.[4]

In the common pathway, the combination of FXa with activated factor V (FVa) alongside calcium on phospholipid surfaces creates the prothrombinase complex, which activates factor II (FII), also called prothrombin, into thrombin (FIIa).[6] Thrombin catalyzes three integral positive feedback mechanisms within the coagulation cascade. Upon its generation, it plays a major role in activating FVIII.[7] Moreover, especially in the presence of platelets, thrombin activates FXI in a long-range feedback loop.[7]Finally, thrombin activates FV, thus allowing it to serve as a cofactor for FXa in prothrombin activation.[8] All three of the aforementioned feedback steps result in a burst of thrombin production and propagates clot formation. Finally, thrombin mediates the cleavage of fibrinogen to fibrin and activates factor XIII (FXIII to FXIIIa) which crosslinks fibrin strands, thus forming a sufficiently stable clot **(Figure 1)**.[6]

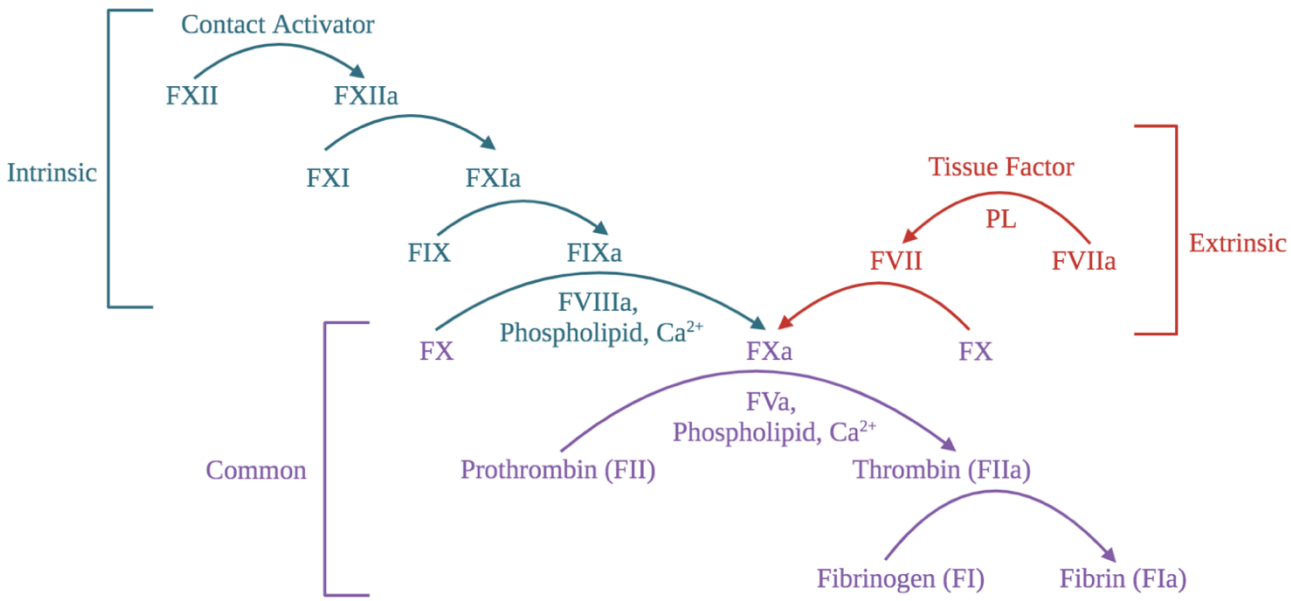

Figure 1: The coagulation cascade

1.1 FVIII: SIZE, STRUCTURE AND IMPORTANC

Coagulation factor VIII (FVIII) is a 2332 amino acid, 293 kilodalton (kDa) glycoprotein synthesized primarily in the liver by sinusoidal endothelial cells. [9] In the bloodstream it is present in its inactive form where it is tightly bound to VWF in a non-covalent complex (k_d <0.5 nmol/L).[10] In circulation, VWF and FVIII exist at concentrations of roughly 40 nM and 0.8 nM respectively, thus, approximately 95 to 98% of FVIII molecules are bound to VWF, while the remaining 2-5% circulate freely.[10] VWF protects FVIII from premature proteolysis and aids in its transfer to sites of endothelial injury.[11] Upon triggering of the coagulation cascade, thrombin-mediated activation of FVIII via proteolytic cleavage occurs, resulting in conformational changes that alter the binding affinity of FVIIIa such that it releases VWF and integrates into the "tenase" complex composed of FIXa, FX and FVIIIa along with calcium ions and phospholipids.[12] FVIIIa acts as a non-enzymatic

cofactor by increasing the catalytic efficiency of FIXa towards FX within the tenase complex.[11] Thus, FVIII plays an integral role in the generation of FIIa.

FVIII is comprised of six covalently bound domains: A1-A2-B-A3-C1-C2.[11] In circulation, FVIII appears as a heterodimer consisting of two chains: a heavy chain (A1-A2-B) with a molecular weight of 200 kDa and a light chain (A3-C1-C2) of 80 kDa.[13] Proteolytic activation of FVIII involves three critical cleavage sites. On the heavy chain, cleavage occurs after Arginine 372 and Arginine 740 and on the light chain cleavage occurs after Arginine 1689.[13] The activated heavy chain molecular weight can range from 90 kDa to 200 kDa, while the light chain molecular weight remains constant at 80 kDa.[11] FVIIIa exists as a heterogenous mixture depending on the position on the heavy chain at which proteolytic cleavage occurred. Additionally, FVIIIa does not contain the B domain. In fact, the exact function of the B domain remains unknown, as it is not involved in the process of blood coagulation. B-domain deleted (BDD) human and recombinant FVIII heterodimers have also exhibited equivalent or even higher activity.[11]

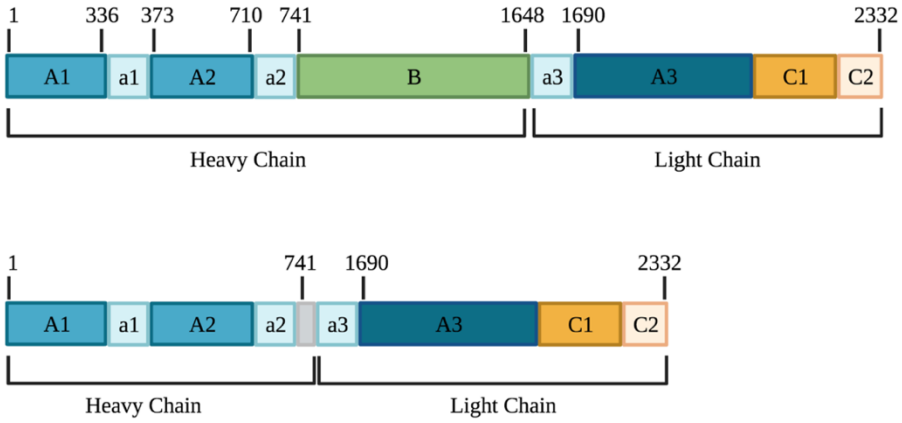

Figure 2: FVIII Structure

Adapted figure of domain organization of full length human FVIII and B-domain deleted FVIII.[13]

1.2 HEMOPHILIA A

Hemophilia A (HA) is a hereditary hemorrhagic disorder caused by a decrease or complete absence of plasma clotting FVIII as a result of mutations in the F8 gene.[11] This leads to excessive bleeding into the muscles and joints either spontaneously or after insignificant trauma.[14,15] FVIII concentration in blood is expressed as international units (IU), with 1 IU being the concentration of FVIII in 1 mL of normal human pooled plasma.[11] A healthy FVIII concentration ranges from 50% to 150%.[11] The hemorrhage severity in HA is determined by the amount of FVIII deficiency in the system. Patients are classified based on their levels of FVIII activity (IU/mL), with 5-40% activity indicating mild disease, 1 - <5% indicating moderate disease and less than 1% indicating severe disease.[11] This X-linked disease affects roughly 1 in 5000 live male births.[16] FVIII plays an integral role in the intrinsic coagulation pathway. In its absence, the generation of FIIa is impaired and the hemostatic platelet plug that forms upon injury is not sufficiently strengthened by fibrin.[16] This results in a weak clot that easily disintegrates which leads to excessive bleeding.[11]

The F8 intron 22 inversion appears in over 50% of severe HA patients making it the most common mutation associated with severe disease.[17] The inversion occurs via an intra-chromosomal recombination event between a 9.6 kilobase (kb) sequence within intron 22 of the F8 gene and one of two related sequences positioned distal on the F8 gene about 300 kb away. This results in the inversion of exons 1 and 22 and it is the most deleterious large-scale DNA rearrangement, leading to the formation of a truncated transcript and subsequently a non-functional FVIII protein (figure 3).[17] Additional mutations associated with HA include smaller inversions, such as that which occurs with a 1 kb sequence in intron 1 and an inverted 5' repeat sequence – this inversion is also associated with severe disease.[17] Missense, splice site and single base changes

in the 5' promoter region also occur within the F8 gene and are often associated with moderate or mild forms of the disease.[17]

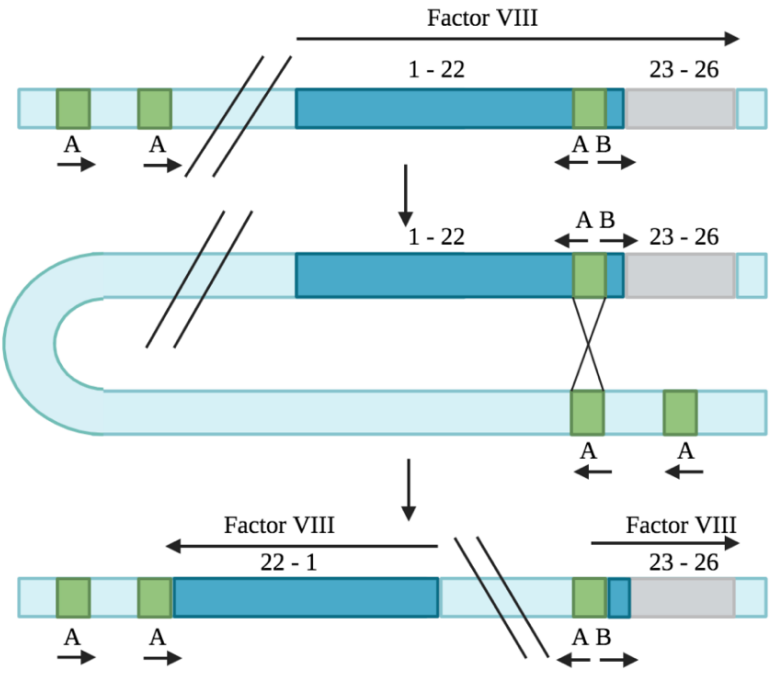

Figure 3: Inversion and disruption of F8 gene - intron 22 inversion

Adapted figure of intron 22 inversion mutation mediated by the F8A gene. **A)** Organization of F8 gene with three copies of F8A gene and one copy of F8B gene, direction of transcription indicated by arrows.[18] **B)** Two homologous F8A sequences located outside F8 gene that mediate intrachromosomal recombination with proximal (left) or distal (right) A gene. [18] **C)** F8 gene after inversion - this is the recombination product. [18]

1.3 DIAGNOSIS

A person may be suspected to be hemophilic due to family history or after bleeding excessively in response to a minor injury. One common diagnostic measure employed to detect hemophilia is the activated partial thromboplastin time test (aPTT).[19] This is a routine coagulation test that measures how long it takes for blood to form a clot. The aPTT test is used to assess the integrity of the intrinsic pathway of the coagulation cascade and accounts for all factors involved

except for FVII and FXIII.[19] During the test, decalcified blood is centrifuged to separate plasma. Then, calcium and the activating substances Kaolin and cephalin are added to the plasma to initiate the intrinsic pathway. Kaolin is used to activate the contact dependent FXII molecule and cephalin serves as a phospholipid substitute.[19] A healthy clotting time for the aPTT test is around 25 to 35 seconds, however patients with HA have a prolonged aPTT indicating that a factor from the intrinsic pathway is missing or defective.[19]

1.4 TREATMENT: REPLACEMENT THERAPY

At the moment, unless a liver transplant is performed, no cure exists for HA, however it can be managed through replacement therapy. One current mainstay of treatment in developed countries is prophylactic FVIII infusions to patients with HA to prevent bleeding episodes.[20] Infusion usually starts at one year of age with patients completing 75 exposure days (EDs) by the time they reach the age of 2 or 3.[20] Replacement therapy products are either pooled human plasma derived FVIII (pdFVIII) or fall into one of three generations. First generation products are obtained from cultures containing Bovine Serum Albumin (BSA) and/or human serum albumin (HSA) as a stabilizer.[21] Second generation drugs contain human plasma protein solutions in culture and third generation drugs contain no human or animal protein whatsoever.[21]

PdFVIII concentrates are highly purified using ion exchange, monoclonal antibody or heparin ligand chromatography followed by viral inactivation.[22] One major drawback of these products is the risk of transmitting blood-borne pathogens found in plasma and HSA which often deters patients from choosing this therapy.[22] Some examples of commercially available plasma derived products include Hemofil M, Koate-DVI and Monoclate-P.[21]

Secondly, there are recombinant FVIII (rFVIII) products which are further subdivided into full length products or B-domain deleted (BDD) products. Full length recombinant FVIII concentrates are synthesized from baby hamster kidney cells (BHK) or Chinese hamster ovary (CHO) cells.[22] These products have undergone solvent/detergent viral inactivation. Some brands names of rFVIII products include Kovaltry and Kogenate, to name a few.

BDD products are another popular choice. As mentioned earlier, the B-domain of FVIII is not required for clotting activity, hence its deletion in recombinant products is not associated with significant discrepancies in procoagulant properties. In fact, B-domain deletion increases FVIII secretion in cell expression systems and enhances its stability.[22] Some commercially available BDD products include ReFacto, Xyntha and Nuwiq.[21] Furthermore, some BDD products have an extended half-life (EHL) by the addition of polyethylene glycol (PEG) groups to the protein or fusing the rFVIII to fragment crystallizable (Fc) portion of immunoglobulin (IgG). These include the brands Adynovate and Eloctate respectively.[21]

EHL FVIII products are an exciting advancement in Hemophilia care as they reduce the number of scheduled infusions for patients. The standard half-life of FVIII ranges from 8 to 12 hours, however PEGylation and FC fusion can help increase the half-life up to 19 hours.[21] Currently, the rFVIII-FC fusion product Eloctate has the longest half-life of 19.7 hours in adults. Unfortunately, in children the half-life for these products is much shorter and may not be worth the increased cost associated with these products.

Prophylactic replacement therapy has made living with HA a manageable feat for patients of all ages. With regular infusions of the FVIII products mentioned above, a child diagnosed with HA can live a normal lifespan without restricting their day to day activity.[21] However, this does

not come without significant burdens. First and foremost, due to the short half-life of FVIII, intravenous infusions are needed at least three times per week to ensure pharmacokinetic trough levels are higher than 1%.[21] This imposes a significant liability on all patients, especially children who may have poor venous access. Additionally, replacement therapy is financially draining as FVIII products cost up to $300,000 USD per year.[21]

Table 1: Commercially available FVIII products

Generation	Products	FVIII	Technology	Half-life
Plasma derived	Antihemophilic factor (Hemofil M®, Koate DVI®, Monarc-M® , Monoclate-P®)	Full length	Pooled human plasma	14.8 – 17.5 hours
Recombinant: first generation	Antihemophilic factor recombinant (Recombinate ®)	Full length	BSA in culture HSA stabilizer	14.6 ± 4.9 hours
Recombinant: second generation	rFVIII-FS (Helixate ®, Kogenate ®)	Full length	Human plasma protein solution in culture	13.74 hours
Recombinant: third generation	Antihemophilic factor recombinant (Kovaltry ®, Advate ®)	Full length	No added human or animal protein	12 – 14.2 hours
Recombinant: third generation EHL	Octocog alfa pegol (Adynovate ®)	BDD-PEGylated	PEGylation to Advate	14.69 ±3.79 hours
Recombinant: fourth generation	Simoctocog alfa (Nuwiq ®)	BDD	HEK cells – allow human glycosylation	17.1 ± 11.2 hours
Recombinant: fourth generation EHL	rFVIII-FV (Eloctate ®)	BDD-rFVIII-Fc	HEK cells - allow human glycosylation	19.7 ± 2.3 hours

1.5 TREATMENT: NON-FACTOR THERAPY

The burden of replacement therapy along with the risk of inhibitor development prompted the development of non-factor therapies. One such treatment is the FVIII mimetic known as emicizumab. This is a humanized bispecific monoclonal antibody that mimics the function of FVIII, but since it is not FVIII, there is no immune response to this therapy.[23] Emicizumab works by bridging FIXa and FX enhancing FX activation. This interaction is depicted in figure 4 below. Emicizumab is widely used for prophylaxis in HA patients with and without inhibitors.[23] Also, emicizumab can be given subcutaneously allowing for at home treatment and it has a far longer half-life than any replacement therapeutic, at 4 to 5 weeks.[24]

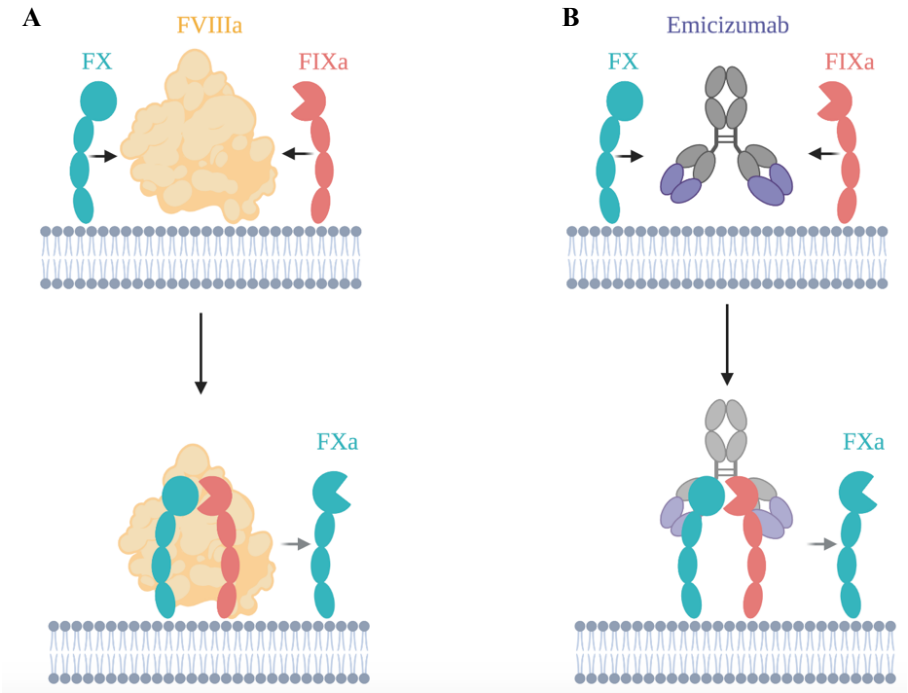

Figure 4: Emicizumab Mechanism of Action

A) adapted figure depicting the role of FVIIIa in bridging FIXa and FX to produce FXa. **B)** Emicizumab performing the same mechanism. (adapted figure) [23]

The second class of non-replacement therapies are known as bypassing agents which are used to treat bleeding episodes in patients with high titer inhibitors. Instead of replacing the missing FVIII, these agents bypass the inhibited FVIII to confer a hemostatic effect.[25] Recombinant activated Factor VII (rFVIIa) is an example of a bypassing agent. It works by bypassing the absence of FVIII and directly activating FX at the site of a vascular insult.[25] A high dose of rFVIIa (around 90 μg/Kg) is needed in HA patients with inhibitors to replace the FVII zymogen that is bound to tissue factor (TF). The rFVIIa-TF complex aids in the conversion of prothrombin to thrombin and ultimately enhances fibrin formation leading to a stable cross-linked fibrin clot.[25]

1.6 TREATMENT: GENE THERAPY

Another emerging treatment option is gene therapy. This treatment would allow for the repair or addition of a functional copy of the F8 gene through the delivery of a transgene to its physiologically relevant target tissue, the liver. [26] Ideally, the gene is stably expressed and does not interfere with cellular function within the liver.[26] Gene therapy entails a one-time infusion that would result in the long-term expression of FVIII, thus providing lasting relief to severe HA patients while bypassing the side effects associated with replacement and non-replacement therapy. The adeno-associated viral (AAV) vector has provided the greatest clinical success for in vivo gene delivery and it is highly unlikely to cause oncogenesis, unlike lentiviral vectors, making it the vector of choice to target hepatocytes.[26] One example of a gene therapy currently in clinical trial is Valoctocogene Roxaparvovec which implements a codon-optimized AAV5 vector that expresses the B-Domain deleted human FVIII transgene with a liver specific transcriptional promoter.[26] In an ongoing clinical trial, 4 of 7 severe HA patients given $6x10^{13}$ viral genomes/kg

of Valoctocogene Roxaparvovec had FVIII levels higher than 5 IU/dL 5 years after administration![27] This therapy is the first to be approved in Europe.

1.7 DRAWBACKS OF NON-REPLACEMENT THERAPY

As HA patient care evolves, treatment options such as non-factor and gene therapy have gained popularity; however, they too have their limitations. First and foremost, non-replacement therapy options are extremely expensive, making prophylactic FVIII infusions the economically preferable strategy. In fact, one study following severe HA patients reported that not only was replacement therapy the less costly treatment option, when compared to emicizumab, but it was also more effective in disease management.[28] Over an estimated 70-year lifespan, the study projected prophylactic FVIII to cost 2.8 million dollars less than emicizumab.[28] With respect to gene therapy, in addition to its high cost, some disadvantages include neutralizing antibody formation to the AAV delivery system, the potential for a non-permanent response and adverse liver reactions.[29] Finally, all of the aforementioned non-replacement therapy options are a luxury that are rarely available in developing countries. Over 75% of the HA patient population resides in these nations, thus, their only treatment option, if they can afford it, is replacement therapy.

1.8 INHIBITORS: WHAT ARE THEY?

The most serious complication of replacement therapy is the development of anti-FVIII antibodies.[30] These inhibitors are polyclonal IgG alloantibodies that impede the normal activity of endogenous and exogenously administered FVIII.[30] Inhibitors develop in up to 40% of severe HA patients and in up to 15% of patients with mild or moderate HA.[30] The development of these neutralising antibodies usually occurs within the first 20 EDs to FVIII drugs.[20] Ultimately, patients

with inhibitors develop resistance to conventional replacement therapy, thus; there is great interest in uncovering new and effective strategies to prevent immunologic responses towards FVIII.

1.9 INHIBITOR DEVELOPMENT: GENETIC AND NON-GENETIC RISK

The exact cause of inhibitor development is not well understood as it is a multifactorial process with a complex interplay of various genetic and environmental factors. Aside from family history, a patient's ethnicity can also hint at whether or not they will develop inhibitors, as Black and Hispanic patients have higher prevalence of inhibitors than Caucasian patients.[20] In general, the risk of developing inhibitors is also greater in patients who have no circulating FVIII as a result of null mutations as opposed to those with non-null mutations.[20] Additionally, patients with major gene deletions or nonsense mutations have a 75% chance of developing inhibitors while this risk drops to 20% to 30% for those with an intron 22 inversion.[20] The compelling relationship between FVIII genotype and inhibitor risk is likely due to HA patients lacking a central tolerance to FVIII.

In terms of replacement therapy, patients given second or third generation FVIII concentrates are at a higher risk of inhibitor development than those receiving full length recombinant FVIII (rFVIII). However, rFVIII administration is associated with more cases of inhibitors than plasma derived FVIII.[20] In fact, a recent randomized control study (SIPPET) carried out in previously untreated patients (PUPs) found that 44.5% of patients receiving rFVIII developed inhibitors compared to 26.8% in those treated with pdFVIII.[31] The reduced immunogenicity seen in pdFVIII may be due to the presence of VWF in plasma. VWF plays a protective role by shielding epitopes on FVIII and preventing uptake by dendritic cells (DCs).[20] Moreover, the glycosylation pattern of each factor concentrate also plays an important role in immunogenicity. FVIII has 25 potential N-linked glycosylation sites and the use of different cell

lines results in various FVIII glycomes that can affect the uptake of FVIII by antigen presenting cells (APCs).[20] Since pooled human plasma contains human FVIII there is no change in the glycosylation patterns, which reduces pdFVIII immunogenicity. Conversely, due to the production of rFVIII in non-human cell lines (BHK and CHO), differences in post translational modifications are observed including differences in the degree of glycosylation of the FVIII concentrate which can cause an immune response. Glycans can promote inhibitor formation by acting as epitopes that are recognized by endocytic receptors on APCs.[20]

The intensity of FVIII treatment at first exposure also plays a role in inhibitor development. The discontinuity theory states that a fluctuation of a given molecular motif over time drives an immune response.[31] Patients with severe HA are constantly subjected to varying doses of FVIII which could boost inhibitor formation. This theory was tested in the RODIN study where the incidence of inhibitors was examined in PUPs exposed to high dose intensive FVIII therapy and low dose prophylactic treatment. [32] The findings indicated that the frequent administration of FVIII at low doses resulted in less inhibitor formation than those receiving high doses.[32] This was especially true in patients with low-risk F8 mutations such as missense and splice site mutations.[32] Patients who received FVIII doses of less than 35 IU/Kg had the lowest risk of inhibitor development while patients treated with doses between 35 to 50 IU/Kg had a 2.4 times higher risk. [32] Moreover, patients infused with >50 IU/Kg of FVIII had a 2.3 higher risk than those treated with 35 to 50 IU/Kg. [32] Prophylactic treatment with FVIII within the first 75 EDs had a 32% lower risk of inhibitor formation than those treated on demand for a surgery or trauma.[32]

1.10 QUALITY OF LIFE WITH INHIBITORS

Inhibitor development is also linked to an onslaught of medical problems, as well as higher morbidity and a decreased quality of life. When compared to their inhibitor free counterparts, patients with inhibitors experience over double the average annual number of joint bleeds, require more hemophilia related hospitalizations, outpatient visits and consultations.[33] Additionally, Patients with inhibitors also require more pain-alleviating medication than those without inhibitors.[33] A retrospective cohort study also concluded that the odds of death increase by 40% in HA patients who experience inhibitor development.[34] All of these aspects combined further prove just how essential it is to unravel better treatments for HA patients suffering from FVIII inhibitors.

1.11 INHIBITOR FORMATION: MECHANISM

Upon first exposure to FVIII, the primary immune response commences. This involves the internalization of the FVIII protein by APCs such as DCs and subsequent presentation of the FVIII molecule to naïve CD4+ T-cells.[35] In the presence of an activating, inflammatory microenvironment, the T-cells become activated and go on to activate FVIII-specific B-cells.[35] These B-cells expand and differentiate into either plasma cells which secrete anti-FVIII IgG or FVIII-specific B memory cells. During the secondary immune response, it is those same FVIII specific B-memory cells that differentiate into antibody secreting plasma cells. [35]

Memory B-cells play an integral role in the maintenance of an established antibody response. With each re-exposure to FVIII, B-cells are re-stimulated resulting in their proliferation and differentiation into plasma cells which secrete high affinity antibodies against FVIII.[36] Moreover, memory B-cells possess high affinity antigen receptors, major histocompatibility complex type II

(MHCII) and co-stimulatory molecules, all of which enable these B-cells to act as highly efficient APC's.[36] Thus, they can stimulate CD4+ T-helper cells which results in the downstream activation of additional B and T lymphocytes, further exacerbating the immune response towards FVIII.

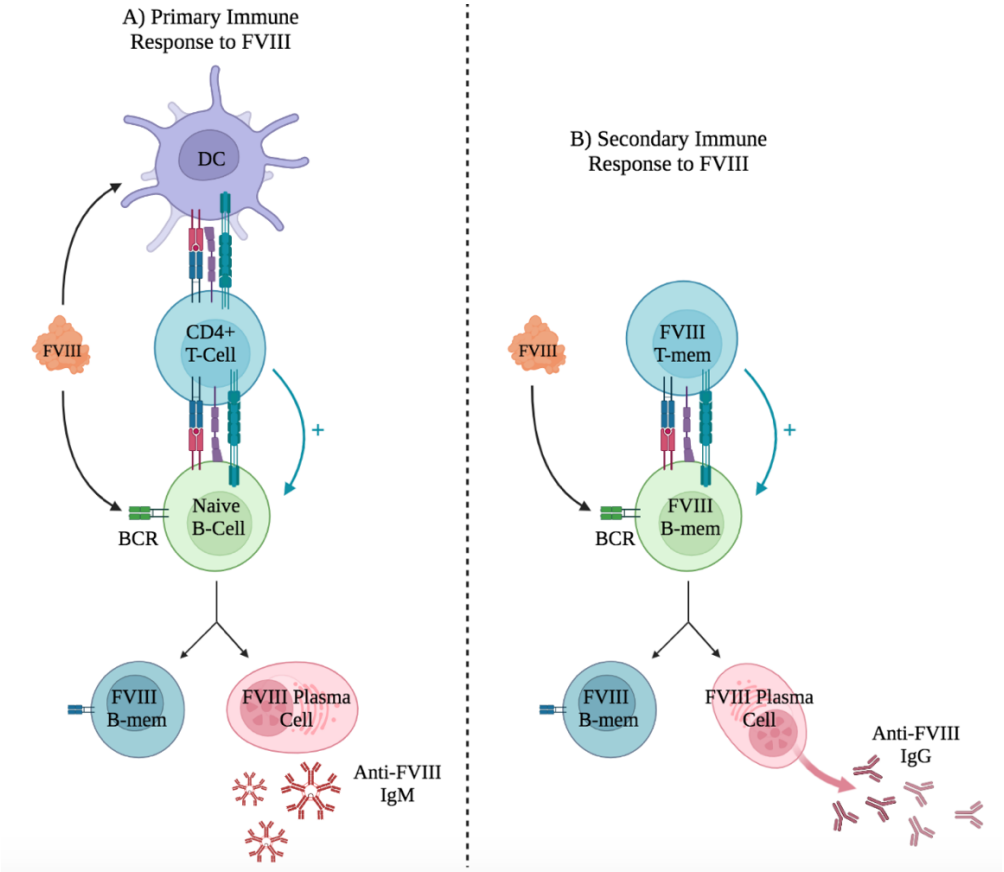

Figure 5: Primary and Secondary Immune Response to Exogenous FVIII

1.12 INHIBITOR DETECTION: THE BETHESDA ASSAY

A physician may suspect inhibitor development in a patient when their bleeding is not controlled by standard doses of replacement therapy. Inhibitor concentrations are measured using a Bethesda assay or a Nijmegen-modified Bethesda Assay (which has improved sensitivity and

specificity). These assays only detect antibodies that reduce FVIII activity.[37] To prepare the assay samples, the patient plasma is serially diluted with equal volumes of normal plasma and incubated at 37°C for 2 hours. Then, the remaining FVIII level after incubation is measured.[37] A decrease in residual FVIII indicates a positive result. This assay gives a numeric value of inhibitor level and is quantified as Bethesda units per milliliter (BU/mL). It is important to know the level of inhibitors in a patient as this provides a basis for determining the best treatment regimen.[37] Inhibitors are divided into two classes, low responding inhibitors which are 5 BU/mL or less or high responding (5 BU/mL or greater).[37] The inhibitor response is determined by the patient's peak inhibitor titer after repeated FVIII administration. Patients with low responding inhibitors generally respond better to replacement therapy than those with high responding inhibitors.

1.13 PORCINE FVIII

Historically, a therapy frequently used in patients with severe HA complicated with inhibitors was plasma derived porcine FVIII.[38] In the 1970's it was observed that there was 30% less cross reactivity between anti-human FVIII inhibitors and Porcine FVIII than there was towards human FVIII.[38] This led to the widespread use of porcine FVIII in inhibitor positive HA patients. By the 1980s, porcine-FVIII products such as Hyate:C and later, the recombinant product known as Obizur were developed. The use of these products allowed patients to undergo FVIII replacement with a reduced incidence of bleeding episodes.[38] Ultimately however; most patients developed inhibitors to the porcine FVIII products as well, thus it is no longer a mainstay for the treatment of HA with inhibitors.

1.14 IMMUNE TOLERANCE INDUCTION

The only known treatment proven to eradicate inhibitors is immune tolerance induction (ITI). [35] This treatment involves frequently administering FVIII at high doses (50 to 200 IU/Kg depending on which protocol is used) under non-inflammatory conditions to re-establish tolerance. [35] The three most popular ITI protocols are highlighted in table 2 below. The mechanism behind ITI largely remains elusive, however it is known that recurring exposure to FVIII down-modulates the anti-FVIII IgG response to the point that immune tolerance is induced. [35] More specifically, evidence suggests that high doses of FVIII inhibit FVIII-specific memory B-cells, resulting in their inability to differentiate into plasma cells that secrete alloantibodies.

Although promising, ITI treatment has some major drawbacks that must be addressed. Firstly, it is extremely demanding for patients as they must be infused with factor anywhere from three times per week to daily. [35] In fact, the Malmö protocol requires patients to be hospitalized. On top of this, the treatment takes extended amounts of time ranging from 1.5 to 3 years. [39] Since ITI requires recurring high dose FVIII administration, this treatment is also profoundly expensive. The patients can expect to pay anywhere from 1.5 to 3 million USD. [40] Finally, ITI is only successful in 45% to 80% of patients.[35,39] With these points in mind and the substantial challenge HA patients with inhibitors face on a daily basis, we sought to investigate a new approach to induce tolerance to FVIII in patients with HA.

Table 2: Treatment regimens for the Bonn, Van Creveld and Malmö ITI protocols

ITI protocol	Regimen	Success rate
Bonn protocol (high dose)	Initially: - FVIII 100-150 IU/Kg twice per day - aPCC 50 IU/Kg twice per day when necessary When inhibitor <1 BU/mL: - FVIII 150 IU/Kg once per day	87%
Van Creveld protocol (low dose)	- 25-50 IU/Kg thrice per week	87%
Malmö protocol	- Continuous FVIII infusion for 10 – 14 days to maintain plasma levels of >30% - Cyclophosphamide at 12 -15 mg/Kg IV on day 1 and 2 and orally on days 3-10 - IV IgG at 2.5-5 g on day 1, 0.4 g/Kg/day on days 4-5 - Protein A adsorption: to reduce inhibitor titer below 10 BU/mL if they surpass 10 BU/mL before therapy commences.	59-83%

1.15 THE EPIC STUDY

The Early Prophylaxis Immunologic Challenge (EPIC) Study is the only clinical trial to date that explored low dose FVIII administration in PUPs with HA. This phase 3b clinical trial included boys less than one year of age who had moderately severe HA (FVIII ≤ 2%). Participants were infused weekly with 25 ± 5 IU/kg ADVATE, a recombinant FVIII product.[41] The first 20 EDs were considered the early prophylaxis period. After this period, participants continued to receive weekly FVIII infusions at the same dose for as long as possible, increasing the frequency of

infusions up to 3 times per week when necessary.[41] Bethesda assays were conducted to track inhibitor levels weekly for the first 10 EDS, every 5 weeks for the next 10 EDs and every 10 weeks thereafter. The purpose of this study was to evaluate whether a once per week prophylactic FVIII infusion at 25 IU/Kg started in babies, therefore before the inception of severe bleeding phenotypes such as joint or muscle bleeds, could reduce the incidence of inhibitors in PUPs with moderate or severe HA. Thus, the primary outcome measure was to assess the number of patients with moderate or severe HA who developed FVIII inhibitors within the first 50 EDs.

Out of the nineteen participants, eight exhibited confirmed inhibitor development, and eight others were pre-maturely dismissed from the study, leaving only three participants who completed the treatment.[41] As a result of the observed inhibitor incidence, the study was terminated, leaving the hypothesis of the EPIC study unresolved. It is still unknown whether or not early prophylaxis at low doses can reduce inhibitor formation in PUPs. Nonetheless, the study provides a foundation for further consideration and indicates that there is potential behind a low dose FVIII regimen that remains to be investigated on a deeper level.

2 STUDY RATIONALE:

2.1 LOW ZONE TOLERANCE

Low zone tolerance (LZT) is a state in which the immune system is unresponsive to a specific antigen induced by prior exposure to very low doses of said antigen.[42] Inducing LZT to FVIII in HA patients could provide a new way to battle inhibitor development in a safe and cost-effective manner. The idea that low doses of therapeutic FVIII can be used to sensitize the immune system of HA patients to FVIII came from several previous studies on immune tolerance. Notably,

Shellam and Nossal successfully proved a similar theory with Salmonella Flagellin in Wistar Rats. They explored tolerance induction in new born rats by injecting sub-immunogenic doses of flagellin into the tail vein of rats daily for two weeks. At the end of the two-week period, the rats developed complete tolerance to flagellin.[42] In addition to Flagellin, low-zone tolerance has been successfully induced in vivo using antigens such as Bovine Serum Albumin, Bovine-γ globulin and Ovalbumin.[43] With this in mind, this thesis sought to explore whether FVIII tolerance can be induced in a similar fashion. If proven possible, this could revolutionize the way autoimmune disorders are treated.

2.2 THE DISCONTINUITY THEORY OF IMMUNITY

In nature, certain biological systems detect the rate of change in a stimulus as opposed to the stimulus itself. The discontinuity theory of immunity proposes that the immune system also functions under this principle. The theory states that sudden, discontinuous antigenic stimulation triggers an effector immune response, while weak or continuous stimulation with an antigen over time leads to immune tolerance.[44] This implies that the immune system can adapt to slow appearing and long-lasting modifications in a host system, thus setting it as a new reference point, but the sudden appearance of an antigen triggers antibody production.[44] In this theory, the immune system detects the change in antigenic variation over time. Thus, as a function of time, a set concentration of antigen given chronically with minimal levels of variation results in a net zero immune response.

The discontinuity theory is rooted in the observation that chronic exposure to an antigen leads to desensitization in both the adaptive and innate immune cells.[45,46] This has been observed in cancer, viral infections and allergies. Specifically, in asthma, rhinitis and food allergies, repeated

allergen injection leads to a degree of desensitization to the allergen hence acting as a form of allergen immunotherapy.[47] Mechanistically, the tolerogenic effects seen through chronic antigen stimulation involves both intrinsic factors such as cell surface receptor internalization and extrinsic factors including self-reactive T-regulatory cell production which suppress autoimmune responses and induce immunoregulatory cytokine production.[44]

With the surmounting physiological and clinical evidence supporting the discontinuity theory of immunity, it is of great interest to investigate this theory within the context of the immune response in HA. This thesis aims to explore whether the slow and repeated exposure of low doses of FVIII can result in immune tolerance to FVIII, as backed by this theory.

3 SPECIFIC AIMS AND HYPOTHESIS

To combat inhibitory antibodies to FVIII, we proposed a protocol that may induce LZT in HA mice. Experimentation involved frequently infusing low doses of therapeutic FVIII to FVIII knockout (KO) mice over the course of several weeks. Plasma was collected at different time points during the experiment and subjected to an enzyme linked immunosorbent assay (ELISA) to detect and quantify anti-FVIII IgG's. Different FVIII doses and injection intervals were explored to determine which treatment regimen – if any, induced FVIII tolerance in HA mice. This process differs from ITI as very low doses of FVIII were administered with the goal of ultimately leading to FVIII tolerance. As such, this procedure could provide a low cost and permanent solution to inhibitor development in Hemophilia A. It should be noted that in a clinical setting the proposed therapy would be carried out on babies with HA, aged one year or younger as this therapy would only work on patients who have not been previously exposed to FVIII therapy.

The aims of this project were as follows:

1. Determine if low dose FVIII injections are a viable strategy to induce immunologic tolerance to FVIII in HA mice and which FVIII dose(s) and injection interval(s) allow for tolerization to occur.
2. Determine whether the induced LZT is maintained upon FVIII re-exposure.
3. Explore the mechanism behind LZT induction by elucidating the anti-FVIII IgG subclass profiles of tolerized and non-tolerized mouse treatment groups.

We hypothesized that a weekly or bi-weekly FVIII dose around 0.25 IU/kg would induce immune tolerance in HA mice, thus resulting in the reduction or absence of inhibitors when compared to the control groups that had not received repeated low dose FVIII injections.

4 MATERIALS AND METHODS

4.1 HEMOPHILIA A MOUSE MODEL

The mouse model used for these experiments is a FVIII knock-out mouse colony that exhibits Hemophilia A. These mice have had exon 17 of the F8 gene knocked out, thus resulting in mice deficient in FVIII.[48] This mouse model is an accurate model system as they exhibit prolonged bleeding and diminished blood clotting capabilities as there is no circulating FVIII in their body, much like a patient with severe HA. Additionally, they develop inhibitory antibodies to exogenously administered FVIII, making them the ideal model for my experiment.

4.2 FVIII SOURCE

The source of therapeutic FVIII used in these experiments is Kovaltry.[49] This is a commercially available third generation, full-length, unmodified human recombinant FVIII (hrFVIII) that is synthesized in BHK cells and manufactured by Bayer.[49] Kovaltry is used by persons with Hemophilia A across the globe, and as such, it is an appropriate drug to address the research question. Additionally, given that it is a full-length drug without modifications, it enables us to obtain a more comprehensive study of the natural immune response to FVIII.

4.3 FVIII INJECTION PREPARATION

All preparatory work was carried out aseptically using autoclaved materials. For the 25 IU/Kg, 2.5 IU/Kg injections, a 0.01 IU/Kg working solution of Kovaltry was prepared in the biological safety cabinet (BSC) by diluting a 1 IU/μL stock solution of Kovaltry into sterile PBS. For the 0.25 IU/Kg injections, a 0.001 IU/Kg working solution of Kovaltry was prepared from the same stock solution. The diluted FVIII solutions were prepared on the day od use and kept on ice until it was time to inject.

4.4 INJECTION REGIMENS AND CONTROLS

4.4.1 WEEKLY FVIII INJECTION + BOOSTER SHOT

During the low dose tolerance phase, naïve FVIII KO mice were treated with intravenous FVIII at doses of 0.25 IU/kg or 2.5 IU/kg weekly for 6 weeks. Then, during the immunization phase, the mice were treated with a prophylactic dose of 25 IU/kg FVIII weekly for 4 weeks. Following a

two-week rest period, all mice received a 25 IU/Kg booster shot on day 78. Blood was collected at weeks 7, 11 and 13 (day 40, 69 and 85) for anti-FVIII antibody titre analysis.

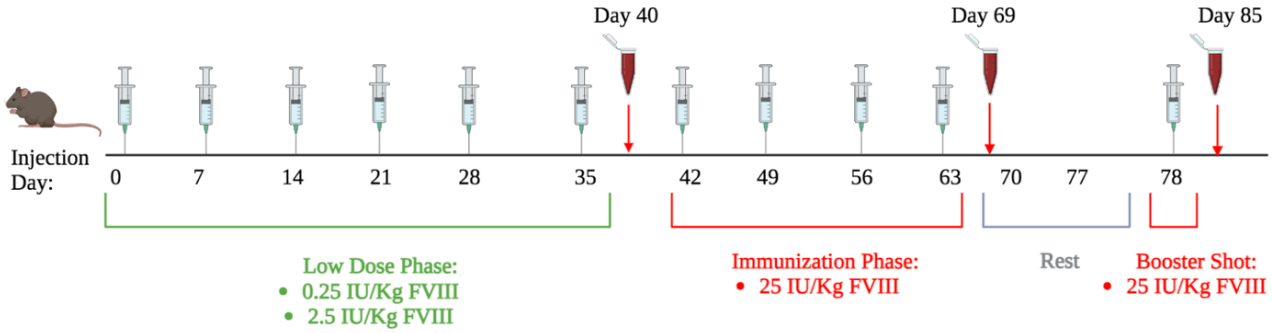

Figure 6: Weekly injection scheme

Each needle indicates the day at which mice were injected, with days 0 to 35 being the low dose injection phase, and days 42 to 63 being the immunization phase. The two-week rest period is shown in grey from days 64 to 77, and the final booster shot was administered on day 78. Blood was collected one week after each phase of the experiment (Day 40, 69 and 85).

Positive Controls

As a positive control, one group of mice received 25 IU/Kg each week for 10 weeks, followed by a two-week rest period and 25 IU/Kg Booster shot. These mice are expected to have the highest anti-FVIII IgG and inhibitor concentrations.

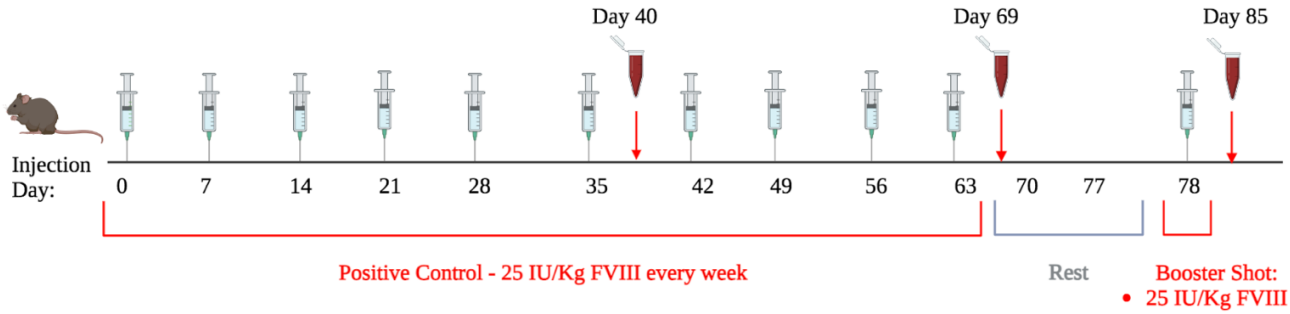

Figure 7: Positive control 1 injection scheme

A second positive control group was included in which mice received PBS for the first 6 weeks of the experiment followed by 25 IU/Kg in the immunization phase of the experiment. Then,

they underwent a two-week rest period and a 25 IU/Kg booster shot like the other mice. These mice were expected to develop high levels of inhibitors and act as a reference to compare inhibitor concentrations with in the low dose injected mice.

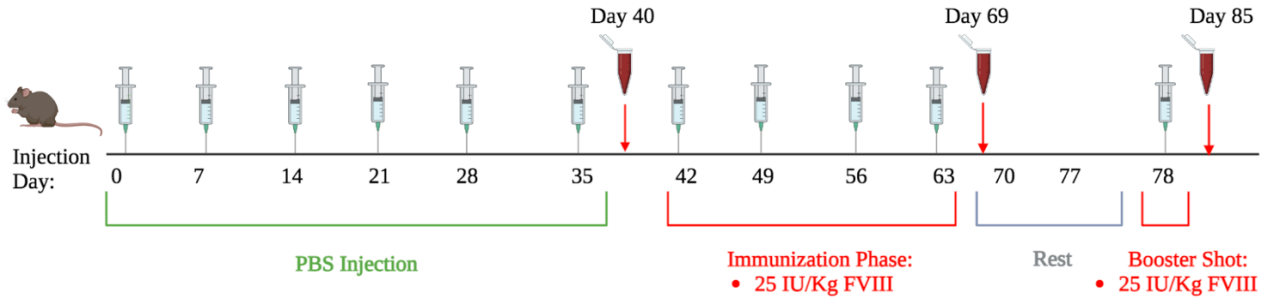

Figure 8: Positive control 2 injection scheme

Negative Control

As a negative control, one group of mice received PBS injections each week for 10 weeks. These mice are expected to have zero anti-FVIII IgG and inhibitors in their plasma.

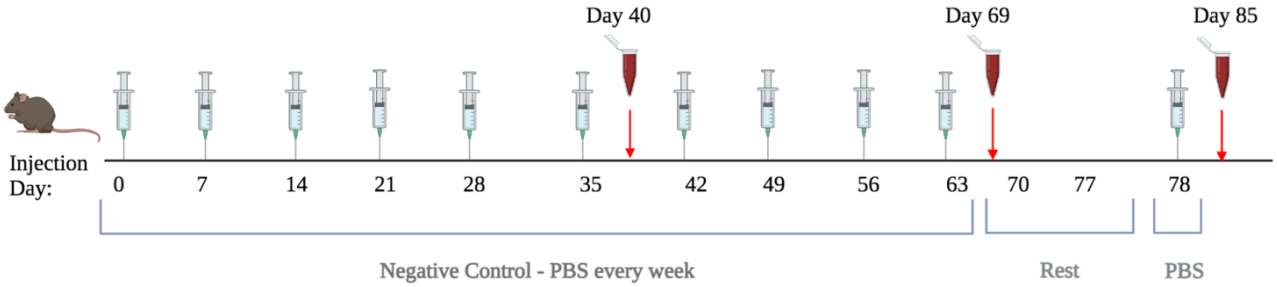

Figure 9: Negative control injection scheme

4.4.2 BI-WEEKLY INJECTION EXPERIMENT + BOOSTER SHOT

In a second experiment, an alternate low dose injection interval was explored. FVIII was administered bi-weekly for ~3 weeks (days 0 to 17). Similarly, control mice were injected biweekly during the first phase of the experiment as well. This was followed by the immunization

phase stated earlier, whereby all mice were treated with 25 IU/Kg FVIII weekly for four weeks (day 23, 30, 37 and 44) and subjected to a booster shot two weeks later (day 59). Blood was collected one week after each phase of the experiment (day 20, 48 and 65).

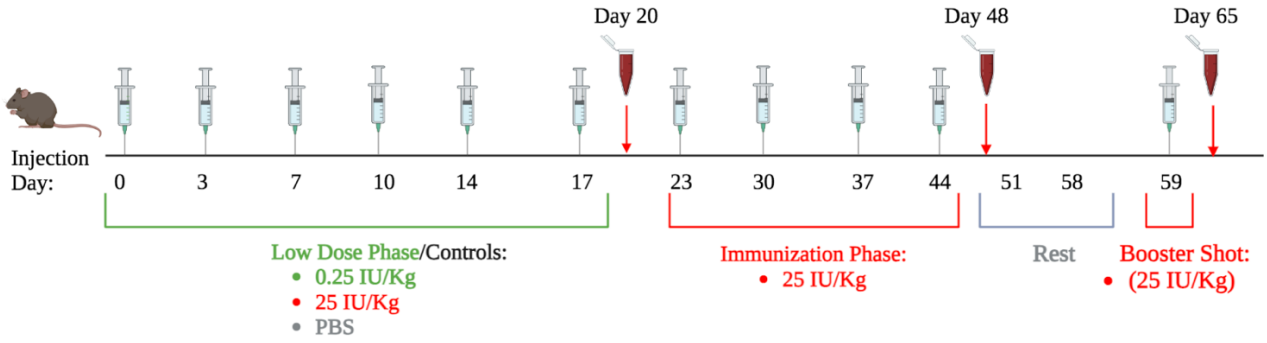

Figure 10: Bi-weekly injection scheme

4.5 INTAVENOUS INJECTION

All injections were administered in an aseptic manner in the BSC. Mice were anesthetized using Isoflurane and weighed. Injections were prepared based on the animal's weight in sterile centrifuge tubes using the working FVIII solutions and PBS as a diluent. The total injection volume was 100 µL (see dosage sample calculation 1 on page 33). FVIII solution was drawn into 27.5-gauge insulin syringes ensuring no bubbles were present in the injection. One drop of Alcaine (local ophthalmic anesthetic) was instilled in the eye to be injected and the injection was administered to the retro-orbital plexus. Bleeding at the injection site was stopped by applying pressure to the eye with a sterile cotton swab. Lubrication and antibiotic ointment (Isathal) were applied to the eye and the mouse was placed on a clean paper towel to recover.

4.6 BLOOD COLLECTION

All blood collections were performed in an aseptic manner in the BSC, seven days after the last dose of FVIII. Mice were anesthetized using Isoflurane and one drop of local anesthetic was applied to the eye that was bled. Glass hematocrit capillary tubes were coated with 3.2% sodium citrate. The coated capillary tube was used to puncture the retro-orbital plexus and four drops of blood were collected into a sterile Eppendorf tube contain sodium citrate (3.2%) at a ratio of 1:9. The blood was gently mixed by inverting the tube and bleeding at retro-orbital sinus was stopped by applying firm pressure to the eye with a sterile gauze pad. Lubrication and antibiotic ointment (Isathal) were applied to the eye and the mouse was placed on a clean paper towel to recover.

4.7 PLASMA ISOLATION

Blood samples were spun at 2000 x g for 10 minutes. The top translucent layer of plasma was removed without disturbing the Buffy coat and placed in a fresh Eppendorf tube. The plasma was spun again at 2000 x g for an additional 15 minutes and removed into a new Eppendorf tube, leaving behind any remaining blood cells. Samples were labeled and stored at -80°C until analysis.

4.8 PREPARATION OF BUFFERS FOR ELISA

4.8.1 COATING BUFFER

To prepare 1 molar (M) Na_2CO_3: 10.6 grams (g) Na_2CO_3 was dissolved in 1 liter (L) of deionized water. Then, the pH was adjusted to 9.6 with HCl and the solution was stored at 4°C for 30 days.

4.8.2 WASHING BUFFER

To prepare 0.01M phosphate buffered saline - 0.1% Tween (PBS-T): 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were dissolved in 1L of deionized water. The pH was adjusted to 7.4 with HCl, followed by the addition of 0.5 mL of Tween 20 to 500 mL of PBS. The solution was mixed and stored at 4°C for 30 days.

4.8.3 BLOCKING BUFFER

To prepare a 0.05% skim milk blocking buffer: 5 g of skim milk powder (Carnation brand) was dissolved in 100 mL of PBS-T. This buffer was made fresh for each ELISA.

4.8.4 SUBSTRATE BUFFER

To prepare a 1M diethanolamine + 0.5 mM $MgCL_2$ buffer: 102 mg of $MgCl_2$ was dissolved in 1 L of deionized water, then 96.6 mL of diethanolamine was added. The pH was adjusted to 9.8 with NaOH. The storage bottle was covered with aluminum foil and stored at 4°C for 30 days.

4.9 DETERMINATION OF ANTI-FVIII IGG BY ELISA

Coating antigen (hrFVIII – Kovaltry, Bayer, Germany) was diluted 1/100 in coating buffer and 100 µL of the solution was applied to each well. The plate was incubated in the dark for two hours at room temperature. To wash the plate, 200 µL of washing buffer was placed into each well using a multichannel pipette and then discarded, this was repeated three times. The plate was blocked by adding 200 µL of blocking buffer to each well and incubated for two hours at room temperature. Plasma samples to be tested were thawed at room temperature and prepared by diluting each mouse

plasma sample 1/100 and 1/500 in blocking buffer. A standard curve was constructed by serially diluting a commercial mouse anti-FVIII antibody (Invitrogen Mouse anti-FVIII monoclonal antibody, stock concentration 1 mg/mL) in blocking buffer starting with a 1/1000 dilution and going down to 1:64,000. Blocking buffer was used as a blank. After blocking was complete the plate was washed as mentioned above. Then, samples and standards were applied to each well and incubated at 4°C overnight. The plate was then washed as stated above, and the detecting antibody (Abcam Goat anti-mouse IgG (H&L) alkaline phosphatase conjugated detecting antibody) was diluted 1/10000 in blocking buffer. 100 µL of the diluted antibody was applied to each well and the plate was incubated for 2 hours at room temperature. After washing the plate as stated above, 100 µL of the substrate solution containing para-Nitrophenyl phosphate (PNPP) was applied to each well. The colour was developed for 20 minutes and the absorbance of each well was read at a wavelength of 405 nm in a Spectramax Plus384 plate reader (Molecular Devices).

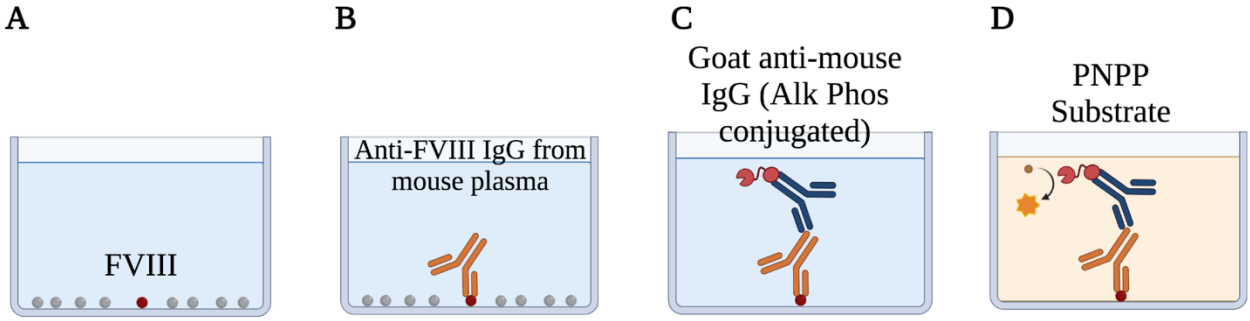

Figure 11: Anti-FVIII IgG Indirect ELISA

A) The plate is coated with FVIII (Kovaltry) and **B)** incubated with the mouse plasma samples or known amounts of commercial anti-FVIII antibody used as a standard which contain anti-FVIII IgG that binds to the Kovaltry. Then **C)** the detecting antibody is added which binds to mouse IgG and is conjugated to Alkaline Phosphatase. **D)** Once the PNPP substrate is added, it is cleaved by Alkaline Phosphatase and produces a yellow colour that absorbs light at 405 nm which can be read on a spectrophotometer.

4.10 DETERMINATION OF ANTI-FVIII IGG SUBCLASSES (IGG1, IGG2 AND IGG3) BY ELISA

All coating, blocking, standard dilutions, sample preparation and washing steps were completed as previously outlined in section 4.9. The next day, secondary antibodies specific for mouse IgG1 or IgG2a or IgG2b or IgG3 (Invitrogen Goat anti-mouse IgG1/IgG2a/IgG2b/IgG3 horse radish peroxidase (HRP) conjugated detection antibody) were diluted 2000-fold in blocking buffer and added to each well. After a one-and-a-half-hour incubation period, the plate was washed three times with PBS-T and 100 µL of Tetramethylbenzidine (TMB, Abcam) solution was added to each well and incubated for 5 minutes. The colorimetric reaction was stopped with the addition of 100 µL of 2.6 molar H_2SO_4 per well. Finally, the absorbance of each well was read at wavelength of 405 nm in a Spectramax Plus384 plate reader.

4.11 BETHESDA ASSAY

The Bethesda assay was carried out at the special coagulation laboratory located in the McMaster Children's Hospital. Human pooled normal plasma was used as the source of FVIII. The plasma samples sent for analysis were obtained from the following LZT experimental mouse groups: 0.25 IU/Kg, 2.5 IU/Kg and 25 IU/Kg FVIII.

5 RESULTS:

5.1 WEEKLY FVIII INJECTION

As expected, the control mouse groups that received solely PBS had no anti-FVIII IgG present in their blood after week 6. Similarly, all mice treated with 6 weekly injections of 0.25 IU/Kg also had no detectable anti-FVIII IgG present in their plasma. Conversely, after 6 weeks of injections, the mouse groups treated with 2.5 IU/Kg and 25 IU/Kg FVIII developed anti-FVIII IgG in a dose dependent manner. For 2.5 IU/Kg: median: 7.25 µg/mL and range: 0 µg/mL - 120 µg/mL. For 25 IU/Kg: median: 103 µg/mL and range 2 µg/mL - 170 µg/mL. (**Figure 12A)**

After week 10, after 4 weekly immunizations with 25 IU/Kg FVIII, the mice that had previously received 6 PBS injections developed elevated amounts of anti-FVIII IgG: median: 12 µg/mL and range: 0 µg/mL - 163 µg/mL. Furthermore, the mice that were treated with 2.5 IU/kg and 25 IU/Kg in the first phase of the experiment developed even higher levels of inhibitors at the end of the immunization phase. The IgG titers increased in a dose dependent manner, similar to what was observed after week 6. For the 2.5 IU/Kg mouse group: median: 47 µg/mL, range: 0 µg/mL - 121 µg/mL) and for 25 IU/Kg: median: 124 µg/mL, range: 82 µg/mL - 171 µg/mL. Conversely, after the immunization phase, the mice treated with 0.25 IU/Kg had the lowest anti-FVIII IgG titers of all the treatment groups: median: 8 µg/mL, range: 0 µg/mL - 96 µg/mL. **(Figure 12B)**

Notably, 50% of the mice treated with 0.25 IU/Kg FVIII did not develop an immune response after four weekly high dose FVIII immunizations. This was indicated by the absence of anti-FVIII IgG in their plasma on week 11. Furthermore, 27% of the mice treated with 2.5 IU/Kg

FVIIII and 38% of the mice treated with PBS during phase 1 had no detectable anti-FVIII IgG after the immunization phase **(Figure 12B)**. The lack of anti-FVIII IgG seen in the low dose groups, especially the 0.25 IU/Kg group points towards possible tolerization through a LZT mechanism.

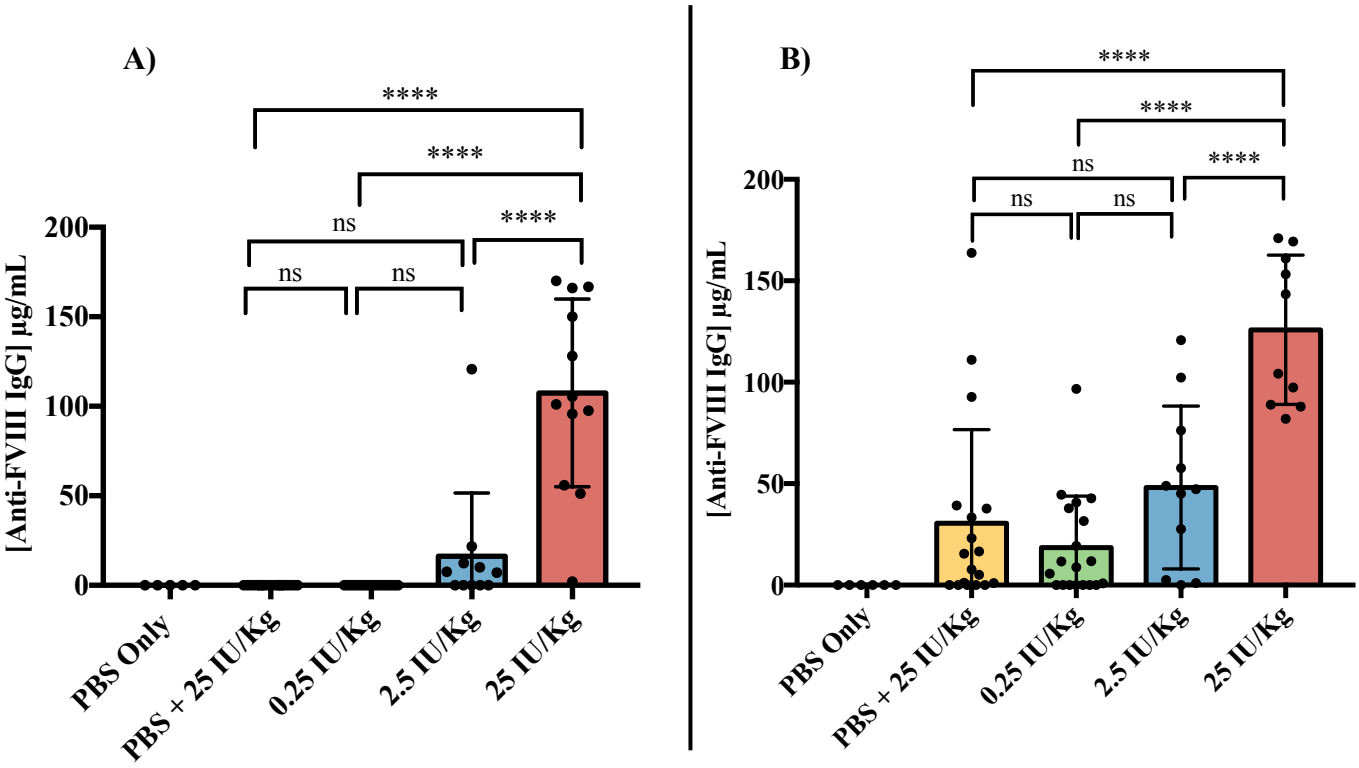

Figure 12: Anti-FVIII IgG titers after week 6 and week 10

A) anti-FVIII antibody titers after injection phase 1/week 6 (low dose phase): This plot displays the antibody titers of each mouse treatment group at the end of injection phase 1 (week 6) of the LZT experiment. At this point in the experiment the "PBS only" group received PBS weekly for 6 weeks, the PBS+25 IU/Kg group received PBS for 6 weeks, and the 0.25 IU/Kg, 2.5 IU/Kg and 25 IU/Kg groups received their assigned FVIII dose weekly for 6 weeks. **B) anti-FVIII antibody titers after immunization phase/week 10:** This plot displays the antibody titers of each mouse treatment group at the end of the immunization phase (week 10) of the LZT experiment. At this point in the experiment all previously treated mouse groups received 25 IU/Kg weekly for 4 weeks, except for the "PBS Only" group which received PBS for 4 weeks. NOTE: n = 6, 17, 18, 13 and 12 from left to right on the graph. Statistical analysis was done via one-way ANOVA with post-hoc Tukey HSD test, $P \leq 0.0001$ = ****, $P \leq 0.001$ = ***, $P \leq 0.01$ = **, $P \leq 0.1$ = *.

To determine whether the tolerance observed after week 10 of experimentation was maintained upon re-exposure to FVIII, all mice were subjected to a two-week rest period and then immunized with an additional dose of 25 IU/Kg FVIII on week 12. One week later their blood was collected and anti-FVIII IgG titers were measured by ELISA. After the booster shot an overall increase in anti-FVIII IgG titers was observed in each treatment group. Upon booster shot administration, the mice treated with PBS + 25 IU/Kg FVIII developed higher levels of anti-FVIII IgG, as seen in **figure 13.** Of the five mice in this group that were tolerized as of week 10, four mice retained tolerance post booster shot, while the other mouse developed low levels of anti-FVIII IgG. Markedly, upon booster injection, 100% of 0.25 IU/kg and 2.5 IU/Kg treated mice that were tolerized at week 10 retained tolerance, suggesting that tolerance through low-dose injections is maintained upon FVIII re-exposure. **(Figure 13)**

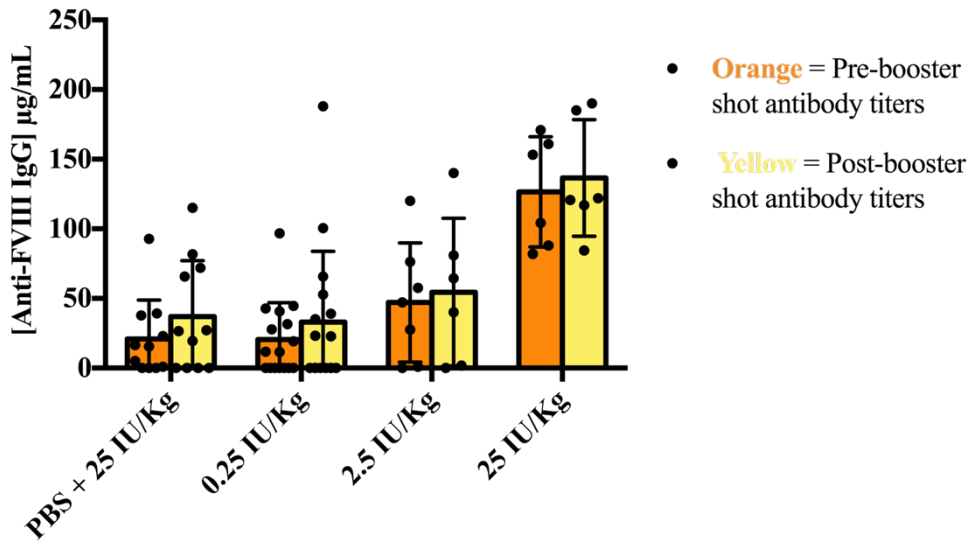

Figure 13: Anti-FVIII IgG titers pre and post booster shot

The **orange** bars display the antibody titers of each mouse treatment group at the end of the immunization phase (week 10) of the LZT experiment. At this point in the experiment all mouse groups received their assigned FVIII dose/PBS for 6 weeks + 25 IU/Kg weekly for 4 weeks. The **yellow** bars display the antibody titers of each mouse treatment group after an additional two-week rest period followed by a booster shot on week 12 of the experiment. N= 6-11.

To determine inhibitory antibody titer, the Bethesda assay was carried out on pooled plasma samples from three mice in each of the following treatment groups: 0.25 IU/Kg, 2.5 IU/Kg and 25 IU/Kg. The plasma used for the assay was collected post-booster shot injection. It is important to note that all of the pooled samples from the 0.25 IU/Kg treatment group came from mice that retained tolerance post booster shot, as this population was a key interest in the study. As shown in **figure 14**, these mice had a Bethesda titer of <0.5 BU/mL, indicating that the tolerized mice did not have detectable inhibitory anti-FVIII antibodies. This data supports the findings shown in figures 12 and 13, which examine the whole anti-FVIII IgG population. Moreover, the 2.5 IU/Kg and 25 IU/Kg treated mice developed inhibitors in a dose dependent manner, with titers of 187 BU/mL and 197 BU/mL respectively, as seen in **figure 14**.

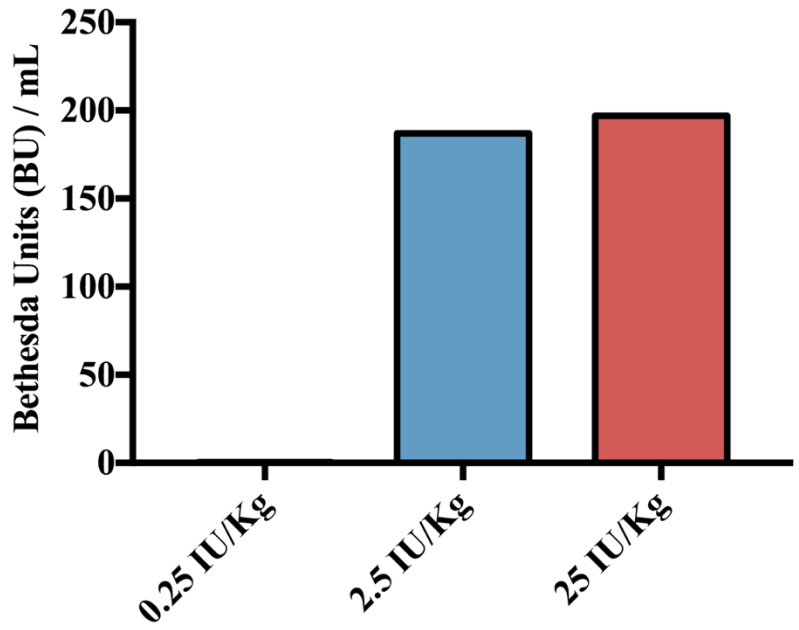

Figure 14: Bethesda Assay

Three samples (n=3) from each treatment group were pooled and tested for inhibitor titer via Bethesda assay. The Bethesda units for the 0.25 IU/Kg, 2.5 IU/Kg and 25 IU/Kg groups were <0.5 BU/mL, 186 BU/mL and 197 BU/mL respectively.

Finally, to further investigate the anti-FVIII IgG immune response in each treatment group, the expression of each of the three unique IgG subclasses was examined. The IgG isotype is further divided into three subclasses: IgG1, IgG2 and IgG3, with IgG2 being further split into IgG2a and IgG2b. Final plasma samples (post booster shot) were collected from mice in each treatment group and were subjected to four ELISAs, each of which detected a different IgG subclass. The results are reported in **figure 15.**

The IgG1 levels in each treatment group is as follows: PBS + 25 IU/Kg: median: 7.8 μg/mL and range 1 μg/mL – 144 μg/mL. In the 0.25 IU/Kg group the median is 0.13 μg/mL and range 0 μg/mL – 6 μg/mL. In the 2.5 IU/Kg group: median: 141 μg/mL and range: 79 μg/mL – 174 μg/mL. Finally, the 25 IU/Kg group had a median of 569 μg/mL and range of 183 μg/mL – 623 μg/mL. Evidently, the expression of IgG1 increases in a dose dependant manner in the 0.25 IU/Kg, PBS + 25 IU/Kg, 2.5 IU/Kg and 25 IU/Kg groups respectively. There is a significant difference in IgG1 expression between each treatment group and the positive control (25 IU/Kg FVIII group). **(Figure 15A)**

No significant differences between groups were observed in IgG2a expression, however there was clearly an enhanced expression in the PBS + 25 IU/Kg and 25 IU/Kg control groups. Select mice from both of these groups expressed detectable levels of IgG2a ranging up to 0.1 μg/mL and 0.4 μg/mL respectively. On the other hand, both of the low dose groups expressed little to no IgG2a. **(Figure 15B)**

Only the 2.5 IU/Kg and 25 IU/ Kg treated mice produced notable levels of IgG2b. The median was 10 μg/mL and the range was 0 μg/mL – 93 μg/mL for the former and the median was 150 μg/mL and the range was 45 μg/mL – 188 μg/mL for the latter. The PBS + 25 IU/Kg and 0.25 IU/Kg groups did not produce significant amounts of IgG2b. **(Figure 15C)**

Lastly, each group except the low dose group produced IgG3. Although the differences between each group were not significant, it is important to note the elevated expression of this subclass in all but the tolerized mouse treatment group. Within the PBS + 25 IU/Kg group, the majority did not express anti-FVIII IgG3, except two which developed anti-FVIII IgG3 to titers up to 11 μg/mL. The mice in the 2.5 IU/Kg group developed the antibody with a median concentration of 10.4 μg/mL and a range of 0 μg/mL – 98 μg/mL. Finally, the mice in the 25 IU/Kg group developed IgG3 with a median concentration of 22 μg/mL and a range of 4 μg/mL – 41 μg/mL. **(Figure 15D)**

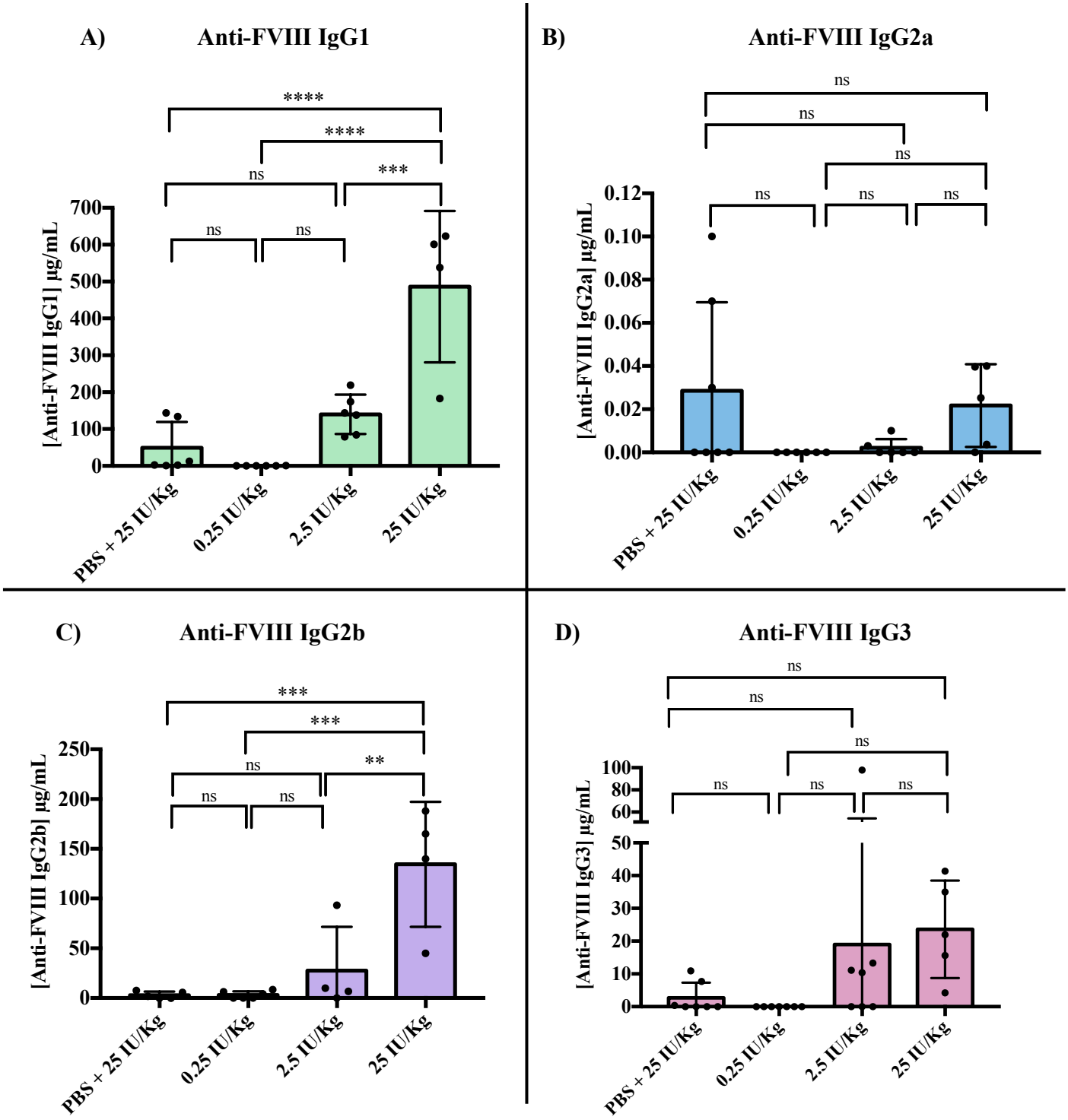

Figure 15: Anti-FVIII IgG Subclass titers

ELISA analysis of post-booster shot plasma samples for IgG subclasses A) IgG1, B) IgG2a, C) IgG2b and D) IgG3 in each mouse treatment group. N=6, 6, 6, 5. Statistical analysis was done via one-way ANOVA with post-hoc Tukey HSD test, $P \leq 0.0001$ = ****, $P \leq 0.001$ = ***, $P \leq 0.01$ = **, $P \leq 0.1$ = *. No significance between treatment groups was observed in figure 15B and 15D.

5.2 BI-WEEKLY FVIII INJECTION

An alternate injection regimen was explored whereby mice received bi-weekly doses of FVIII/PBS, for a total of 6 injections (as depicted in figure 10), mice were bled retro-orbitally after week 3 and their anti-FVIII IgG titers were determined by ELISA **(figure 16A).** Then, from week 3 to 6, all mice received weekly 25 IU/Kg FVIII injections (high dose injection phase), and their blood was sampled for anti-FVIII IgG titers again on week 7 **(figure 16B).** They were then subjected to a two-week rest period, followed by a booster shot of 25 IU/Kg on week 9. One week after the booster shot (week 10), blood was collected via cardiac bleed and the plasma was analyzed as stated above. The pre-booster antibody titers (from plasma samples collected on week 7) were compared to the post-booster antibody titers (from plasma samples collected on week 10), to determine if FVIII tolerance is retained upon re-exposure to FVIII (**figure 17).**

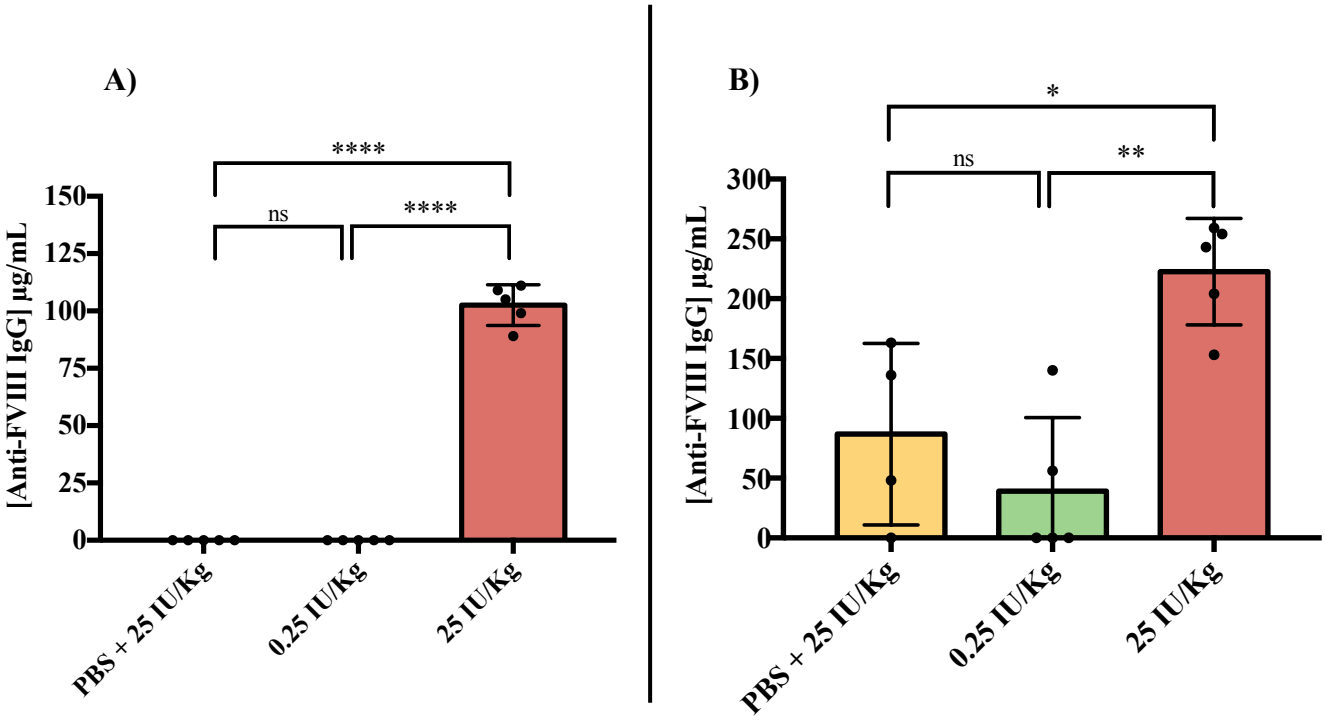

Figure 16: Anti-FVIII IgG titers after week 3 and week 7

A) anti-FVIII IgG titers after injection phase 1/week 3 (low dose phase): bars in this plot display the antibody titers of each mouse treatment group at the end of injection phase 1 (week 3) of the bi-weekly injection LZT experiment. At this point in the experiment the PBS+25 IU/Kg group received only PBS twice per week for 3 weeks, while the 0.25 IU/Kg and 25 IU/Kg groups received their assigned FVIII dose twice per week for 3 weeks. B) **anti-FVIII antibody titers after immunization phase/week 7:** The bars in this plot display the antibody titers of each mouse treatment group at the end of the immunization phase (week 7) of the bi-weekly LZT experiment. At this point in the experiment all previously treated mouse groups received 25 IU/Kg weekly for 4 weeks. N= 5,5,5. Statistical analysis was done via one-way ANOVA with post-hoc Tukey HSD test, $P \leq 0.0001$ = ****, $P \leq 0.001$ = ***, $P \leq 0.01$ = **, $P \leq 0.1$ = *.

The results of the bi-weekly injection regimen follow the same pattern as the results of the weekly injection regimen as seen in **figure 12** versus **figure 16**. After the first phase of injections, mice treated with PBS or 0.25 IU/Kg FVIII did not have detectable levels of anti-FVIII IgG, while those treated with 25 IU/Kg FVIII had significantly high anti-FVIII IgG titers, median: 111 µg/mL and range: 89 µg/mL – 109 µg/mL. After the immunization phase, 75% of the mice treated with PBS developed high levels of inhibitors, with a median of 48 µg/mL and range: 0 µg/mL – 163 µg/mL. The mice within the 0.25 IU/Kg treatment group that did develop anti-FVIII IgG had lower anti-FVIII IgG concentration: median: 0 µg/mL and range 0 µg/mL – 140 µg/mL, when compared to the PBS control mice, and 60% of the mice in this group were tolerant to FVIII as they did not possess detectable levels of anti-FVIII IgG after the immunization phase. Finally, as expected, the anti-FVIII IgG titers of the 25 IU/Kg treated mice considerably increased with a median of 243µg/mL and range 153µg/mL – 259 µg/mL.

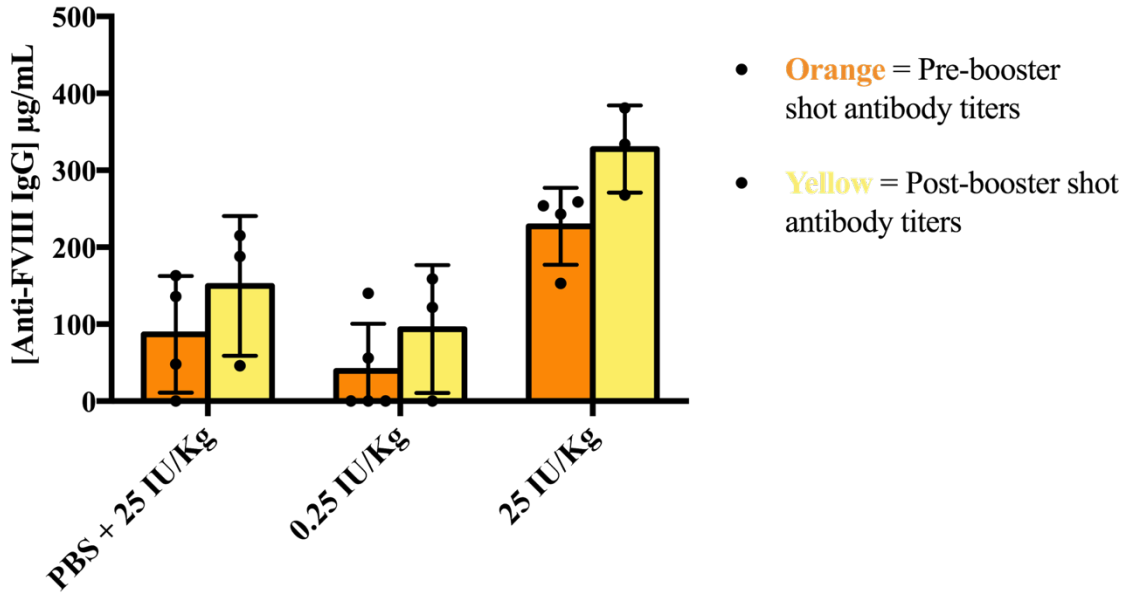

Figure 17: Anti-FVIII IgG titers pre and post booster shot

The **orange** bars display the antibody titers of each mouse treatment group at the end of the immunization phase/pre-booster shot (week 7) of the bi-weekly injection LZT experiment. At this point in the experiment all mouse groups received their assigned FVIII dose/PBS bi-weekly for 3 weeks + 25 IU/Kg weekly for 4 weeks. The **yellow** bars display the antibody titers of each mouse treatment group after an additional two-week rest period followed by a booster shot on week 10 of the experiment. N= 4-5.

Upon booster administration, anti-FVIII IgG titers increased in all treatment groups **(figure 17)**, similar to what was seen in the weekly injection regimen in **figure 13.** Additionally, within the pre-booster shot population, three mice in the 0.25 IU/Kg treatment group had undetectable anti-FVIII IgG titers, and one out of the three mice retained its tolerance post-booster shot. Meanwhile, the sole mouse in the PBS + 25 IU/Kg group that was tolerized pre-booster shot lost tolerance after receiving the booster shot. Due to the small sample size of this experiment, an accurate percent tolerization cannot be determined, however the data shown here indicates that a bi-weekly injection regimen may also effectively induce FVIII tolerance nonetheless.

6 DISCUSSION

Globally, prophylactic FVIII infusions remain a mainstay of HA treatment, which when successful, allow patients to lead a healthy lifestyle. The most serious complication of replacement therapy is the development of inhibitory antibodies which neutralize FVIII, thus preventing the protein from serving its role in the coagulation cascade. To date, inhibitor development remains a largely unresolved concern in HA treatment. With the incidence rate of inhibitors being as high as 40% in severe HA patients, finding new ways to prevent the immune response to FVIII is of immense interest.[30] Tolerizing a patient to FVIII through LZT induction would allow for successful replacement therapy administration, all while serving as a cost effective ITI option when compared to the traditional strategies used, as highlighted in table 2.

6.1 ANTI-FVIII IGG TITERS: WEEK 10

The LZT experiments conducted here show that after ten weekly injections, there is a dose dependent increase in anti-FVIII IgG titer amongst the 0.25 IU/Kg, 2.5 IU/Kg and 25 IU/Kg treatment groups. Although the 2.5 IU/Kg treated mice developed anti-FVIII IgG, it is at similar concentrations to the PBS positive control mice, as seen in figure 12B. Strikingly, 50% of the 0.25 IU/Kg treated mice in the weekly injection experiment developed tolerance to FVIII – which is indicated by an undetectable anti-FVIII IgG titer by ELISA. This conveys that 0.25 IU/Kg FVIII is a promising dose for LZT induction in this model system.

6.2 ANTI-FVIII IGG TITERS: POST BOOSTER SHOT

Furthermore, mice were subjected to a two-week rest period followed by a 25 IU/Kg FVIII booster shot to assess whether tolerance to FVIII is maintained upon FVIII re-exposure. The reasoning behind the two-week break is to allow for germinal centers (GC) to form within the spleen. GCs are specialized microstructures that form in secondary lymphoid tissue such as the spleen.[50] They function to produce B-cells and long-lived plasma cells which secrete antibodies with high affinity to their antigens.[50] Upon immunization, it takes one week for GCs to form and polarize, thus a two-week rest period provides a mouse's spleen ample time to develop an immune response against the infused FVIII. Remarkably, 100% of the 0.25 IU/Kg treated mice that were tolerized after week 10 of the experiment retained tolerance to FVIII after the 25 IU/Kg booster shot. Meanwhile, 20% of the PBS treated mice that did not develop antibodies by week 10 of the experiment developed anti-FVIII IgG after the booster shot. These data further support the notion that the low dose of 0.25 IU/Kg FVIII was sufficient in inducing LZT in our mice.

One of the key risk factors for the development of inhibitors in severe HA patients is the cumulative number of EDs to FVIII concentrates.[51] The majority of patients develop inhibitors early in life, within the first 10 to 15 EDs. In fact, the number of EDs takes precedence over the dose of FVIII being administered, in that a lower concentration of FVIII given for a greater number of days will increase the risk of inhibitor development more than a higher concentration of FVIII given for fewer days. This point has been proven in a separate pilot study carried out in our lab, in which mice who were treated with four weekly doses of 100 IU/Kg FVIII developed approximately 12 μg/mL of anti-FVIII IgG whereas mice treated with 25 IU/Kg FVIII weekly for

10 weeks develop upwards of 170 μg/mL (as seen in figure 12B). With that in mind, the LZT experiments conducted here clearly demonstrate that low dose (0.25 IU/Kg FVIII treated mice) developed less anti-FVIII IgG on average, than the mice treated with PBS + 25 IU/Kg FVIII. This is telling of the power of low-dose FVIII injections in preventing inhibitor formation, as the 0.25 IU/Kg mice were subjected to 11 FVIII EDs while the PBS + 0.25 IU/Kg mice only experienced 5 FVIII EDs, but still developed higher anti-FVIII IgG. Ultimately, the 0.25 IU/Kg treated mice had globally reduced anti-FVIII IgG titers in addition to the noteworthy 50% tolerization rate, despite having experienced 6 more EDs than the PBS control mice.

6.3 BI-WEEKLY FVIII INJECTION

The purpose of the bi-weekly FVIII injection regimen was to explore whether injecting FVIII at a more frequent interval during the low dose phase of the experiment would result in a higher percentage, lower percentage or an absence of tolerized mice. The results highlighted in figures 16 and 17 indicate that the twice weekly FVIII injection interval was capable of inducing tolerance in the 0.25 IU/Kg treatment group. Interestingly, the anti-FVIII IgG trends after the low dose phase, immunization phase and post-booster shot followed similar patterns to those observed in the weekly injection experiment, as seen in figures 12 and 13. Although the results for this alternate injection interval were promising, additional experiments following this regimen were not carried out. This was because the weekly injection regimen resulted in higher tolerization rates and was less arduous on the mice. Also, there was a slight increase in the incidence of eye lesions and fatality in the mice in the bi-weekly injection experiments, as their time under anesthesia and injections were doubled in a seven-day window. Consequently, the Bethesda assay and anti-FVIII

IgG subclasses were not characterized in these mice. Therefore, all the results hereafter reflect the weekly injection regimen.

6.4 BETHESDA ASSAY

Neutralizing anti-FVIII antibodies only reflect a portion of the entire anti-FVIII antibody response. Within the pool of antibodies directed against FVIII, there are many which bind with specificity to FVIII but do not neutralize the protein.[52] Therefore, in order to determine exactly how much of the bulk anti-FVIII IgGs in the treated mouse plasma were inhibitory, a Bethesda assay was performed. This assay requires a sample volume of 1 mL, in order to construct a large range of serially diluted plasma samples for testing. Therefore, multiple mouse plasma samples were pooled in order to carry out Bethesda testing. The pooled plasma samples from each treatment group further verified the findings of the LZT study. The absence of inhibitors in the 0.25 IU/Kg treated mice validates that the plasma of mice treated with low dose FVIII that were tolerized did not have inhibitory antibodies in their plasma, as indicated by the Bethesda titer of <0.5 BU/mL. Accordingly, the non-tolerized mice from the 2.5 IU/Kg and 25 IU/Kg treated groups developed high titer inhibitors as seen in figure 14, with concentrations of 187 BU/mL and 197 BU/mL respectively.

6.5 ANTI-FVIII IGG SUBCLASSES

In addition to neutralizing foreign antigens through fragment antigen binding (Fab) interactions, antibodies perform a host of Fc effector functions by interacting with cellular receptors.[53] The Fc domain is highly conserved, however variations in the constant regions of the heavy chain lead to the production of five isotypes: IgA, IgD, IgE, IgG, and IgM.[53] The IgG isotype

is further divided into four subclasses from IgG1 to IgG4, which are the focus of this portion of the study, since inhibitors are a polyclonal population of IgGs. The secretion of each IgG subclass plays an essential role in the humoral immune responses, thus; studying which IgG subclasses are upregulated or downregulated in the FVIII immune response in HA mice offers important insight into the mechanism behind ITI strategies such as LZT.

The IgG subclasses were measured in mouse plasma samples obtained from each treatment group. IgG is the major isotype within the immunoglobulin family, making up 10% to 20% of the protein found in human plasma.[54] In humans, IgGs are further divided into IgG1 through IgG4, each of which has differing heavy chain structures and downstream functions. Within the context of immune tolerance, IgG4 is a key player. This subclass of IgG often dominates in patients undergoing immunotherapy to induce tolerance to allergens such as pollen, following strategies that are analogous to LZT induction.[54] Consequently, IgG4 is formed upon repeated or long-term exposure to an antigen under non-infectious conditions. Interestingly, in HA patients without inhibitors and healthy patients, IgG4 is completely absent.[55] The absence of IgG4 in HA patients without inhibitors suggests that there is a highly specific immune regulatory pathway that causes FVIII specific IgG4 production in HA patients with inhibitors. Although the IgG subclasses in mice and humans share many properties, it must be noted that mice do not produce IgG4. However; mouse IgG1 is the functional equivalent to human IgG4, as neither subtype is involved in c1q complement activation. [56]

With that being said, as seen in figure 15A, the mouse treatment groups in which high titer inhibitors developed, namely the PBS + 25 IU/Kg group, 2.5 IU/Kg group and 25 IU/Kg groups,

also expressed the highest levels of Anti-FVIII IgG1. The IgG1 concentrations reached up to 144 μg/mL, 174 μg/mL and 623 μg/mL in each of these groups respectively. Among the 0.25 IU/Kg treatment group, the mice that were tolerized had undetectable anti-FVIII IgG1 titers, as expected. Moreover, the mice within this low dose group which produced small, but non-zero levels of total anti-FVIII IgG produced IgG1 at reduced concentrations as well, with a mean titer of 0.22 μg/mL. This average IgG1 concentration is lower than that observed in all the other treatment groups. Strikingly, these findings directly correlate to those seen in human patients. In a study examining IgG subclasses in HA patients that had either successfully undergone ITI (<5 BU/mL) or failed it (>5 BU/mL), it was determined that IgG1 is produced at very low levels in tolerized patients, with an average concentration of 0.35 μg/mL, while it is produced at high levels averaging 46μg/mL in patients with high titer inhibitors.[57] It is important to note that the results seen in the 25 IU/Kg were inflated, as they surpassed the total anti-FVIII IgG titers reported in figures 12 and 13. This was due to an issue with the in house ELISA utilized to obtain the anti-FVIII IgG1 data. Nevertheless, the titers obtained remain of value, as the data accurately reflects the overall ratio of anti-FVIII IgG1 produced between each treatment group, which was the goal of the assay.

As seen in figure 15B, the only treatment groups which produced prominent levels of IgG2a were the PBS + 25 IU/Kg group and 25 IU/Kg positive control mice, with concentrations up to 0.1 μg/mL and 0.04 μg/mL respectively. This observation suggests that upon repeated high dose FVIII infusion, the immune response in mice may be IgG2a mediated. Furthermore, with respect to IgG2b titers, a dose dependent increase was observed in mice from the 2.5 IU/Kg and 25 IU/Kg treatment groups, with concentrations reaching 93 μg/mL and 188 μg/mL respectively. Meanwhile, the PBS + 25 IU/Kg and 0.25 IU/Kg treated mice did not produce significant levels

of IgG2b. It is worth noting that the 25 IU/Kg treated mice had significantly higher IgG2b titers when compared to all other treatment groups, with concentrations averaging 135 μg/mL. This finding coupled with the fact that this high dose treated group also produced substantial amounts of anti-FVIII IgG2a indicates that the IgG2 subclass plays an important role in the immune response against FVIII. This data matches results seen in human patients with HA as well, in which the Anti-FVIII IgG2 subclass was identified as the only hallmark IgG indicative of inhibitor presence. [58] Therefore there is a direct overlap in elevated IgG2 production seen in both humans and mice treated with high doses of FVIII.

Out of all the IgG subclasses, IgG3 is the most multipurpose owing to its vast array of Fc effector functions.[53] In mice, IgG3 is a key activator of the complement cascade in response to infections.[59] It also serves as a potent pro-inflammatory antibody; thus, it is no surprise that the treatment groups with the highest anti-FVIII IgG3 are the 2.5 IU/Kg and 25 IU/Kg treated groups. These mice were infused with the highest doses of FVIII over the course of the 13-week experiment and experienced 6 more exposure days than the PBS + 0.25 IU/Kg treated mice, hence why they had elevated levels when compared to the PBS controls. The 0.25 IU/Kg group had undetectable levels of anti-FVIII IgG when compared to all the other treatment groups. This is especially relevant when compared to the PBS+25 IU/Kg group as the 0.25 IU/Kg mice experienced 6 additional FVIII infusions, yet they still did not possess detectable anti-FVIII IgG3, while the PBS group produced low levels of this antibody. Conversely, in human patients with HA, IgG3 is largely undetectable, irrespective of successful or failed ITI induction.[57] Therefore the IgG3 response seen in mice may be unique to the species.

6.6 NOVELTY OF FINDINGS

This study highlights two major novel findings that have not previously been explored within the context of HA. Firstly, this study revealed that infusing mice with low levels of FVIII at a dose of 0.25 IU/Kg induces LZT that is maintained upon FVIII re-exposure. Although LZT has been explored in depth in diseases such as asthma and food allergy, this thesis investigated LZT in HA for the first time. Seeing as high doses of FVIII up to 200 IU/Kg leads to tolerance in traditional ITI protocols, it made sense to examine whether low dose FVIII could do the same.[60]

The second novel aspect of this study was the stratification of the anti-FVIII IgGs produced by each treatment group into each of the IgG subclasses. Generally, animal studies regarding inhibitors in HA report the antibody titers in total anti-FVIII IgG and BU. In addition to reporting the IgG values in both of the aforementioned forms, this study delved even deeper and further classified which IgG subclasses were upregulated and downregulated in each treatment group. Doing this enriched the study by allowing for a deeper understanding of which IgG subclasses play an integral role in both FVIII tolerance and eliciting a potent immune response to FVIII.

6.7 APPLICATION OF FINDINGS

With gene therapy on the rise, and non-factor therapy options, one may wonder what the long-term applications of LZT in HA treatment are. There is no doubt that alternative approaches to treating HA have greatly enhanced patient care, as they circumvent the issue of inhibitor development. However, this does not change the fact that patients with HA may need FVIII infusions at some point in their life. Whether they are undergoing surgery, dental procedures or they experience a traumatic injury, the first line of defense in these cases is FVIII infusions. Of

course, low dose FVIII alone is not sufficient in protecting against bleeds, so how or why exactly would LZT induction be implemented? If or when a situation arises in which FVIII infusions are necessary, it is extremely advantageous to be tolerized to FVIII, so as to prevent adverse effects upon first exposure to FVIII. Consequently, LZT induction to FVIII could provide a new means to tolerize PUP HA patients to FVIII early in life in an efficient, safe and cost-effective manner, which may benefit them later in life. For instance, LZT induction can be done in conjunction with emicizumab treatment, which would allow for bleed prevention and tolerization to occur simultaneously. Another important aspect to consider is that treatment options such as emicizumab and gene therapy are not currently readily available in developing countries. Hence, having LZT induction as an option for patients in this demographic would allow them to undergo a cost effective ITI treatment option, and later receive prophylactic FVIII as needed.

7 LIMITATIONS

A limitation of this study was the fact that FVIII was not given to the mice continuously over the course of the experiment, rather, it was given repeatedly. This was done by retro-orbital IV injections, which could only be performed a maximum of 12 times, as per the allowance of the animal utilization protocol. This limitation prevented continuous FVIII administration. Ideally, the FVIII should be infused continuously to best represent the discontinuity theory of immunity, which states that the continual administration of a foreign antigen leads to the downregulation of the immune response.[44] It is in fact possible to continuously infuse laboratory animals with substances. For instance, a well-known practice in mice is the use of an osmotic pump.[61] This small cylindrical pump is surgically implanted subcutaneously or intraperitoneally, allowing for the continual administration of a set flow rate of drug for up to 6 weeks.[61] Multiple pumps can also

be placed sequentially to allow for longer drug administration.[61] After the pump is placed, there is no need to handle the animal, which further simplifies the study. However, since HA mice are prone to excessive bleeding, subjecting them to a surgery to place the pump would be very difficult. If it is done successfully, this would accurately represent a continuous low dose injection.

On a similar note, due to the bleeding phenotype of HA mice, it was exceedingly challenging to keep the mice alive for extended periods of time. This made it difficult to further explore the downstream effects of the observed immune tolerance. There is potential to overcome this issue by singly or doubly housing the mice, at most. This would assist in extending their lifespan as the mice would be less prone to experiencing injuries and fight wounds from their cage mates. Additionally, more stringent animal monitoring may also aid in increasing the mouse's lifespan.

Another limitation of this study was the large sample size (1 mL) required for the Bethesda assay. At most, 200 to 400 μL of plasma can be collected via cardiac bleed from a single mouse, therefore, the subsequent data was a combination of multiple mouse plasma samples. This resulted in a reduced sample size reported in the graph, urging the use of more animals to obtain the desired sample size required for statistical analysis. Additionally, since the resulting Bethesda titer reflected a pooled sample, it was not possible to discern the exact inhibitory titer of each mouse. To overcome this issue, an alternate blood sampling method could be utilized to maximize the amount of blood collected from each mouse. For example, for a single mouse, cannulation of the carotid artery allows for a maximum volume of 1 mL of blood to be collected.[62] If this method is employed, larger plasma volumes could be obtained from each mouse, reducing the need for pooled plasma samples for Bethesda testing.

The various ELISAs utilized throughout this thesis were created in house, and normally these assays work well. However, as mentioned in the discussion, the anti-FVIII IgG1 values obtained for the 25 IU/Kg treated mice were inflated, despite having been diluted to fit well within the range of the standard curve of the ELISA. Although the resulting values obtained were still usable as the key goal was to assess the relative ratios of the anti-FVIII IgG1 subclass produced within treatment groups, it would still be beneficial to further optimize this ELISA. Some suggestions to enhance the results and prevent inflated values from appearing in the future include using commercial ELISA buffer packs instead of preparing reagents in house. This removes the degree of human error associated with measuring chemicals and reagents, thereby reducing variance in results. Moreover, when preparing the standard curve, it may be worthwhile to use a mouse anti-FVIII IgG1 to enhance specificity as opposed to the currently used mouse anti-FVIII monoclonal antibody. Although the standard antibody presently used is sufficient, using an antibody with increased specificity would more accurately reflect the target IgG1 and likely improve the results.

Finally, due to limitations in the number of IV injections that could be given to each mouse, it remains unclear whether more FVIII immunizations would have eventually caused the PBS + 25 IU/Kg treated mice that did not produce anti-FVIII IgG, to produce antibodies later on. There is a considerable degree of variation in each mouse's immune response. This means that not all mice will produce anti-FVIII antibodies after 5 FVIII immunizations, as their body may take a few added weeks to fully trigger a response. Therefore, if additional immunizations could be given to the mice, it would allow for a better understanding of just how many mice react to FVIII, and how many are truly tolerized. To explore this, the animal utilization protocol can be amended, to allow for more injections to be administered. If additional injections are not approved, the existing

allowance of 12 injections can be spaced out over longer periods of time. For instance, each FVIII dose can be given 1.5 to 2 weeks apart, as opposed to 1 week apart in the immunization phase of the experiment. This added time will allow for additional GC formation in the spleen and consequently, more antibody production.

8 FUTURE DIRECTION

A question that frequently surfaces regarding these LZT experiments is: how long does the tolerance last? This question can be further explored in future experiments, by keeping the tolerized mice alive, instead of sacrificing them after the booster shot. Thereafter, the mice can be given additional booster shots over the course of several months or even years, to investigate the robustness of FVIII tolerance over a longer time course. Furthermore, the mice can be injected with alternate doses of FVIII ranging from 50 IU/Kg up to 150 IU/Kg to examine if the tolerized mice can withstand higher doses of the protein.

Another avenue worth investigating is whether or not the tolerized mice are more effectively protected from bleeding when compared to non-tolerized mice. It is well known that patients with HA are highly susceptible to joint and muscle bleeds brought on spontaneously or by exercise.[14,15] Therefore, to assess the effects of FVIII tolerization on bleeding, a physiologically relevant mouse bleeding model such as a musculoskeletal treadmill bleeding model or a tail clip bleeding assay can be performed. This would involve the administration of prophylactic FVIII at a dose of 50 IU/Kg to both tolerized and non-tolerized LZT mice followed by subjecting them to the treadmill procedure or tail clip procedure, while the FVIII is still present at pharmacokinetically relevant levels in their circulation. If the results reveal that the tolerized mice bleed less than the non-tolerized mice, this would suggest that the circulating prophylactic FVIII was not neutralized by

inhibitors and thus protected against bleeding more effectively than it did in the non-tolerized mice who have circulating inhibitors against FVIII.

In order to further enhance the LZT study, additional steps should be taken to elucidate the mechanism behind LZT. This would entail in vitro studies using primary splenocytes harvested from tolerized and non-tolerized LZT mice. With these cells, one can examine whether the observed tolerance is B-cell or T-cell mediated. This would involve re-stimulating the cells with FVIII in vitro and profiling which key pro and anti-inflammatory cytokines are produced by the tolerized and non-tolerized cells, as performed previously by Waters et al.[63] Another possible experiment to explore involves incubating the splenocytes with FVIII overnight to allow for immune cell differentiation. Then, the cells could be labelled with fluorescent antibodies specific for plasma cells and T-regulatory cells and analyzed via flow cytometry. Depending on the fluorescence intensity observed, it can be determined whether the FVIII mediated activation of the splenocytes is mediated by regulatory T-cells.

9 CONCLUSION

Inhibitor development in patients with HA remains the primary concern in treating the disease. With the burden, expenses and suboptimal success rates of traditional ITI therapy, investigating other strategies to induce immune tolerance to FVIII in HA are of great value. The LZT experiments conducted here shine light on a novel approach to prevent FVIII inhibitors. The studies outlined in this thesis suggest that frequently administering low doses of FVIII weekly or bi-weekly effectively induces tolerance to FVIII in HA mice. Also, treatment through LZT induction may confer long lasting protection against inhibitor development as evidenced by the complete retention of tolerance in low dose treated mice that were subjected to a rest period and

post-treatment booster shot. These findings are further backed by the inhibitor titers and unique IgG subclass profiles observed in the low dose treated mice, which largely parallel results seen in human HA patients that have successfully undergone ITI. To build on this work it is imperative that the LZT phenomenon be elucidated further by investigating the mechanisms behind LZT in vitro. Moreover, it is worthwhile to explore the upper time bound of the LZT strategy by examining how long tolerance is maintained upon additional FVIII exposures. Finally, studies pertaining to the downstream physiological implications of LZT would greatly enhance this project by determining if LZT to FVIII protects against bleeding by using mouse bleeding models. In sum, this thesis has successfully set up the ground work for future in vivo and in vitro LZT induction studies in a HA mouse model.

Sample Calculations:

Sample calculation 1: determining FVIII dosage based on mouse weight

For a mouse that weighs 22 g and is receiving a 0.25 IU/ Kg injection:

$$0.25\,\frac{\text{IU}}{\text{Kg}} \times \left(\frac{1\text{kg}}{1000\text{g}}\right) = 0.00025\ \frac{\text{IU}}{\text{g}}$$

$(0.00025\ \text{IU/g})(22\text{g}) = 0.0055\ \text{IU}$ **(Dosage)**

***Working solution is 0.001 IU/ µL**

$$\frac{0.0055\ \text{IU}}{0.001\text{IU/uL}} = 5.5\ \mu\text{L}$$

∴ Mix 5.5 µL of working solution FVIII with 94.5 µL PBS

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