EFFECT OF FVIII CO-ADMINISTRATED WITH IVIG IN IMMUNITY TO FVIII IN HEMOPHILIA A MICE

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TITLE: Effect of FVIII co-administrated with IVIG in immunity to FVIII in hemophilia A mice

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Abstract

Background Hemophilia A is X-linked recessive congenital bleeding disorder. Exogenous infusion of FVIII is the treatment of choice in hemophilia A patients. However, inhibitor development remains the major problem in management of Hemophilia A. It has been showed that IVIG has immunomodulatory effects and it has been being used in the treatment of several autoimmune and inflammatory disorders. Here, we investigated the effect of co-administration of FVIII with IVIG on the development of inhibitor in naive and previously immunized hemophilia A mice.

Methods Initially, hemophiliac mice were immunized by weekly intraperitoneal injection of human recombinant FVIII (rFVIII). The mice then were treated, either by rFVIII/IVIG co-injection or rFVIII alone. In the other experimental group, naive hemophiliac mice were treated with rFVIII/IVIG co-injection for four weeks followed by injection of either rFVIII or rFVIII/IVIG. Plasma's anti-FVIII Ab titer was measured using ELISA.

Results Weekly injection of rFVIII led to the development of anti-FVIII Ab in all previously untreated mice. Treatment of those immunized mice with rFVIII/IVIG co-injection did not reduce the level of pre-existing Ab. On the other hand, naive mice treated with rFVIII/IVIG co-injection showed significantly less Ab titer compared to the mice received rFVIII alone after 4 weeks (mean Ab titre of 1 compared to 39, in rFVIII/IVIG co-injection and rFVIII groups respectively). Although the rFVIII/IVIG-treated mice developed immune response following the injection of rFVIII alone, Ab titer in those that kept receiving rFVIII/IVIG co-injection remained lower compared to other groups during the whole twelve weeks of the experiment.

Conclusions Co-injection of rFVIII with IVIG decreased the anti-FVIII immune response in previously untreated hemophilia A mice. These findings suggest that IVIG co-administration can be effective in management of hemophilia A patients at risk of inhibitor development.

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List of Abbreviations and Symbols

FVIII	factor eight
%	per cent
TF	tissue factor
FVII	factor seven
FIXa	activated factor nine
aPTT	activated partial thromboplastin time
PT	prothrombin time
TEG	thrombin generation test
TGT	thromboelastography
IU	international unit
pdFVIII	plasma derived factor eight
rFVIII	recombinant factor eight
APC	antigen presenting cell
TCR	T cell receptor
Th1	T helper type 1
Th2	T helper type 2
IgG	immunoglobulin G
BCR	B cell receptor
Treg	regulatory T cell
vWF	von Willebrand factor
ITI	immune tolerance induction
aPCC	activated prothrombin complex concentrate

IVIG	intravenous immunoglobulin
FcRn	neonatal Fc receptor
MHC	major histocompatibility complex
° C	degrees Celsius
kg	kilogram
g	gram
mg	milligram
ml	milliliter
µl	microliter
M	mole
min	minute
ELISA	enzyme linked immunosorbent assay
Ab	antibody
Ag	antigen
ng	nanogram
nm	nanometer
SD	standard deviation
PBS	phosphate buffered saline
p-NPP	p-nitrophenyl phosphate disodium hexahydrate
DEA	diethanolamine
OD	optical density
4PL	4 parameter logistic
wk	week

Declaration of Academic Achievement

The following is a declaration that the content of the research in this document has been completed by Sajjad Afraz and recognizes the contributions of Dr. Gonzalo Hortelano, Dr. Anthony Chan, Dr. Howard Chan and Dr. Jianping Wen in both the research process and the completion of the thesis. I, Sajjad Afraz, performed the animal experiments, and was responsible for data collection, data analysis and writing of the manuscript. Dr. Gonzalo Hortelano provided study design and guidance on testing and analysis and editorial suggestions during writing of my thesis. Dr. Jianping Wen assisted with the study design and the animal experiments and data collection. Dr. Howard Chan and Dr. Anthony Chan provided insightful advice and manuscript review.

CHAPTER ONE: BACKGROUND

Hemophilia A

Hemophilia A is defined as deficiency of the functional endogenous procoagulant factor VIII (FVIII). It is a genetic disorder and the disease is inherited in an X-linked recessive manner, or it can be resulted from a new mutation in up to 30% of cases [1]. In a healthy individual, vessel injury leads to the platelets activation, as well as the activation of coagulation cascade through extrinsic pathway following the exposure of subendothelial tissue factor (TF) to factor VII (FVII). Thrombin derived from the initial activation of extrinsic pathway cleaves and activates FVIII, which in turn, together with activated factor XI (FIXa), take part in amplification of the cascade and boost thrombin generation [2], [3]. While, the lack of sufficient FVIII in hemophiliacs decreases and delays thrombin generation, that may significantly alter clot formation and result in serious mucosal and internal bleeding.

Hemophilia A affects approximately 1:5000 male births in all races and ethnic groups [4]. In the United States, around 400 babies are born with hemophilia annually [5], and there are approximately 3000 hemophiliacs in Canada [6]. Since it is inherited in an X-linked recessive pattern, the disease occurs almost only in males, with rare exceptions in females. Generally, female individuals are asymptomatic carriers or they may be mildly affected [7]. Clinical symptoms of the disease vary based on the severity of the disease, from easy bruising to more severe spontaneous soft tissue hemorrhage and hemarthrosis [8]. The

disease is classified according to the baseline level of residual FVIII activity in plasma (FVIII:C). The normal range for FVIII clotting activity is 50~150%, whereas the level of FVIII:C in plasma in patient with severe hemophilia A is <1%, which represents up to 70% of cases. FVIII:C level is 1~5% and 5~40% in moderate and mild hemophilia A respectively [1], [8].

Diagnosis

Most people with hemophilia are diagnosed at an early age. They usually present with excessive bruising or intra muscular hematoma with a very little impact [8]. However, people with mild hemophilia may be remain undiagnosed until they are adults. The disease should be suspected in any male patient with a history of easy bruising or excessive bleeding following injury, especially in the presence of frequent bleeding into joint spaces and muscles. Blood coagulation tests will reveal abnormal activated partial thromboplastin time (aPTT), however, the test may be normal in some patients with mild hemophilia. Other laboratory values like prothrombin time (PT), platelet count and bleeding time will be within normal range in hemophilia [1]. A definitive diagnosis will be made following FVIII activity assays, which will show low FVIII:C level [9]. Positive family history of hemophilia will also be frequently present in patients [7].

Precise measurement of FVIII level is curtail for the diagnosis and management of hemophilia A patients [10]. Different types of assays are available for this purpose. The most common method being used is the one-stage activity assay based on the aPTT [11]. However, those conventional clotting assays are not always accurate in determining plasma

level of FVIII:C [7], [10], [12]. Nowadays, other methods that asses global hemostasis including thromboelastography (TEG) and thrombin generation test (TGT) have been introduced in evaluation and monitoring of hemophilia patients.

Treatment

The decreased level of FVIII activity can cause an increased bleeding tendency in hemophiliacs. Degree of the bleeding depends on the level of FVIII:C. The hallmark is deep internal bleeding in joints and muscles which is mainly seen in severe hemophilia [13]. If untreated, frequent bleeding into joint spaces results in hemophilic arthropathy and permanent disability. Most common affected joints are knee, elbow and ankle [9]. In addition, hemophiliac are at risk of excessive life threatening bleeding following major surgeries and trauma [8]. Hence, management of acute bleeding (on demand), as well as prevention of bleeding (prophylaxis) are both crucial in treatment of hemophiliacs [13]. The principal treatment of hemophilia is factor replacement therapy through intravenous administration of exogenous FVIII in both on demand and prophylactic therapy [7]. The factor can be administered during an episode of bleeding or as a prophylaxis in the absence of bleeding. Injection of FVIII during a bleeding episode will stop hemorrhage and secure hemostasis. FVIII dosage needed for this purpose depends on patient profile, site and degree of the bleeding. In most cases more than a thousand international unit (IU) of FVIII is needed for each injection [14]. Management of initial hemarthrosis before occurrence of chronic joint degeneration will prevent the development of arthropathy substantially [13],

[15]. FVIII replacement therapy is started at an early age or with the first episode of bleeding. Treatment regimen varies according to the region [16].

Currently two major types of FVIII products are available. Plasma derived FVIII (pdFVIII), manufactured from human plasma pooled of thousands of donors, was first introduced in 1970s. This was hugely replaced by safer recombinant FVIII (rFVIII) in 1990s in order to minimise the risk of pathogen transmission [17]. Different generations of rFVIII are now available. Furthermore, progress in viral inactivation procedures has been resulted in the development of safer pdFVIII products [9]. rFVIII is being used to a great extent in some countries like Canada, whereas pdFVIII is a product of choice in some European countries [17], [18]. With FVIII products readily available and a proper treatment, there is no significant difference between life expectancy of people with hemophilia and general population [19], [20].

Nonetheless, factor replacement therapy is still very costly [17]. Moreover, FVIII administration can lead to the development of immune responses in the form of allergic reaction or immunoglobulins generation [21]. Production of immunoglobulin against FVIII that inhibit the functions of therapeutic FVIII (inhibitor) remains the major problem in the management of hemophilia A patients.

Factor VIII

FVIII is synthesised mainly in the liver by Kupffer cells and sinusoidal endothelial cells [22]. ProFVIII is a complex glycoprotein with a heavy chain comprising A1-A2-B domains and a light chain comprising A3-C1-C2 domains that interact through a noncovalent link with a metal ion at domains A1 and A3 [22], [23]. FVIII is present in blood in association with vWF which prevents the proteolytic breakdown of FVIII. It is activated following specific proteolytic cuts of B domain residue and one of vWF binding site by thrombin, that results in formation of A1-A2/A3-C1-C2. FVIIIa has a mean life time of 12 hours in humans (8 hours in mice), it is rapidly inactivated by proteolysis and dissociation of the A2 subunit via activated protein C (Fig 1).

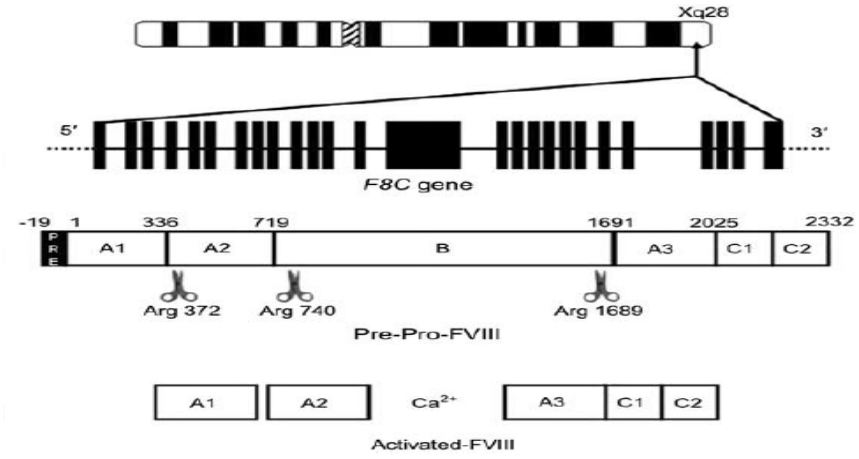


Figure 1 FVIII:C gene and FVIII protein structure [23]

FVIII is a large gene with 186 kb pairs. The encoding gene is located on the long arm of the X chromosome (Xq28) [22]. A large number of molecular defects result in hemophilia A and more than 2000 mutations have been identified [24], [25]. The inversion of intron 22 is the most frequent mutation in patients with severe hemophilia, which cause a complete absence of the protein [22]. Further, double or even multiple mutations in the gene may occur in individual that can result in varied phenotype among patients sharing the same types of mutation [26]. The genetic defects can cause a quantitative decrease in FVIII expression or a qualitative decrease in protein function or both, that result in dysfunctional clot formation. Moreover, patients with severe gene alterations frequently develop a higher antibody titre following exogenous injection of FVIII products [21], [27]. Genetic analysis in all patients with haemophilia is recommended [28]. Molecular genetic testing is used to determine the carrier status, for prenatal diagnosis and for prediction of the likelihood of inhibitor development. However, type of the mutation is not a single determining factor for clinical outcome in individuals. Other factors such as coinheritance of prothrombotic genes and the presence of antiphospholipid antibodies are known to contribute to the clinical outcome and phenotypic heterogeneity among patients [26].

Inhibitor

Inhibitors are specific antibodies that develop against FVIII and neutralize the infused factor. Inhibitor development is the most problematic and costly complication of haemophilia treatment today [29], [30]. The occurrence of an inhibitor can result in high

morbidity and a significant reduction in quality of life in patients [31]. Inhibitor development depends on a complex multifactorial immune response that is influenced by several factors [32]. Risk factors for the development of inhibitor to FVIII include the severity of the disease, the genetic mutation responsible for haemophilia, family history of inhibitors, ethnicity, age of first exposure to FVIII, molecular modifications of the FVIII molecule and the number of exposure days to FVIII [29]. Generally, those with large deletions, severe disease, early exposure to FVIII and family history of inhibitor are at the greatest risk [33]. The development of inhibitor antibody occurs in 10~15% of all patients with haemophilia and it is more common in patients with severe hemophilia A. This event is more frequent in hemophilia A than in other inherited bleeding disorders [13].

Generation of inhibitor

The immunology of inhibitor development is complex and not completely understood [34]. The alloimmune response to therapeutic exogenous FVIII products is believed to develop as a classical T-dependent immune response to an external antigen [35]. The immune system in severe hemophilia A patients is activated after FVIII treatment, since no circulating normal FVIII is present in these patients the infused FVIII may be recognized as a foreign protein. The protein will be internalized by antigen presenting cells (APCs). After being processed, oligopeptides will be formed and these peptides bind to the MHC class II molecules. The complex is then transferred to the cell membrane and FVIII derived peptides are presented to the antigen specific CD4+ T cells through T cell receptors (TCRs) on the surface of CD4+ T cells. Activated T helper cells provide activation signals to antigen

specific B cells that in turn proliferate and differentiate into secreting plasma cells and memory B cells (Fig 2) [21], [23], [36]. APCs probably play a key role in directing the immune system toward either tolerance or immunity through activation of T helper 1 (Th1) and T helper 2 (Th2) cells. Secreted immunoglobulin G (IgG) antibody binds to epitopes in FVIII protein (A2, A3, C1 and C2 domains) and prevent its interaction with other coagulation proteins. Inhibitors typically appear following 10~15 exposure days and they rarely develop after 100 exposure days [37]. Generally, there is an increased risk of inhibitor formation following an initial exposure to high dose FVIII. This is because in intensive FVIII treatment, like following surgical procedures or repeated high dose treatment, high doses of FVIII in combination with tissue damage and inflammation stimulate APCs and amplify immunologic response which could promote inhibitor [38].

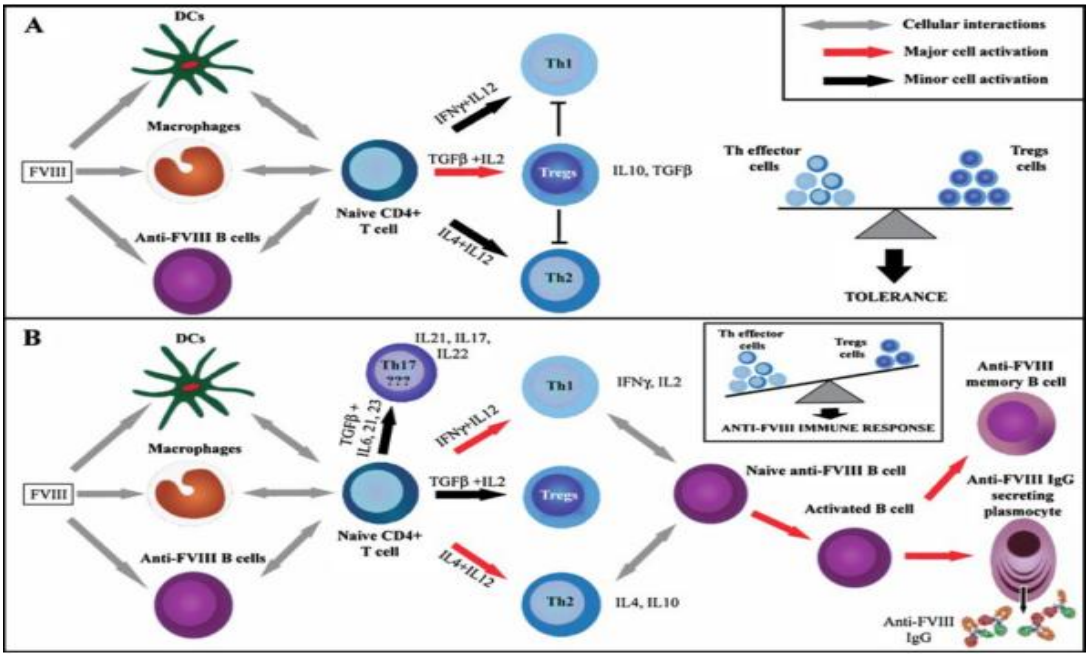


Figure 2 Mechanisms of tolerance induction (A) and the anti-FVIII immune response (B)[21]

Immune cells involved in inhibitor development

Studies from autoimmune diseases demonstrated that antigen specific auto-reactive B cell are very important auto-antigen presenting cells that activate auto-reactive T cells and break T cell tolerance in autoimmunity [39]. B cells from transgenic mice unable to secrete antibodies can function in disease pathogenesis as important APCs for development of proinflammatory T cell responses. Further, the unique position of marginal zone B cells in spleen allows them to play an important role in immune responses to antigens delivered via the blood [34]. Marginal zone is an important transit area for cells leaving the bloodstream and entering the splenic white pulp and contains specific macrophage populations and non recirculating B cells. The initial event in antibody response against proteins is the recognition of proteins by specific B cells receptors (BCRs) expressed on naive B cells [40]. Most of these B cells reside in and circulate through the B cell follicles of peripheral lymphoid organs such as spleen and lymph nodes. BCR binding of proteins initiates the activation of intracellular signal transduction pathways, which can eventually lead to B cell activation and clonal expansion and differentiation into antibody producing plasma cells. Given sufficient stimulatory signals, activated marginal zone B cells can differentiate into short lived antibody-secreting plasma cells independently of T cell help [40].

The presence of circulating CD4+ T cells that proliferate when stimulated *in vitro* with FVIII derived peptides has been reported in inhibitor positive patients with hemophilia A [41]. Moreover, loss of FVIII alloantibodies was seen with infection of HIV in patients with

a history of high inhibitor titers following a drop in CD4+ cell count [42]. Additionally, culturing CD4+ splenocytes from FVIII infused hemophilia A mice exhibited Th1/Th2 cytokine patterns. By analysis of the IgG isotypes, in one study authors concluded that patients who exhibited a higher anti-FVIII immune response had Th2 driven IgG4 antibodies. They suggested that Th1 cells could play a role in initiating FVIII immune response by the release of IFN-γ, and that Th2 has a role in the development of strong inhibitor development by the release of IL-4 [43]. These finding indicate that both B and T cells (Th1 and Th2) are actively involved in inhibitor development and can be potential targets in inhibitor treatment studies.

Factors affecting inhibitor generation

The type of causative mutation is an important risk factor in inhibitor development [27]. Mutations that are expected to cause complete absence of protein would be associated with a higher risk of inhibitors, whereas those that may result in some protein synthesis would be associated with a lower risk of inhibitor development. An immune response against FVIII may occur due to lack of central tolerance to FVIII protein. Patients with FVIII mutations that allow the production of some non-functional FVIII protein may be able to develop partial tolerance to FVIII protein. In these patients, T cells specific for fewer FVIII epitopes are present in the periphery, and the generation of FVIII specific regulatory T cells (Tregs) is possible. In patients with missense mutations and small deletions and insertions, some production of parts of the FVIII protein may occur, and therefore these patients face a lower risk of inhibitor development. Conversely, in patients with FVIII mutations that result in

complete absence of FVIII protein, central tolerance is lacking. Anti-FVIII specific T and B cells are not negatively selected, and no FVIII specific regulatory T cells can be generated. Subsequently, anti-FVIII specific lymphocytes enter the periphery and may react against infused FVIII product [32].

Type of FVIII concentrate is another determining factor that has been implicated in several studies [44]–[46]. However, controversies remain probably due to heterogeneity of the study populations and different data collection systems in those studies. In overall, findings show no significant difference between recombinant and plasmatic FVIII products with regards to inhibitor development [44]. Nonetheless, in one of the most recent finding reported by SIPPET trial, higher incidence of inhibitor generation was seen in previously untreated patients with severe hemophilia A who were treated by rFVIII as compared to those were treated by pdFVIII [47].

The role of vWF in immune protection is not consistent [48]. However, it is shown that vWF was able to block antigen presentation of FVIII and reduced the immunogenicity of FVIII in mice model of hemophilia A [49]. In addition, in a study by Qadura et al, the mice that received pdFVIII treatment that contained vWF had more splenic CD4+CD25+Foxp3+ regulatory cells than mice that received purified rFVIII [50].

Effect of polymorphism on inhibitor formation

Another possible risk factor in inhibitor development is potential mismatched FVIII replacement therapy due to FVIII polymorphism. Six wild type FVIII proteins have been

introduced [48]. Among which, two types , which are the only types seen in white people, have been wildly used to produce FVIII concentrates. As a result, inhibitor incidence can be higher in non-white ethnic groups due to the mismatch between the endogenous FVIII haplotype and the infused product [51].

Furthermore, genetic polymorphisms in immune system genes may affect inhibitor development following the factor replacement therapy. Polymorphism in gene coding MHC molecules, T cell surface molecules and immunoregulatory genes, such as IL-10 and TNF-α cytokine genes, potentially influence the risk to develop inhibitors [52].

Current treatments for patients with inhibitor

Once an inhibitor developed, two general treatment options are available for the hemophilia A patient; Using FVIII bypassing agents to treat acute episodes of bleeding, and immune tolerance induction (ITI) to permanently eradicate the inhibitor. Bypassing agents, like activated rFVII and activated prothrombin complex concentrates (aPCC), are effective in management of acute bleeding. They are important tools to reduce morbidity in patients before they undergo ITI and in those with persistent high inhibitors titer [53], but the cost of the treatment limits the extensive use of these agents [54]. Because of the substantial burden causes to the patients and the health care system, inhibitor eradication should be performed in as many patients as possible which this by itself often requires the expensive treatment strategies [37].

ITI currently is the best method to successfully eradicate inhibitors. It is the treatment of choice for patients with inhibitors particularly those with severe hemophilia A. Tolerance induction can eliminate the neutralizing antibodies and allow patients to receive regular FVIII treatment following that. It can prevent the development of haemophilic arthropathy and results in improvement in quality of life and also health economic benefits [33]. The process of ITI involves repeated and long term exposure to FVIII concentrate with the goal of inducing peripheral tolerance to FVIII.

ITI outcome is influenced by different variables. Host related factors like lower inhibitor level before performing ITI and peak titers during ITI, or treatment related variables like bleeding, central venous catheter infection, FVIII product type, and dosing regimen are all potentially correlated with ITI success and time to achieve success [55]. Successful tolerance is defined as the elimination of inhibitor and the ability to use FVIII for the treatment of bleeding without developing inhibitor by patient. This is usually achieve after 14~16 months of treatment [56]. Exact mechanism of action of ITI is not fully understood, some possible mechanisms include inhibition of B cell memory and induction of T cell anergy and suppressor T cells [57]. Immunosuppressive medications can be added to the ITI regimens of patients with poor responses. However, they are currently not considered as a routine part of ITI in patients with hemophilia A [55].

Nonetheless, ITI is not successful in some patients. It leads to a return to a normal FVIII response in 60% ~ 70% of patients underwent induction [58]. Additionally there would be a risk of recurrence in part of patients after successful tolerance induction [57]. Such a risk varied between several studies, and clinical factors that influence the inhibitor recurrence

have not yet been fully understood [57]. Accordingly, the likelihood of success and longer term benefit of ITI needs to be weighed against the risks and costs associated with ITI therapy. Moreover, inhibitors development in patients with mild or moderate hemophilia A can be more challenging than in those with severe hemophilia. The bleeding pattern of these patients often becomes more severe and the benefit of ITI for them not demonstrated [59], [60]. Hence, alternative therapeutic methods are demanded for the group of patients who failed to respond to ITI.

Intravenous immunoglobulin (IVIG)

IVIG is the concentrated antibodies solution purified from thousands of donors [61]. This number of donors help IVIG solution to maintain polyclonal nature of antibody and antibody diversity seen in healthy individual. The main content of IVIG solution is IgG [61]. It was firstly administrated as immunoglobulin replacement therapy to prevent infection in immunocompromised patients in 1950s [62]. Immunomodulatory effects of IVIG was reported for the first time by Imbach et all. in 1980s. They observed elevation in platelet count in ITP patients receiving IVIG [62]. Since then much attention has been paid to this effect of the drug and it has been used in the treatment of increasing number of autoimmune and inflammatory disorders as well as immunodeficiency disorders [63], [64]. IVIG treatment currently is approved in number of diseases. It is also being administrated off-label in variety of the disease [63]. Although IVIG is widely being used in clinics, its mechanisms of action are not fully understood yet [65]. Several mechanisms of action have

been proposed for the multiple beneficial effects of the IVIG. Indeed, IVIG may have multiple mechanisms for its action [62]. However, further investigation are demanded in order to clarify the exact mechanisms [65]. Potential mechanisms of action of IVIG include; a) inhibition of phagocytosis by blocking the binding of immune complex to Fcγ receptors, b) modulation of antibody half life through saturation of neonatal Fc receptor (FcRn),c) anti-idiotypic antibodies that downregulate autoreactive B cells, d) upregulation of inhibitory Fcγ receptor, e) inhibition of deposition of activated complement on target tissues[62], [66], [67]. The IgG molecules induce IVIG therapeutic effect by targeting the specific pathway in immune system through each mechanism. Although some of the mechanisms have been proven *in vitro* and *in vivo*, the obtained results were not always consistent [66].

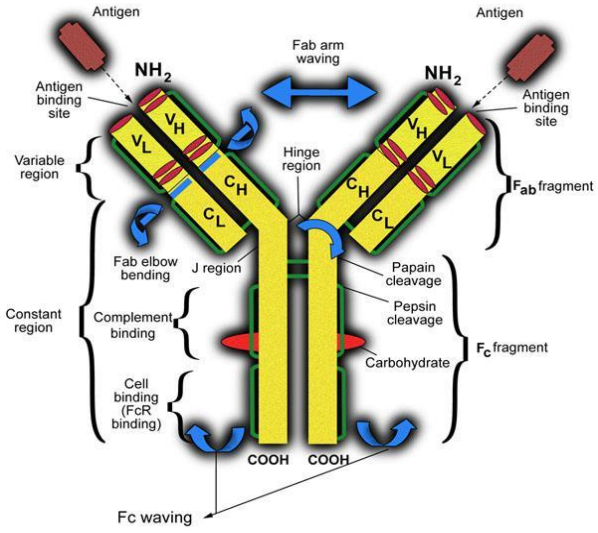


Figure 3 **Schematic view of IgG molecule**. CH, constant region of heavy chain; CL, constant region of light chain; VH, variable region of heavy chain; VL, variable region of light chain [63].

Effects of IVIG on regulatory T cells

Another proposed mechanism for the effect of IVIG is its effect on the expansion of Tregs [62]. In several mouse model of experiments, IVIG induced proliferation of Tregs in the spleen [68], [69]. Tregs are effective immune cells in tolerance induction [70] and tolerance induction by stimulating the proliferation of Tregs is a potential therapeutic targets in autoimmunity and regulation of the anti-FVIII reactive immune response. A study suggests that the inhibitor positive or negative status of the patients may reside in the inability or ability of the patients' immune system to induce regulatory TGF-β producing Th3 cells in the periphery [71]. Furthermore, when CD4+ CD25+ Tregs from tolerized mice were transferred to syngeneic hFVIII hemophilia A mice, there was tolerance induction in the recipient mice [72]. These observations suggest that both natural and adaptive Tregs may be associated with the control of the anti-FVIII immune response under physiological conditions. Adding to this, there is an epitope for Tregs called tregitope located in IgG molecule [73]. It has been showed that tregitopes induce expansion of CD4+ CD25+ Tregs and enhance immunoregulatory effects. It induced tolerance induction *in vitro* and *in vivo*, and had therapeutic effects on mice model of autoimmune diseases [69], [74].

Taking all together, addition of IVIG will potentially enhance immunoregulatory effect following the infusion of FVIII and may inhibit the inhibitor development in hemophilia A treatment.

Antigenic competition

Antigenic competition is the phenomenon in which inhibition of the immune response to one antigen occurs following its administration in the presence of another antigen [75]. The competition occurs at several levels of the interaction between antigen-immune cells complex and will lead to the reduction of antibody production [76], [77]. As it has been described, related and unrelated peptides compete each other to the MHC binding site, thus in a presence of the ideal amount of second antigen peptides derived from two antigens compete to bind to the related MHC molecule, as a result fewer peptide from each antigen are presented on the surface of the APCs, results in fewer T cells priming thus fewer antibody production [76]. Several factors are involved in this phenomenon. The most important one seems to be a antigen 1 : antigen 2 concentration ratio [78]. In fact, studies have reported the effect of antigenic competition in a decreased level of inhibitor development in hemophilia A mice [75], [79]. Administration of FVIII products in presence of another protein (FIX/vWF) led to the reduction in inhibitor formation and they concluded this reduction is due to the competition occurred between FVIII and the other protein. Same effect can be seen by the addition of IVIG to FVIII in hemophilia A mice in this project. Both human IVIG and FVIII can be recognized as foreign antigens by mice immune system, thus the system will react to each protein. Addition of suitable amount of IVIG to the hrFVIII may obstacle the immune response against FVIII and decrease the inhibitor formation.

Objectives

The main objective of this project is to investigate the potential effect of FVIII co-administration with IVIG on inhibitor formation. Considering the co-injection of additional protein (IVIG) with FVIII, and moreover, based on immunomodulatory effects of IVIG, this can prevent the inhibitor development in mice model of hemophilia A. This project will aim to develop alternative therapeutic method in inhibitor treatment.

Specific Aims

1. To investigate the effect of IVIG co-injection on inhibitor level in pre-immunized hemophilia A mice

2. To investigate the effect of IVIG co-injection on inhibitor development in naive hemophilia A mice

CHAPTER TWO: MATERIALS AND METHODS

Animals

Hemophilia A mice (C57Bl/6$^{FVIII-/-}$) housed in the McMaster University Central Animal Facility were used at age of 8wk ~. The mice were maintained under controlled lighting (12:12 light:dark) and temperature (22° C) with access to food and water. All animal experiments were approved by the Animal Research Ethics Board at McMaster University in accordance with the guidelines of the Canadian Council for Animal Care.

Experimental groups

The experimental groups are shown on Table 1. Groups of hemophilia A mice (G1 ~ G3) were immunized by weekly intraperitoneal injection of human rFVIII (Kogenate® FS, Bayer, Germany) for four consecutive weeks. The mice then either received rFVIII alone or co-injection of rFVIII with human IVIG (G2 and G3, respectively) for another eight weeks. In the other experimental design (G4, G5), naive hemophilia A mice were weekly treated with co-injection of rFVIII with IVIG for four weeks followed by eight weeks injection of either rFVIII (G4) or rFVIII and IVIG co-injection (G5). The treatment period was 12 weeks in total. FVIII administrated dose was 2 IU/mouse and IVIG dosage was 1g/kg. Blood samples were collected 50 ~ 100µl each time prior to each injection via retro orbital plexus using heparinised capillary tubes. Plasma was separated and stored in -20°C [80].

	1st four weeks	2nd four weeks	3rd four weeks	Animal number
G1	FVIII	-	-	5
G2		FVIII	FVIII	6
G3		FVIII + IVIG	FVIII + IVIG	6
G4	FVIII + IVIG	FVIII	FVIII	7
G5		FVIII + IVIG	FVIII + IVIG	8

Table 1 Experimental groups

Anti-FVIII Ab ELISA

Mouse anti-hFVIII Ab was detected by enzyme linked immunosorbent assay (ELISA) [81]. Appropriate ELISA assay was designed and mice immune response to human rFVIII were investigated by measuring the titre of anti-FVIII Ab of plasma. The whole procedure was as below:

Coating

Coating buffer (0.1M/L Na_2CO_3, pH 9.6) was warmed to room temperature and desired number of ELISA strips (Corning® clear polystyrene high bind Stripwell, Corning, New York) were assembled in a frame. Previously reconstructed rFVIII solution (1 IU/10µl, equal to 100ng/10µl) was defrosted at room temperature. The coating antigen (rFVIII) was then diluted 1/100 in coating buffer and each well was coated with 100µl of the solution (containing 50ng of rFVIII) using multipipettor. The plate was covered and incubated 2h at room temperature.

Blocking

The plate was emptied and washed with 200µl phosphate buffered saline with Tween 20 (PBS/T; 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.4, 0.1% Tween 20) 3 times and it was let dry. Tween 20 was added for more effective washings, to prevent non-specific background staining. To wash, washing buffer was applied with multipipettor, then shook the plate gently, empty plate over the sink and vigorously banged on paper towel. Blocking buffer (5g of skim milk powder in 100ml PBS/T) was made fresh and 200µl was applied to each well with multipipettor. The plate was covered and incubated 2h at room temperature.

Adding samples

The plate was emptied and washed as before. Plasma samples were defrosted at room temperature and were diluted in blocking buffer and mixed on vortex. Then, 100µl of each diluted sample was applied to each well in plate with single channel pipette. The plate was covered and incubated over night at room temperature.

Detecting Ab

The plate was emptied and washed again as before. Detecting Ab (goat anti-mouse IgG (H&L) alkaline phosphatase conjugated antibody, Aviva Systems Biology, San Diego, CA) was diluted in blocking buffer and 100µl of mixed solution was applied to each well with multipipettor. Diluting the antibody in blocking buffer will help minimise non-specific binding and background. The plate was covered and incubated 2h at room temperature.

Chromogenic substrate

The plate was emptied and washed. Developing solution was freshly prepared by dissolving one p-Nitrophenyl phosphate disodium hexahydrate 5mg tablet (p-NPP, Sigma-Aldrich, Oakville, ON) per 5ml DEA buffer (1M Diethanolamine, 0.5mM $MgCl_2$, pH 9.8). 100µl of the solution was added per well with multipipettor.

Reading the absorbance

Developing yellow color was evaluated and compared with the standards to get the optimal development time. Color reaction was then stopped by adding 50µl/well of 3M NaOH using multipipettor, with same timing used in the step before. At the end, the absorbance was read at 405nm wavelength using SpectraMax® Plus microplate reader.

Standard curve

Commercial mouse polyclonal anti-human FVIII antibody was purchased (Abcam, Toronto, ON) and used as a standard on each ELISA plate. Two series of two-fold serial dilutions of the commercial antibody was prepared and were applied on plates. Average absorbance value of each set of duplicate standards was calculated, then standard curve was created by plotting the mean absorbance (y axis) against the corresponding dilution (x axis) and drawing a best fit trend line using 4 parameter logistic (4PL) regression model in SigmaPlot (Systat Software, San Jose, CA).

Optimization of anti-FVIII Ab ELISA

Several series of ELISA with varying conditions were performed in order to obtain suitable optical density (OD) amount with the lowest background (Table 2). In each run of experiment, secondary Ab dilution and reading time were changed and the results were compared. Based on the results from Table 2, ELISA with secondary Ab dilution of 1/1000 and the reading time of 20min gave the most desirable result.

Abcam anti-FVIII polyclonal Ab dilution	Secondary Ab dilution	Reading Time (min)	Obtained O.D	Background
1/20	1/1000	20	1.58	0.17
1/20	1/1000	30	2.27	0.23
1/20	1/1000	40	2.77	0.3
1/20	1/1000	50	3.01	0.38
1/20	1/1000	60	3.14	0.42
1/20	1/1000	90	3.33	0.63
1/20	1/2000	20	1.07	0.14
1/20	1/2000	30	1.48	0.16
1/20	1/2000	40	1.88	0.2
1/20	1/2000	50	2.17	0.24
1/20	1/2000	60	2.42	0.26
1/20	1/2000	90	2.86	0.36
1/20	1/5000	20	0.46	0.12
1/20	1/5000	30	0.66	0.14
1/20	1/5000	40	0.88	0.16
1/20	1/5000	50	1.05	0.14
1/20	1/5000	60	1.2	0.2
1/20	1/5000	90	1.69	0.25

Table 2 Optimization of anti-FVIII Ab ELISA

Anti-IVIG Ab ELISA

Similar method was used to detect mouse anti-human IVIG Ab in the sample plasma. Briefly, all wells were coated with hIVIG 100ng/well in 100 µl of coating buffer. Blocking steps were under taken following incubation period and washing the plates. The samples then were applied to each well same as above, incubated and the plates were washed. Next, 100µl of goat anti-mouse IgG with a 1:10000 dilution was applied to each well and incubated. The plates were washed and 100µl of substrate was loaded into the wells. The reaction was stopped at 20min by adding 50µl of 2.5M H_2SO_4 and the plates were read at 450nm wavelength.

Calculations and Statistical Analysis

To make the results obtained from different ELISA comparable, OD values were converted to titres based on a standard curve on each plate. By creating a 4PL regression line in SigmaPlot, the equation of the graph would be calculated as:

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

In which, y = absorbance, x = concentration, a = minimum asymptote, d = maximum asymptote, c = inflection point, b = Hill's slope of the curve.

By solving the equation for x and multiply it by the dilution factor, the titre of each given OD was calculated.

$$x = c \times \left(\left(\frac{a-d}{y-d} - 1 \right) \right) \text{^} \left(\frac{1}{b} \right)$$

All titre values in the result section are shown using the same calculation.

A cut-off point was calculated as an average of negative samples plus 3 Standard Deviation (SD) [82]. Plasma samples from untreated hemophilia A mice (i.e. samples from day 0 of mice in G2~G5) in addition to negative controls (dilution buffer) on each plate were considered as negative samples. There were total of 37 negative samples. Values below the cut-off points were considered negative.

Data are expressed as mean + SD. Graphs were produced in Microsoft Excel 2007. Comparison of data was performed using IBM SPSS Statistics 22 using One Way ANOVA and Tukey post hoc test. Values $p < 0.05$ were considered significant.

CHAPTER THREE: RESULTS

Therapeutic effect of IVIG co-injection

The effect of FVIII and IVIG co-administration in previously immunized hemophilia A mice was evaluated by comparing anti-FVIII Ab level between G2 and G3 (Fig 5a). Fig 4 indicates a standard curve of related ELISA. The absorbance cut-off was 0.82, any value below that was considered negative. The hemophilia A mice were first immunized in both groups through weekly injection of rFVIII 2IU for four weeks. FVIII inhibitor level is deemed to be high at this point. The animals then received mixed solution of FVIII (2IU) and IVIG (1g/kg) for eight weeks in G3 to assess the treatment's effects on already developed inhibitor level. The mice in G2 kept receiving the same dose of rFVIII for another eight weeks as a control. One mouse from each group expired during the experimental period due to the complication from the sample collection procedure. Anti-FVIII Ab development pattern of each mouse in G2 and G3 is demonstrated in Fig 5b. Anti-FVIII Ab was developed in all mice after four weeks. As it is shown, the level of antibody increased gradually during the first four weeks and kept increasing in both groups following that. The differences between two groups were not significant (Fig 5a).

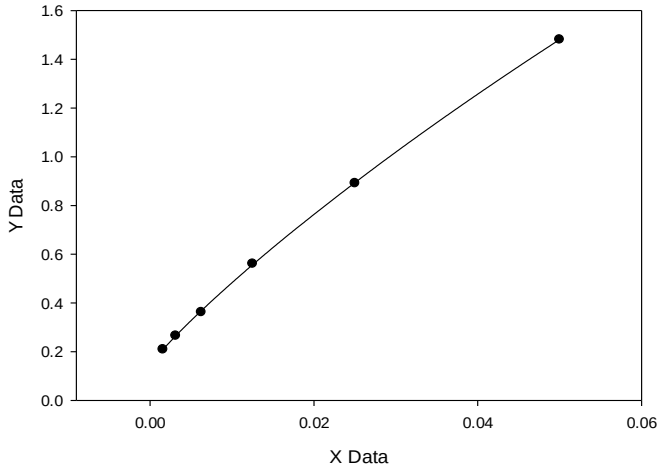
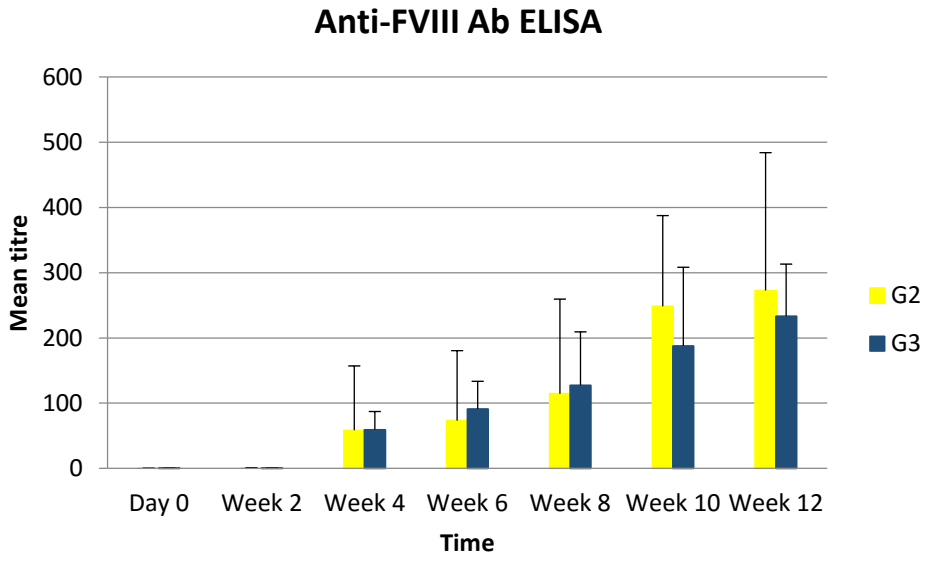
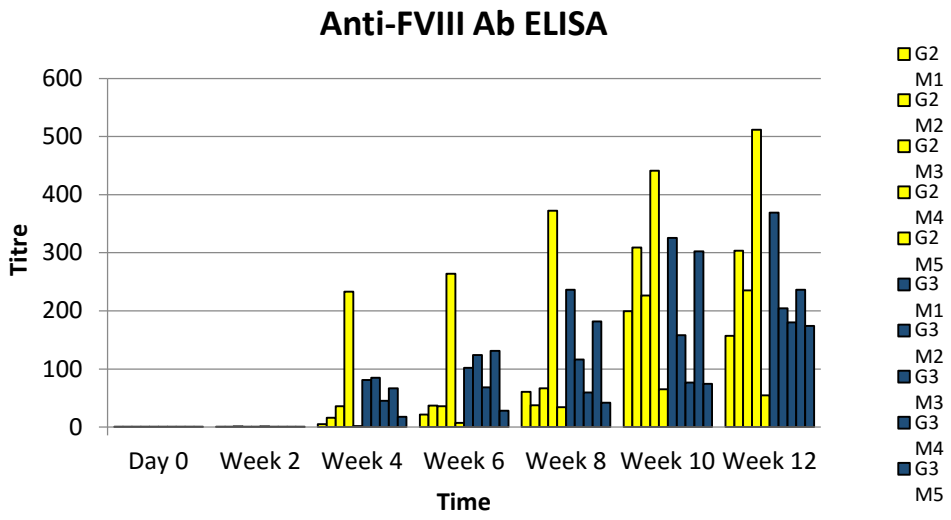


Figure 4 Standard curve from the ELISA for samples from G2 and G3. X axis is concentration and Y axis is absorbance. $R^2 = 0.9999$.

a)



b)



c)

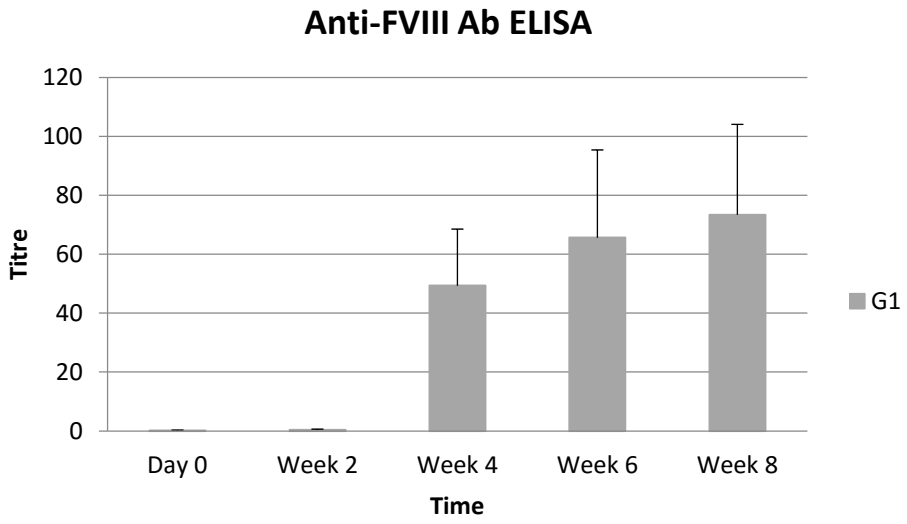


Figure 5 Anti-FVIII Ab development following the co- injection of FVIII and IVIG in previously immunized hemophilia A mice. a) First, mice were immunized with FVIII during the first four weeks, then they started receiving FVIII + IVIG (G3), or kept receiving FVIII alone (G2). The differences between two groups are not significant. b) Antibody development pattern of each mouse in G2 and G3. c) Antibody level in mice in G1 as another control group, in which animals did not receive any drug following the four weeks of immunization with FVIII. Error bars indicate SD.

Prophylactic effect of IVIG co-injection

Effect of FVIII and IVIG co-injection on inhibitor development in naive hemophilia A mice was evaluated. Naive mice were treated with FVIII + IVIG for four weeks. Comparison of anti-FVIII Ab level between treatment and the control groups is indicated in Fig 7. Comparing to the mice received FVIII alone, the Ab level was significantly lower in the treatment group at week 4 ($p<0.001$). While all animals in control group produced anti-FVIII Ab, only two mice in the treatment group developed antibody against FVIII and no antibodies were detected in the other mice following four weeks of the treatment.

Mice then received FVIII for the remaining eight weeks in G4 to evaluate the tolerance induction effect of the treatment, whereas mice in G5 were treated with FVIII and IVIG co-injection for another eight weeks. G2 (12 wk injection of FVIII) and G3 (4 wk injection of FVIII followed by 8 wk of FVIII/IVIG co-injection) were used as control groups. One animal from G4 was euthanized during the experiment due to the complication following the bleeding. In addition, anti-FVIII Ab development in each individual mouse is shown in Fig 10. Cut-off point was calculated as 1.24 and 1.06 for the relevant ELISA for G4 and G5 respectively. All mice in G4 developed antibody following the injection of FVIII. Antibody level against FVIII was still negative in two mice from G5 after six weeks. However, the antibody level became positive in all animals in G5 after eight weeks.

Anti-FVIII Ab generation rate increased in G4 following the exclusion of IVIG from the treatment. There was no significant difference in antibody levels in G4 compared to those of

G2 and G3 (Fig 8 & Fig 9). Although the antibody level gradually started to increase in mice in G5, the level was significantly lower compared to G2 over the whole experimental period except at week 8 (Fig 8, $p < 0.05$). This difference was not significant in G5 compared to G3, however, the Ab level remained lower in G5 during the whole experiment (Fig 9; mean titre of 87 compared to 233 at week 12).

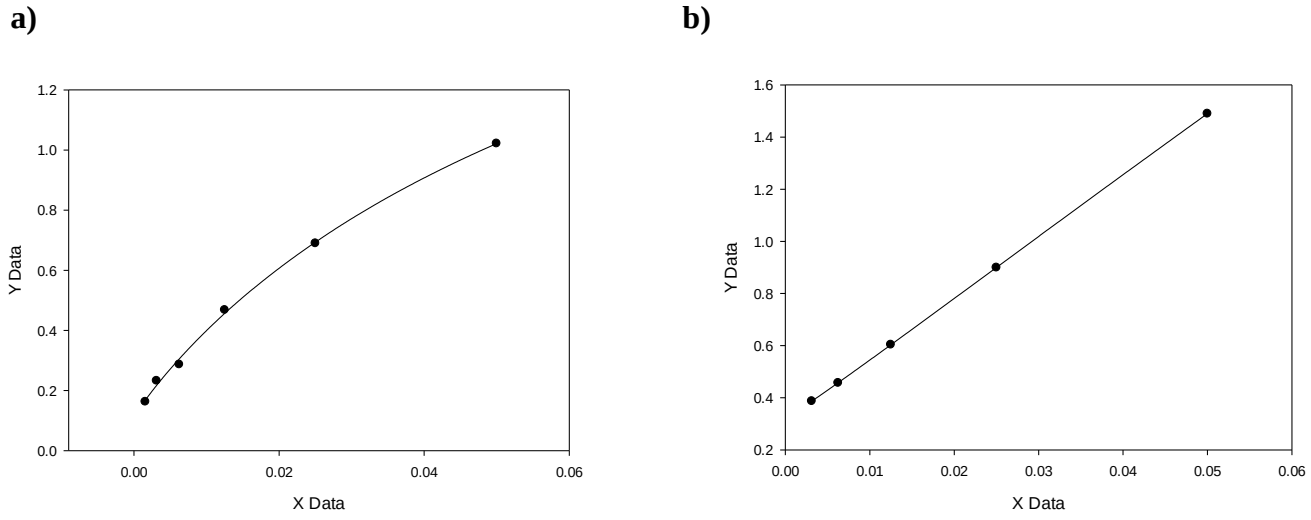


Figure 6 4PL regression line of the ELISA on samples from G5 (a) and G4 (b). X axis indicates concentration and Y axis indicates absorbance. $R^2 = 0.9986$ and 0.9999 respectively.

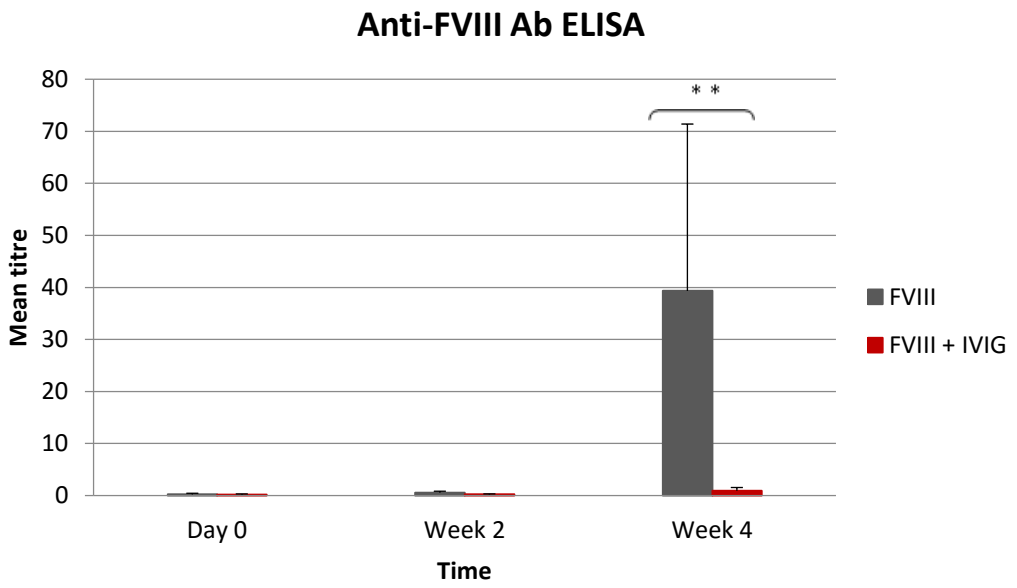


Figure 7 Comparison of anti-FVIII Ab level between the treatment and the control groups. Mice in treatment group received FVIII + IVIG. Mice in control group received FVIII alone. The antibody level was significantly lower in the treatment group at 4 weeks (** $p<0.001$). Error bars indicate SD.

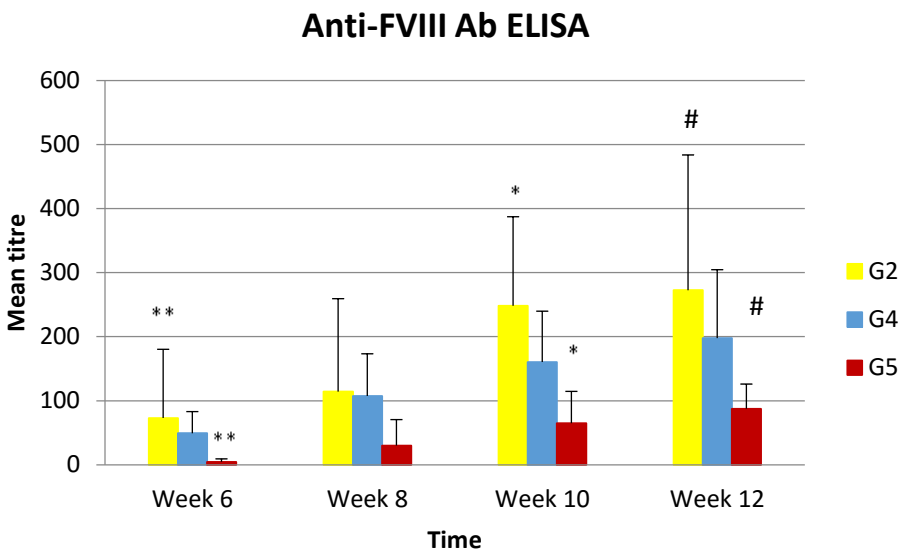


Figure 8 **Anti-FVIII Abs development in G4 and G5 compared to G2**. Mean titre at each time point is compared to mean titer of G2. Antibody level was significantly lower in G5 compared to G2 at 6 (** p=0.005), 10 (* p<0.05) and 12 weeks (# p<0.05). The Data was not significantly different in G4 compared to G2. Error bars indicate SD.

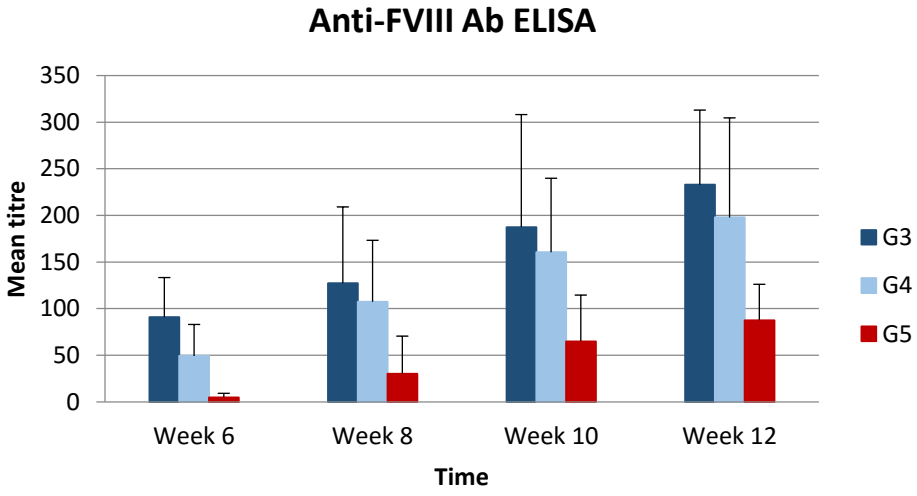


Figure 9 **Anti-FVIII Abs development in G4 and G5 compared to G3.** Mean titre at each time point is compared to mean titer of G3. The difference was not significant. Error bars indicate SD.

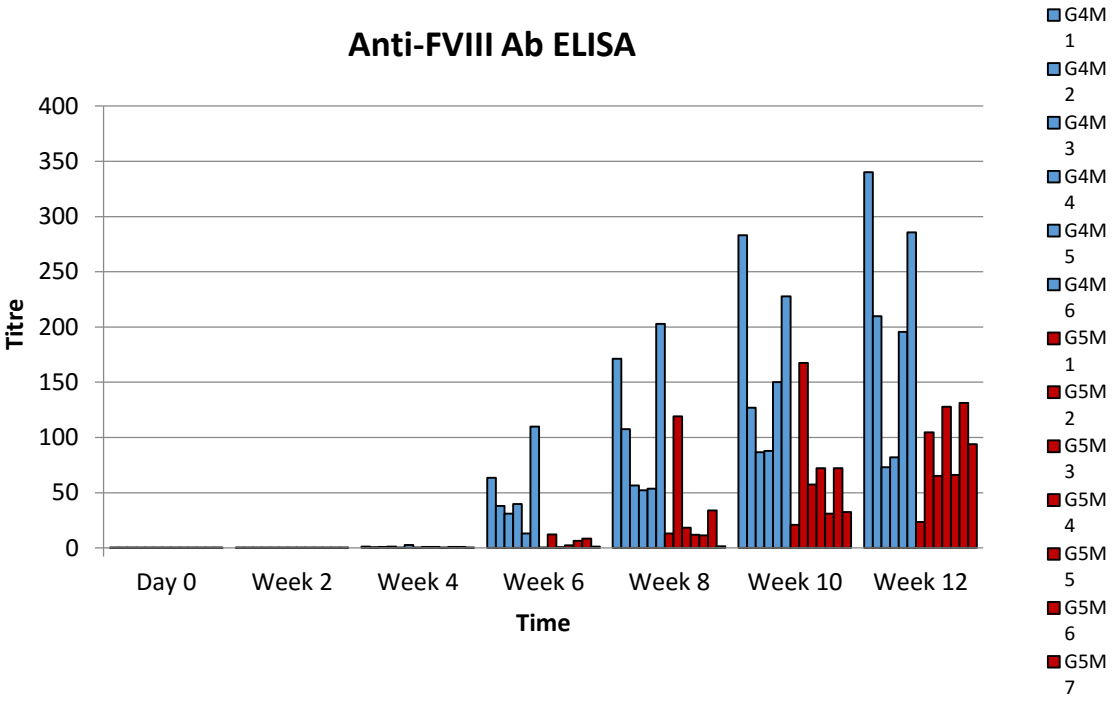


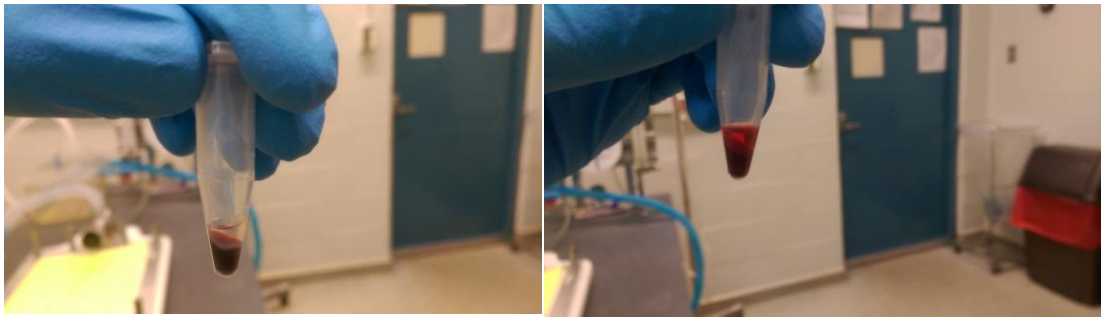
Figure 10 Anti-FVIII Ab development pattern of each mouse in G4 and G5. Mice in both groups were treated with FVIII and IVIG co-injection for the first four weeks. Mice in G4 then received FVIII alone, while mice in G5 kept receiving FVIII together with IVIG. Data are shown as titre.

Evaluation of probable effect of hemolysis on ELISA results

Since some degree of hemolysis were inevitable during collection of blood samples, another experiment was conducted so as to investigate the possible effect of hemolysis on the obtained results. For this, two blood samples were collected from a naive hemophilia mouse. Hemolysis was induced mechanically in one of the samples by repetitive suction using a core needle during the blood collection (Fig 11a). Normal and hemolyzed samples then were mixed together with different ratio into several eppendorf tubes. Each tube

contains different concentration of hemolyzed sample from 0% ~ 100%. Anti-FVIII Ab ELISA was performed for all mixed samples based on the same protocol as before. As it is shown, there was no significant difference on the ELISA results obtained from the normal sample and the sample with hemolysis (Fig 12).

a)



b)

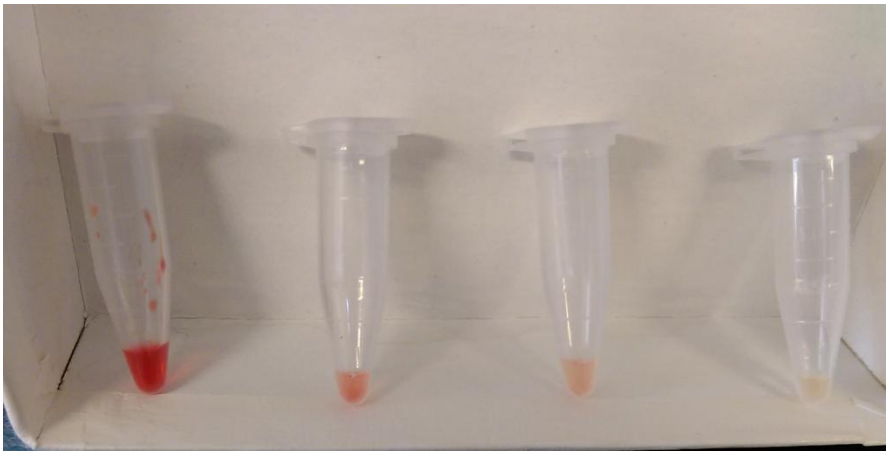


Figure 11 Normal and hemolyzed plasma samples. a) Normal and hemolyzed blood samples are shown. Plasma of these two samples were separated and then were mixed in different ratio and ELISA was performed. b) Left tube is related to the plasma sample with intentional hemolysis, other three tubes are from experimental samples which had some level of hemolysis. As it can be seen on the picture, degree of hemolysis in experimental samples was far less compared to the sample with intentional hemolysis.

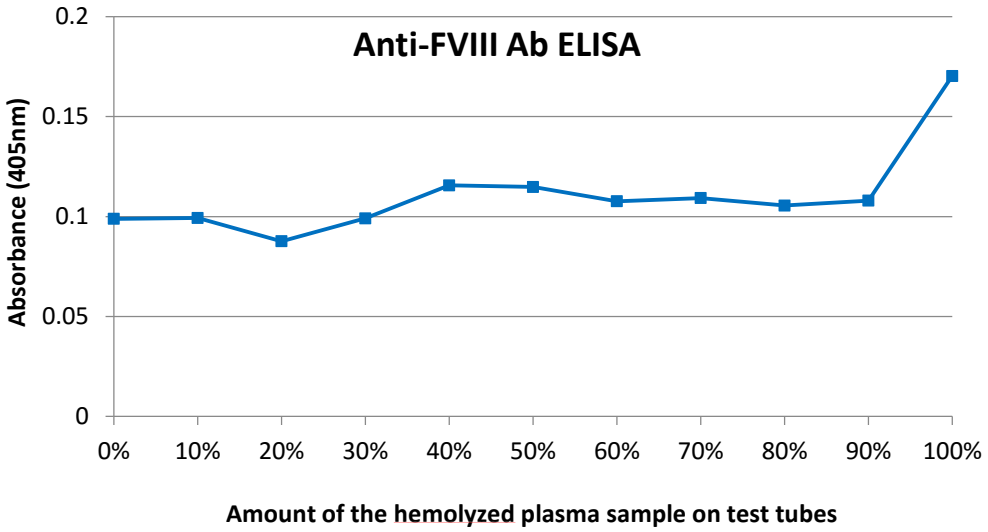


Figure 12 Comparison of hemolyzed samples and the real samples. ELISA was performed on several test tubes, each tube contains varying ratio of normal and hemolyzed plasma samples. Degree of hemolysis did not affect the absorbance.

Evaluation of probable effect of IVIG molecules on ELISA results

In order to evaluate possible binding and interactions between human IVIG and FVIII molecules in plasma samples, and to rule out the possibility that those potential interaction would affect the results from ELISA, a different ELISA assay was designed. The well was first coated with FVIII s before. Next, human IVIG was applied to the well, and then goat anti-human IgG, as a detecting Ab, was added to the plate and absorbance was read at 405nm wavelength. The absorbance was similar to the negative control (buffer) and no signal was obtained (Fig 13). There was no binding between IVIG and FVIII molecules, human IVIG did not bind to the coated rFVIII in anti-FVIII Ab ELISA.

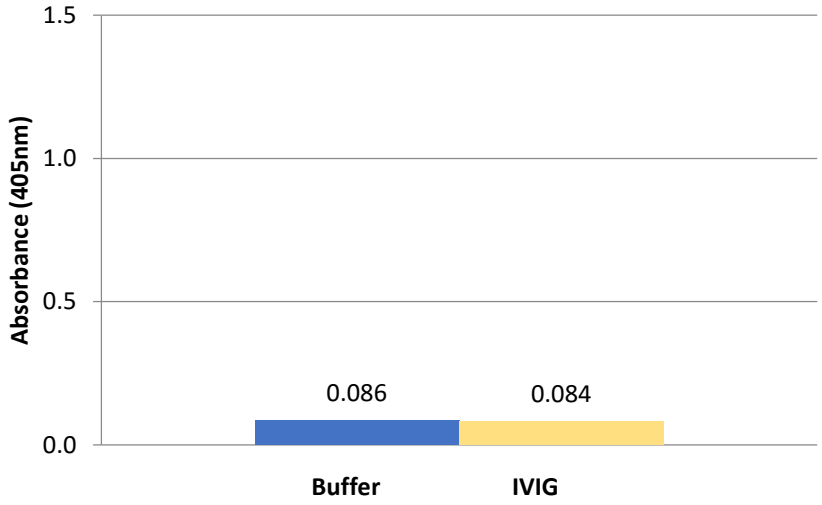


Figure 13 Anti-FVIII Ab ELISA on IVIG solution. There was no difference in OD compared to the control.

Additional ELISA assay was performed to specify how the presence of IVIG molecules in the test sample may affect the ELISA results. Plate were coated with rFVIII once again. Next, in one well human IVIG was applied. In another well only diluting buffer was applied (negative control). Plasma sample deemed to be containing high amount of inhibitor level (G2M4 at week 8) was applied into the third well as a positive control. For the last well, the same sample as the third well plus human IVIG were applied. Goat anti-mouse IgG was added as a detecting Ab in next step into the all wells. The absorbance was read at 405nm wavelength (Fig 14). Based on the results from, addition of human IVIG did not change the absorbance of the test samples. Presence of IVIG molecules in the test environment did not cause a significant difference in OD obtained from anti-FVIII Ab ELISA.

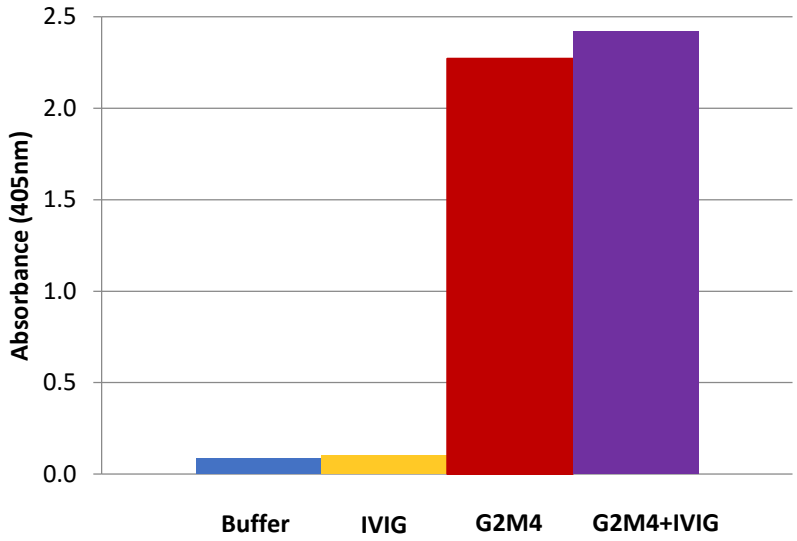


Figure 14 Results from anti-FVIII Ab ELISA. ELISA was performed on four different samples to specify whether the presence of IVIG molecules in samples would affect the obtained OD or not.

Evaluation of tolerance induction following the treatment

After 12 weeks of treatment, mice in G5 received weekly injection of FVIII alone for another 8 weeks in order to evaluate whether extended treatment with FVIII and IVIG co-injection for 12 weeks would induce active tolerance to FVIII or not. The results are indicated in Fig 15. Following the exclusion of IVIG from the treatment, anti-FVIII Ab generation rate increased and the mice produced more Ab against FVIII. No reduction or inhibition in anti-FVIII Ab development was seen (Fig 15).

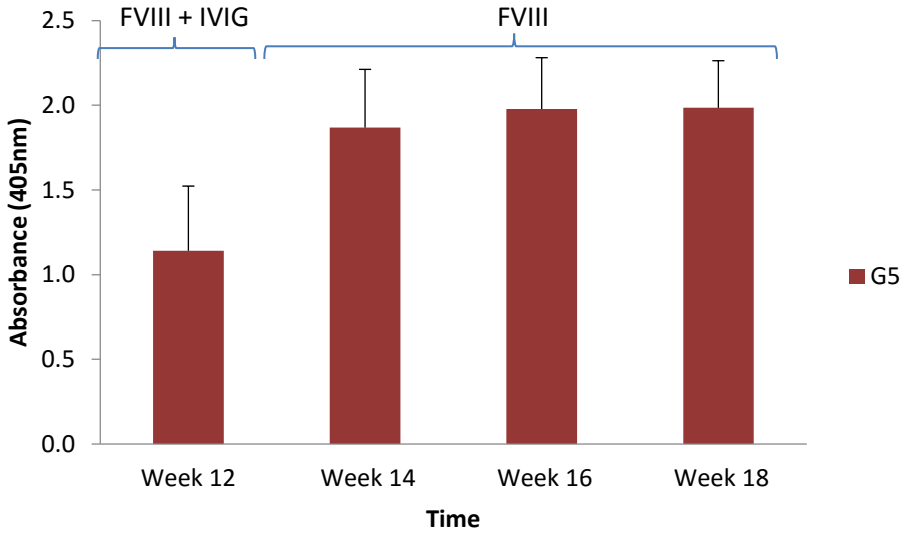


Figure 15 Anti-FVIII Ab development following 12 weeks of treatment with FVIII and IVIG co-injection in naive hemophilia mice. Mice were first treated with FVIII + IVIG for 12 weeks and then, they received FVIII alone. Data are shown as OD. Error bars indicate SD.

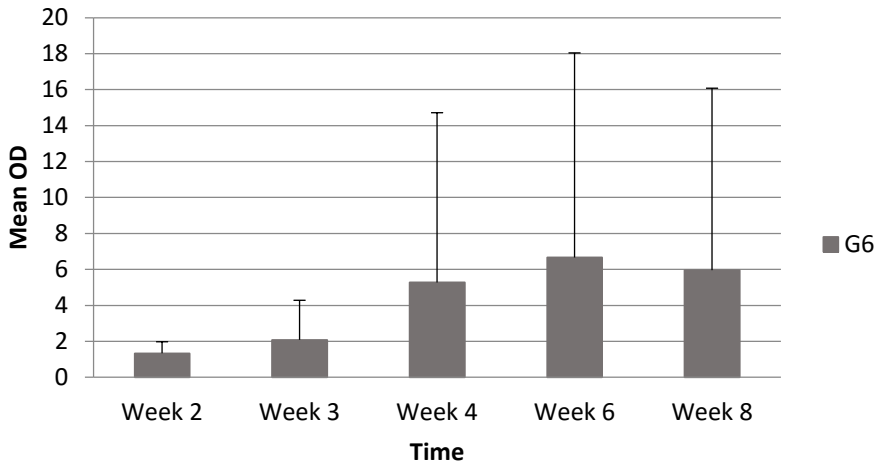
Anti-IVIG Ab ELISA

To assess mouse immune response against human IVIG, new experimental group was designed (G6). The group of mice were treated with weekly injection of IVIG (1g/kg) for four consecutive weeks and anti-IVIG Ab levels were measured by ELISA (Fig 16a). One mouse out of five expressed a very high level of aniti-IVIG Ab, while remaining mice developed lower Ab level (Fig 16b; absolute OD amount of 22.1 for mouse 1 compare to average absolute OD of 1.1 for other four mice at week 4).

The same assays were conducted to measure anti-IVIG Ab level of mice in G3, G4 and G5 (Fig 17a). In addition, Ab developing patterns of each individual mouse are indicated in

Fig 17b, c, d. Approximately half of mice in each group didn't seem to generate anti-IVIG response or developed very low Ab level (Fig 17b, 17c and 17d, for G3, G4 and G5 respectively).

a)



b)

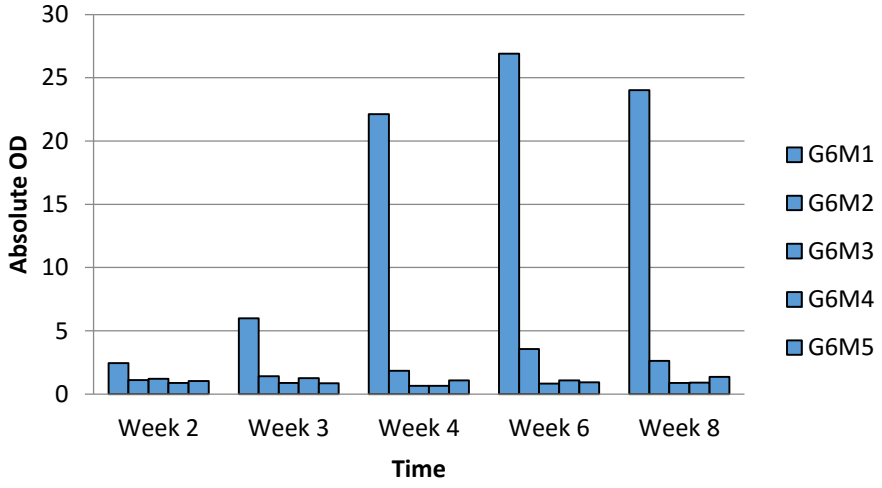
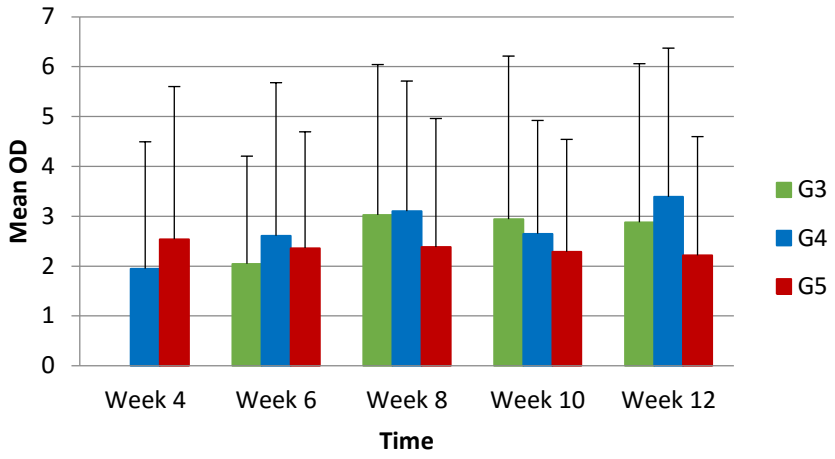
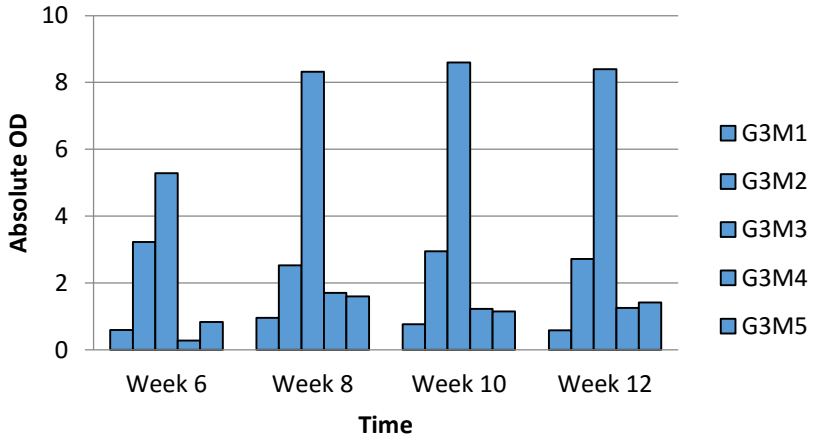


Figure 16 Anti-IVIG Ab development following four weekly injection of human IVIG. a) The data are shown as Means OD for each week. Error bars indicate SD. b) Ab developing pattern of each individual mouse in G6.

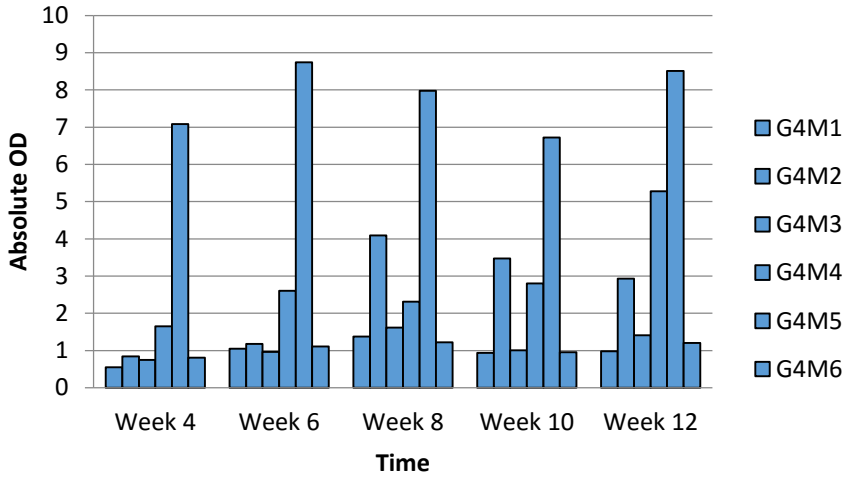
a)


7
6
5
4
3
2
1
0
Mean OD
Week 4
Week 6
Week 8
Week 10
Week 12
Time
G3
G4
G5

b)


10
8
6
4
2
0
Absolute OD
Week 6
Week 8
Week 10
Week 12
Time
G3M1
G3M2
G3M3
G3M4
G3M5

c)



d)

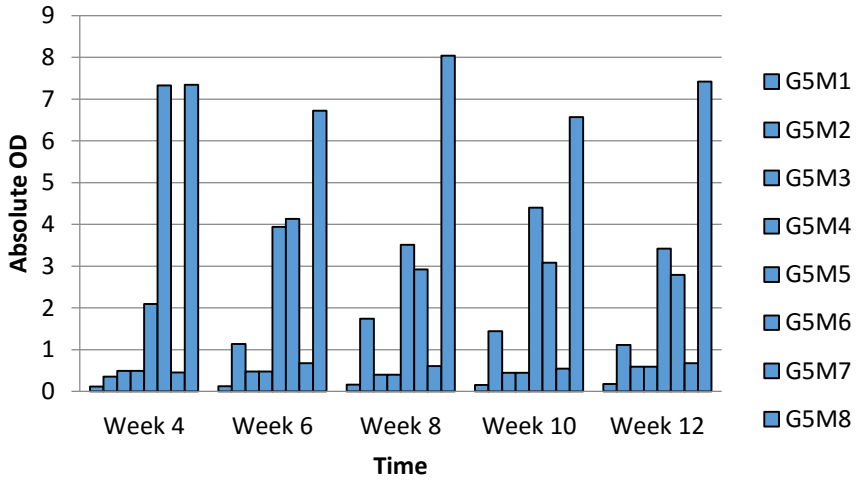


Figure 17 Anti-IVIG Ab ELISA. a) Anti-IVIG Ab development in mice in G3, G4 and G5. Error bars indicate SD. b), c), d) Ab developing pattern of each individual mouse in G3, G4 and G5 respectively.

CHAPTER FOUR: DISCUSSION AND CONCLUSION

The aim of this project was to assess how the co-administration of FVIII with IVIG will alter the nature of the immune response generated in hemophilia A mice. The hypothesis was that infusions of FVIII protein together with IVIG will reduce the immune responses to human FVIII, both in naive and hemophilia A mice with inhibitors. Based on the preliminary results, co-injection of FVIII and IVIG will reduce the immune responses to human FVIII in hemophilia A mice. In that previous experiment, treated mice developed significantly low level of anti-FVIII Ab at third week of the experiment. This project's goal was to reproduce those results and furthermore, to evaluate the effect in therapeutic and prophylactic model in longer time period in order to assess the potential clinical relevance of any observed effect. Also it aims to determine tolerance to infused FVIII, and possibly determine the involved mechanism of such effect.

The findings of the study were in part supported by the hypothesis but not fully. Both therapeutic and prophylactic effects of FVIII/IVIG co-injection were investigated in this study. Therapeutic effect was evaluated by investigating the effect of co-injection on already developed inhibitor level in previously immunized hemophilia A mice, while prophylactic effect was evaluated by investigating the effect of co-injection on inhibitor development in previously untreated hemophilia A mice. The findings indicate that FVIII co-administrated with IVIG did not fully inhibit the inhibitor formation, however, it significantly reduced and

delayed anti-FVIII immune response in previously untreated hemophilia A mice compared to controls.

Comparison to other studies

Based on the results obtained from ELISA for anti-FVIII Abs, antibody level in naive hemophilia mice treated with FVIII/IVIG co-injection was significantly lower than hemophilia mice received FVIII alone during the first 4 weeks (Fig 7). This was consistent with the results observed in previous studies. Modulation of immune response to hFVIII by co-administration of other Ag was previously reported [75], [79]. Qadura et al., compared immune responses to human FVIII in hemophilia mice [79]. They treated Balb/c E16 hemophilia A mice with 4 weekly intravenous injection of 2 IU hFVIII either alone or mixed with different doses of human FIX. Total anti-hFVIII Ab was measured using ELISA. They observed that mice generated significantly lower anti-FVIII Ab titres in the presence of high concentration of FIX. Moreover, they did not observe high anti-human FIX Abs in these mice. The authors suggested that antigenic competition might be responsible for the observed effect. Reduction in FVIII inhibitor level was also reported in a study comparing immunogenicity of different human FVIII products in hemophilia A mice [75]. Here mice received FVIII products containing high amount of human-vWF elicited lower anti-FVIII Abs level compared to mice treated with FVIII products without vWF. Unlike patients, the murine immune system recognize human proteins (vWF) contained in FVIII product as a foreign antigen and therefore reacts to them. As a result, competition occur in recognition

and presenting of two antigens to the immune cells, and consequently influence the development of the immune response against FVIII [75].

The role of antigenic competition

Previous studies on antigen competition emphasize that competition happen mainly at the level of peptide binding to the MHC molecules in antigen presenting cells [76], [77]. This may partially explain the negative results obtained in this project, where the therapeutic effect of FVIII and IVIG co-injection was investigated on already developed inhibitor level. Co-injection of FVIII and IVIG did not cause a reduction in inhibitor level in previously immunized mice (Fig 5a and b) . Possible explanation for this could be, the mice were immunized following four weeks injection of FVIII prior to the addition of the IVIG to the regimen. In this case proliferation of the cognate T cells had been occurred following the prior immunization, specific antibody producing B cells and memory cells had been formed [40]. Thus the antigenic competition probably did not occur here and the treatment didn't have any effect in the inhibition of the already developing inhibitors. Furthermore, here we used IVIG with the dosage of 1g/kg which mimics the dosage used in clinics. However, studies suggested that sufficient amount of the competitor is needed in order to observe the antigen competition effect [76], [78], [79]. Co-administration of IVIG probably with higher dose may show different effects. Additional experiment with a dose escalation of IVIG is needed to get the ideal amount of IVIG in this regard.

Effect of IVIG in tolerance induction

In order to assess the tolerance to FVIII, naive hemophilia mice which were previously treated with FVIII and IVIG co-injection for four weeks started receiving weekly injection of FVIII alone (G4). Following the discontinuing IVIG and injection of FVIII alone, the inhibitor generation rate increased and the level of anti-FVIII antibody went high in them (Fig 8, 9 and 10). In another experiment, mice in G5 received FVIII injection after being treated with FVIII and IVIG co-injection for twelve weeks. Here again, exclusion of IVIG from the treatment regimen resulted in increased rate of anti-FVIII immune response in mice (Fig 15). In this study, treatment of naive hemophilia mice with FVIII and IVIG co-administration did not induce active tolerance to FVIII. Nonetheless, presence of IVIG in the treatment regimen reduced anti-FVIII Ab levels. Mice in G5 which were treated with FVIII and IVIG co-injection for the whole twelve weeks, produced significantly lower anti-FVIII Ab compared to other groups. (Fig 8 and 9). Although the antibody level gradually increased in them, the treatment effects last longer comparing to the mice in G4 and the level remain significantly lower over the whole experiment time. This effect can be ascribed to the presence of IVIG. The presence of IVIG in the treatment reduced the anti-FVIII immune responses, although this effect was caused by other potential mechanisms and not through tolerance induction.

The IVIG solution used in this experiment was human IVIG. The Imuunomodulatory effects of human IVIG in mice were shown in several studies [68], [83], [84]. Repeated injections of high dose (1.5 g/kg, IP) human IVIG twice a week for three months improved behavioral

function in mice model of Alzheimer's disease, and there was no significant increase in anti-human IgG antibody [83]. In other study, injection of human IVIG, 0.4 g/kg/day for two weeks, increased the proportion of the Tregs in the spleen in mice [68]. IVIG induced minimal immune response in above study. Nevertheless, mouse IVIG is likely to have more significant immunoregulatory effect in mice [67], [68] . In this project, around half mice from the experimental groups which had IVIG in their therapeutic regimen (G3, G4, G5 and G7) did not develop high anti-IVIG immune response, while other produced relatively high anti-IVIG Ab (Fig 16b, 17b, c and d). No logical relation was found between anti-IVIG and anti-FVIII immune response in them. However, there seems to be mice which developed significantly high immune response against human IVIG also had a high anti-FVIII immune response. The immune responses to IVIG in mice suggests the need to evaluate the co-administration of FVIII with mouse IgG, rather than human IVIG. Conducting an experiment in which hemophilia mice are treated with mouse IVIG ,instead of human's, could be beneficial in this regard.

Limitations

One of the limitations in this study was variation in anti-FVIII immune responses seen among mice of the same group. The experimental mice were highly inbred, hence they are considered to share almost identical immune system characteristics. Hemophilia mice in the same group which had received the same treatment should exhibit very similar immune response. In this project ,however, there were at least 1 ~ 2 mice in each group that behaved

in a different way and detected antibody level was not consistent (too high or too low) in them compared to other mice in the group. This may be because of a complex immunology of inhibitor generation. Inhibitor is generated as a result of a cascade of interactions between different cells of the innate and the adaptive immune system [21], any mutation or event that modulates and changes the activation or migration pattern of immune cells will therefore potentially influence the risk to develop inhibitors [34]. Additionally, variations related to the procedure, especially drug injection, could be another explanation for the differences seen among mice.

Mice were injected with the fixed dose of 2 IU rFVIII each time during the whole experiment. FVIII dose was the same for both immunization and treatment period. It is equal to 100 IU/kg for each injection, which is effective dose for immunization but it is higher than the dosage being used in hemophilia treatment [13]. This is important since amount of antigen is one determining factor in stimulating host immune response and antibody production [40]. Treating mice with lower dose of FVIII similar to treating dose in hemophilia patients may show different results.

Due to a limitation in the access to human FVIII products for research purpose, recently expired rFVIII products donated from a hemophilia clinic were used in this project. Since they were expired products FVIII protein might be denatured or inactivated to some degree. Nevertheless, to make sure they were functioning, FVIII protein was tested and they fully interacted with two different commercial anti-FVIII antibodies from different sources.

Presence of hemoglobin derived from hemolysis in the test environment could interrupt an absorbance measurement and affect the results from ELISA. To exclude this supposition additional experiment was conducted, in which there was no change in absorbance based on the result obtained from ELISA on a sample that had intentional hemolysis (Fig 12). Only slightly increase in absorbance was seen (maximum 0.07 in OD) which was ignorable. All experimental samples had less hemolysis compared to the test sample when visually assessed. Also, ELISA plates were frequently and vigorously washed between each step so that any irrelevant molecule would be washed out. Possible binding between IVIG and FVIII molecules, and potential cross reactivity between anti-FVIII Ab and IVIG were also excluded (Fig 13 and 14).

Further, not all anti-FVIII Abs are neutralizing inhibitory antibodies. There are low affinity non-neutralizing specific antibody against FVIII that can be found in healthy individual and hemophilia patients without inhibitor [32]. Some value of anti-FVIII Ab generated in mice could be non-neutralizing antibody. However, anti-FVIII Ab levels shown on the Result section are total antibody amount since ELISA cannot discriminate between inhibitor and those non neutralizing antibodies [85]. To distinguish between neutralizing and non neutralizing antibodies another experiment should be done. Bethesda assay specifies inhibitory antibodies level by incubating test samples with normal pooled plasma and measuring residual FVIII activity in the mixture [86], [87]. Performing Bethesda assay on the experimental samples will define true inhibitor level and will give a better understanding of the nature of anti-FVIII immune response in hemophilia A mice.

Conclusion and future work

In this thesis experiment, I looked at the effect of IVIG co-administration on the immune response to FVIII in hemophilia A mice. The results obtained from anti-FVIII Ab ELISA indicated that prophylactic treatment with FVIII and IVIG co-injection reduces anti-FVIII Ab level in previously untreated hemophilia mice, suggesting that IVIG co-administration will alter the immune response and alleviate inhibitor generation. Findings in this project suggest that IVIG co-administration has no effect on pre-existing inhibitor levels. Co-injection of FVIII with IVIG didn't reduce anti-FVIII Ab level in previously immunized hemophilia mice.

In conclusion, FVIII co-administrated with IVIG decreases the anti-FVIII immune response in previously untreated hemophilia A mice. IVIG co-administration may be effective in the management of hemophilia patients at risk of inhibitor development. However, the exact mechanism of the immunoregulatory effect of the treatment remained unclear. Considering the fact that various cells and pathways are involved in the anti-FVIII immune response, further studies should be conducted to determine the mechanisms of this effect. Performing Bethesda assay on experimental samples should be considered in future studies in order to distinguish between neutralizing and non-neutralizing antibodies to specify the level of inhibitors. Also, more experiments should be done to investigate the characterization of type of immune response by isotyping immunoglobulins and determining cytokines profile and immune cells, especially CD4+ T cells, profiling.

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