Structural and functional aspects of factor viii in the initiation of the anti-factor viii immune response

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To cite this version:

Bagirath Gangadharan. Structural and functional aspects of factor viii in the initiation of the anti-factor viii immune response. Immunology. Université Pierre et Marie Curie - Paris VI, 2014. English. ¡ NNT : 2014PA066257 ¿.

HAL Id: tel-01089105
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THESE DE DOCTORAT DE L'UNIVERSITE PARIS VI

PIERRE ET MARIE CURIE

Spécialité :

Immunologie

Ecole doctorale : Physiologie et physiopathologie

Présentée par :

Bagirath Gangadharan

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE PARIS VI

Sujet de la thèse

STRUCTURAL AND FUNCTIONAL ASPECTS OF FACTOR VIII IN INITIATION OF ANTI-FACTOR VIII IMMUNE RESPONSE

Soutenue le 22 septembre 2014

devant le jury composé de :

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ACKNOWLEDGEMENTS

The completion of this thesis would have been impossible without the help of numerous people. First of all, I am thankful to Dr. Isabelle CREMER for kindly accepting the invitation to be the president of the jury. I wish to convey my sincere gratitude to Dr. Peter LENTING and Dr. Bernard MAILLERE for agreeing to be rapporteurs. I am grateful to Dr. Veronique OLLIVIER and Dr. Roseline d'OIRON for accepting the invitation to be the examinateurs. All of their advice has been valuable.

I would like to express my deepest appreciations to Dr. Srinivas Kaveri for agreeing to provide me with this opportunity to pursue my doctoral degree in his team and for his continual leadership, and support throughout this research journey. The experience I gained from working in his team provided me valuable experiences and has allowed me to continue my scientific research career ahead.

I am grateful to my supervisor Dr. Sebastien Lacroix-Desmazes for agreeing to be my mentor knowing my limitations and his constant encouragement and support that made this journey enjoyable. I hope this collaboration can continue to provide more fruitful contributions to field of hemostasis.

To Dr. David Scott who has always been there to guide me through, with his valuable advice and help at appropriate times. I am every grateful for your constant presence in my research life.

I would like to thank everyone of 'Team Srini Kaveri', present and past. They made me feel at home and being patient with my gibberish talks in Anglais! I had the great pleasure to work with them all, for the conversations, thought provoking disagreements, and challenges and of course the time chatting over a few drinks. Special Thanks to Mme Veronique Barraud and Mme Marie-Francoise Bloch, for

their constant support and much needed efforts in handling the complicated administrative business end of my research.

I am thankful to everyone at Emory University, Atlanta in particularly members of Dr. Chris Doering and Trent Spencer for having given me research opportunities, advices, mentorship and continued support in all aspects of my career. I am also thankful to Dr. Pete Lollar and his team John Healy and Erine Parker for teaching me the nuances regarding FVIII.

I owe this to my parents; my sister and my success would have been little without their constant prayers and well wishes. Special thanks to my sister, though much younger to me for understanding my complex career and agreeing to support my family. My thanks to all my friends who always have been there and will continue to be there for their encouragement and advice.

Thanks to Grifols and Pfizer for having faith in my thoughts and providing necessary funds to support my research.

Abstract

Immunogenicity of Factor VIII *(FVIII)* is a major hurdle that affects about 30% of severe hemophilia A patients. Though a significant advancement has been accomplished in the development of newer FVIII molecules, the factors that drive FVIII immune responses remain elusive. Many genetic and environmental risk factors have been identified or suggested but a complete understanding of the immunological basis for the antibody formation and the mechanism(s) behind tolerance induction, in the 30% of the patients that never develop anti-FVIII antibodies, are not understood. My thesis involves overlapping aspects important for initiation of an anti-FVIII immune response in a mouse model of hemophilia A. The primary role of FVIII is its participation in coagulation-associated events and thus, the first part of my thesis addresses whether coagulation events *per se* are implicated in the initiation of anti-FVIII immune responses. The second part of my thesis focuses on the importance of the membrane binding residues within the C2 domain of FVIII in antigen uptake and presentation by antigen presenting cells *in vitro* and discusses its relevance *in vivo*.

List of Publications

Article 1: Role of coagulation-associated processes on FVIII immunogenicity in a mouse model of severe hemophilia A. Bagirath Gangadharan[1,2,3], Sandrine Delignat[1,2,3], Véronique Ollivier[4], Nimesh Gupta[1,2,3], Nigel Mackman[5], Srinivas V Kaveri[1,2,3], Sébastien Lacroix-Desmazes[1,2,3]

Manuscript under revision. Journal of thrombosis and hemostasis

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Article 2: A role for phospholipid-binding residues in the C2 domain of factor VIII for endocytosis. Bagirath Gangadharan[1,2,3], Mathieu Ing[1,2,3], Sandrine Delignat[1,2,3], Ivan Peyron[1,2,3], Olivier Christophe , Srinivas V Kaveri[1,2,3], Sébastien Lacroix-Desmazes[1,2,3]

Manuscript Under preparation.

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Abbreviations

ADP	Adenosine-5'-diphosphate
Anti-FVIII	Factor FVIII antibodies
Anti-VWF	von Willebrand factor antibodies
APCs	Antigen-presenting cells
APTT	Activated partial thromboplastin time
BHK	Baby hamster kidney cells
BP	Base-pairs
BMDC	Bone marrow derived dendritic cells
BU	Bethesda units
CTLA-4	Cytotoxic T lymphocyte antigen-4
CD	Cluster differentiation
CD11c	Cluster Differentiation 11 c
CD25	Cluster Differentiation 25
CD28	Cluster Differentiation 28
CD40	Cluster Differentiation 40
CD80	Cluster Differentiation 80 DCs Dendritic cells
DAMP	Damage associated molecular pattern
DC	Dendritic cell
E16-C57BL/6	Exon 16 knockout C57BL/6 mice
ELISA	Enzyme-Linked ImmunoSorbent Assay
FEIBA	Factor VIII Inhibitor Bypassing Activity
FIIa	Thrombin
FIX	Factor IX
Foxp3	Forkhead transcription factor 3
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
GPIb	Glycoprotein 1b

GPIIb/IIIa	Glycoprotein II b/IIIa
H2B Major	Histocompatibility Haplotype 2D
H2D	Major Histocompatibility Haplotype 2B
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigens
iDCs	Immature Dendritic cells
IFN-γ _	interferon gamma
IgA I	immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL2	Interleukin 2
IL4	Interleukin 4
IL6	Interleukin 6
IL10	Interleukin 10
ITI	Immune tolerance induction
LDL	Low density lipoprotein
LRP	Low density lipoprotein receptor
LPS	Lipopolysaccharide
MHC-I	Major Histocompatibility Class I
MHC-II	Major Histocompatibility Class II
MMR	Mannose macrophage receptor
MoDc	Monocyte derived dendritic cells
PAR	protease activated receptor
PRR	Pattern recognition receptor
pdFVIII	Plasma derived Factor FVIII
rFVIII	Recombinant Factor FVIII
TCR	T cell receptor
TF	Tissue factor
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
TNF-α	Tumor necrosis factor-alpha
Treg	T regulatory cells
VWF	von Willebrand factor

INTRODUCTION

Section I. Introduction

Section 1.01 Hemophilia A

Hemophilia A is a rare congenital genetic bleeding disorder that occurs in 1:5000 males. The disease is due to mutations within the factor VIII (FVIII) gene resulting in a deficiency of functional FVIII. The lack of functional FVIII results in impaired thrombin generation required to generate stable fibrin and hence results in a bleeding phenotype[1].

The FVIII gene is situated on the long arm of the X chromosome, band Xq28. The gene is 186,000 base-pairs (bp) long and consists of 26 exons. There are over 2000 unique mutations reported within the FVIII gene that causes hemophilia A phenotype. These mutations can be due to insertions, deletions, duplications or point mutations within the FVIII gene[2, 3]. FVIII mutations cause varying severities depending on the amount of functional FVIII available in circulation. More commonly (approximately 45%), patients have plasma FVIII protein reduced to an extent roughly comparable to the level of FVIII activity and are designated cross-reacting material (CRM)-reduced. Additionally, there also exists about 5% of hemophilia A patients that have normal amounts of a dysfunctional FVIII and are termed CRM-positive. The most frequent mutation causing severe FVIII deficiency is an inversion mutation affecting intron 22 (found in ~50% of individuals with severe hemophilia A). This arises from a recombination event between homologous sequences in intron 22 and the terminal region of the long arm of the X chromosome.

Hemophilia A patients can be broadly classified based on their disease severity as (1) severe hemophilia A patients have less than 1% functional FVIII in the circulation, (2) moderate hemophilia A patients, between 1% and 5% and (3) patients with FVIII

levels between 5% and 25% are classified as mild hemophilia A patients. Depending on the severity of the disease, bleeding episodes may be frequent to rare or may only occur with surgery or other procedures. Thus, prevention of bleeding remains the primary goal in managing patients with a bleeding disorder. The current treatment of hemorrhages is FVIII replacement therapy using plasma-derived or recombinant FVIII. The treatment regimen can be prophylactic (i.e. given at 25-50 IU/kg every 48 hours to maintain physiological levels of circulating FVIII) or on demand (i.e. during bleeding episodes either as low or high dose FVIII). The economic impact of prophylaxis has been described [4], and it is more costly, even when one corrects it for improvements in health-related parameters. However, in addition to the exorbitant costs[5] associated with the treatment (over $60,000 to $150,000 per person per annum), replacement therapy with exogenous FVIII leads to the development of inhibitory anti-FVIII antibodies (inhibitory antibody or inhibitors) in up to 30% of the patients. Clinically, it is defined as the inverse of dilution that inhibits FVIII activity by 50% and termed as Bethesda titer and expressed as bethesda units per milliliter (BU/ml). It is postulated that CRM status of a patient can modulate immunogenicity of FVIII, as they are patients that are immunologically educated to exhibit a state of non-responsiveness to exogenous FVIII. Most CRM negative patients tend to develop inhibitors perhaps due to the bleeding severity and probably also due to lack of immune education. Thus these aspects are of immunological relevance in terms of inhibitor development, but may lack clinical significance in terms of disease severity. Interestingly, only 20% of severe hemophiliacs due to inverse mutation affecting intron 22 develop inhibitory antibodies. A recent study by Pandey [6] et al. () demonstrated that these patients express *FVIII* mRNA and intracellular FVIII protein in B lymphoblastoid cells and liver biopsies, supporting the hypothesis that most

individuals with the intron 22 inversion may be tolerant to FVIII and thus do not develop inhibitors.

Table 1, summarizes the historical evolution in advances related to FVIII products. Additionally, hemophilia A patients also develop severe joint damage or „hemophilic arthropathy" and suffer from painful, debilitating joint bleeds, associated-mobility issues that severely impede their quality of life. Bleeding episodes in patients with inhibitors are treated using activated prothrombin complex concentrates (APCC) or administration of activated FVII (FVIIa) [7] activated clotting factors in APCC restore hemostasis through their ability to directly activate the clotting system downstream of FVIII and, in the near future, using recombinant porcine FVIII.

The current preferred strategy to eliminate inhibitors in alloimmunized patients is immune tolerance induction (ITI) therapy. This involves the continuous infusion of FVIII over extended periods of time (few months to several years) and results in a decline in circulating inhibitor levels. Additionally, several modifications of ITI have been proposed and in some cases, may also involve the use of immune-suppressive agents such as chemotherapeutics, corticosteroids and/or anti-CD20 monoclonal antibodies (Rituximab) to aid the outcome. ITI is successful in over 70% of the cases and yet the underlying mechanism(s) of tolerance induction is unknown. Identifying factors that influence the development of an anti-FVIII immune response, strategies that can modulate anti-FVIII immune responses and novel FVIII therapeutics that can moderate development of an anti-FVIII immune response are attractive and active areas of research interests.

Table 1. Milestones in treatment of hemophilia A

Year	Treatment
1964	Cryoprecipitate
1970s	Lyophilized plasma-derived coagulation factor concentrates
1977	Desmopressin for mild hemophilia A
	Introduction of immune tolerance induction therapy
1985	Virus-inactivated plasma-derived coagulation factor concentrates
	Virus-inactivated activated prothrombin complex concentrate
1989	First recombinant factor VIII
1996	Recombinant activated factor VII
	Antiretroviral therapy for HIV
1997	Recombinant factor IX
2000	First gene therapy trials
2002	HCV eradication by interferon plus ribavirin
2010	Long-acting coagulation factors
Current	Renewed interest in gene therapy, continued development of extended life products

** Adapted from Massimo Franchini, Pier MannuccioMannucci· Semin Thromb Hemost 2014*

Section 1.02 Hemostasis

Hemostasis is a process that causes bleeding to cease and thus maintain vascular integrity. Hemostasis involves three steps that occur in a rapid and overlapping fashion. Vascular spasm or constriction is the first response as the blood vessels constrict to allow less blood to be lost[8]. The second step is formation of a platelet plug, and the last step is coagulation or blood clotting[8-10]

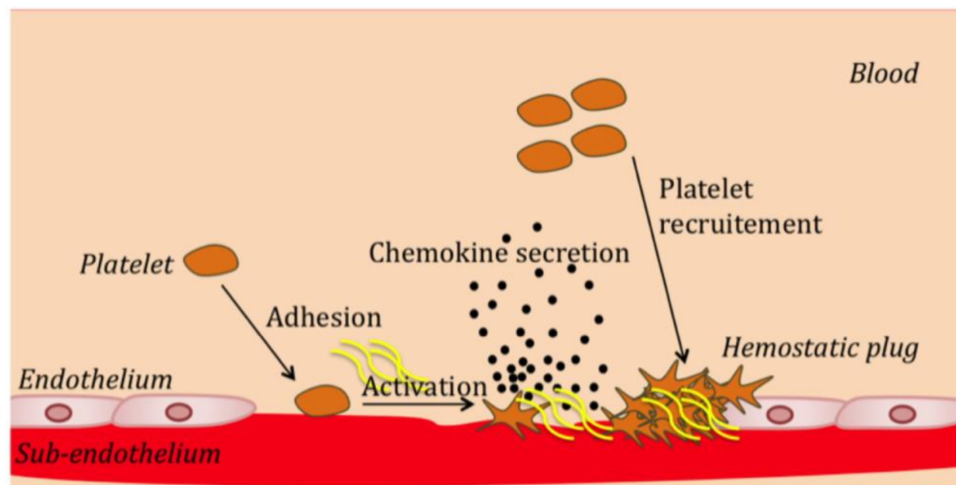

Figure 1. Formation of hemostatic plug. *During primary hemostasis, following vessel wall contraction, activated or injured endothelium releases several chemokine factors that recruit platelets and inflammatory mediators to the site of injury. Platelets play a primary role in this process by interacting with subendothelium-bound von Willebrand factor (VWF and represented as yellow strings in the figure) via the membrane glycoprotein (GP) Ib complex. This initial interaction (platelet adhesion) sets the stage for other adhesive reactions that allow the platelets to interact with each other to form an aggregate. The platelet GP IIb/IIIa complex mediates platelet-to-platelet interactions (platelet aggregation).*

(a) Primary Hemostasis

Primary hemostasis is triggered in response to damage to the vascular wall and results in exposure of the subendothelial tissue[8]. First steps following a vessel wall contraction are the activation and adhesion of platelets to the site of injury. This requires von Willebrand factor (VWF), a large multimeric plasma protein[9]. VWF mediates platelet adhesion by serving as a bridge between the tissue and the platelets, binding both to collagen exposed at the sites of vascular injury and to the platelet

membrane glycoprotein Ib-V-IX [11] (GPIb-VIX) [12]. Additional interactions between VWF and platelets enhance platelet cross-linking that results in the exposure of the GPIIb/IIIa integrin (also known as platelet fibrinogen receptor) receptor. The GPIIb/IIIa receptor enhances platelet aggregation thus enabling the formation of the platelet plug [9]. In addition to forming the platelet plug, activated platelets also provide phospholipid surfaces for the assembly of blood clotting factors, although direct anchoring of prothrombinase complex on activated platelets has recently been challenged. [10].

(a) Secondary Hemostasis

i) History of Coagulation

Our understanding of blood coagulation can be traced back to about 400BC and the father of medicine, Hippocrates, who observed that the blood of a wounded soldier congealed as it cooled, as well as bleeding from a small wound stopped as skin covered the blood. This “cooling theory” survived many years until in 1627, when Mercurialis observed clots in veins occurring at body temperature. In 1770, William Hewson challenged the cooling theory and proposed that air, lack of motion were important in the initiation of clotting and also demonstrated that the clot comes from the liquid portion of blood, the “coagulable lymph”, and not from the cells thus disproving the cooling theory. In 1905, Paul Morawitz (Morawitz P. Die Chemie der Blugerinnung. Ergebnisse der Physiologie. 1905;4:307–423) was the first to assemble coagulation factors into the scheme of coagulation and demonstrated that in the presence of calcium and thromboplastin, prothrombin (FII) was converted to thrombin (FIIa), which in turn converted fibrinogen (FI) into a fibrin clot. In 1936, Patek and Stetson [13] reported of a substance present in normal plasma and markedly deficient

or unavailable in hemophilic plasma, that in small amounts effectively shortened the clotting time of hemophilic blood. This agent was later termed antihemophilic factor (AHF), antihemophilic globulin (AHG), or factor VIII. During the years of World War II and later, several other coagulation factors were discovered as agents that were functionally deficient on individuals that exhibited prolonged coagulation time [14] [15, 16].

ii) Coagulation Cascade

The discovery of newer clotting factors led to the revision of the old classic coagulation scheme proposed by Morawitz. Coagulation process was modeled to occur as a "cascade" that involves sequential activation of several serine proteases by Macfarlene [17] [18] [19] and shortly followed by Davie and Ratnoff"s "Waterfall" model [20]. This model provided simplicity and represented a major advance in our understanding of blood coagulation processes. It described clotting factor(s) to be proenzyme(s) that gets converted into an active enzyme. It was also proposed to involve two distinct pathways: 1) extrinsic and 2) intrinsic pathway. The extrinsic pathway is initiated by tissue factor (TF) and the intrinsic pathway is so named because all the components are present in blood. Initiation leads to the activation of factor X (FX) and eventual generation of a fibrin clot through a common pathway. The extrinsic pathway is initiated by TF, that functions as the cofactor/activator for FVII. This can further activate FIX/FX resulting in thrombin generation. In the intrinsic pathway, the available thrombin activates FXI, FIX and FVIII. On platelet surface, thrombin will activate FXI, which will then activate FIX. At each step there occurs amplification in the amount of product formed. Finally, thrombin converts soluble fibrinogen to fibrin. Activated FXIII covalently cross-links fibrin monomers

to form a stable clot. This “cascade” also modeled very well the screening coagulation laboratory tests, such as prothrombin time (PT) and activated partial thromboplastin times (aPTT). These clinical tests were designed to differentiate deficiencies within the intrinsic and extrinsic arms of the coagulation cascade. Although several of these proposed concepts represented a major advance in our understanding of coagulation, it failed to address clinical and experimental observations and did not reflect the hemostasis events occurring *in vivo[21]*. For instance, this model does not provide explanations for the absence of a clinical bleeding tendency in deficiencies of factor XII (FXII), prekallikrein, or high molecular weight kininogen, all essential and important components of the intrinsic pathway. Additionally, FXII deficiency does not result in bleeding problems and the absence of FXII protects against pathological thrombosis (Kleinschnitz et al., 2006). It also cannot explain why in hemophilia patients the extrinsic pathway does not bypass the need for FVIII and factor IX (FIX) (Hoffbrand et al., 2005). Similarly, this model also fell short of explaining why deficiencies in intrinsic factors fail to cause embryonic lethality.

iii) A cell based model of coagulation

Thus, in recent years, the coagulation model has been revised with the discovery that exposure of blood to cells that express TF and FVIIa/TF complex is sufficient to initiate blood coagulation *in vivo[22]*. This “cell-based model”, put forth by several investigators together, requires that formation of an impermeable platelet and fibrin plug at the site of vessel injury and the procoagulant factors remain localized to the site of injury. It is proposed to involve amplification, during which platelets and cofactors are activated to set the following overlapping steps 1) initiation, that occurs on tissue factor bearing cells; 2) the stage for large scale thrombin generation; 3)

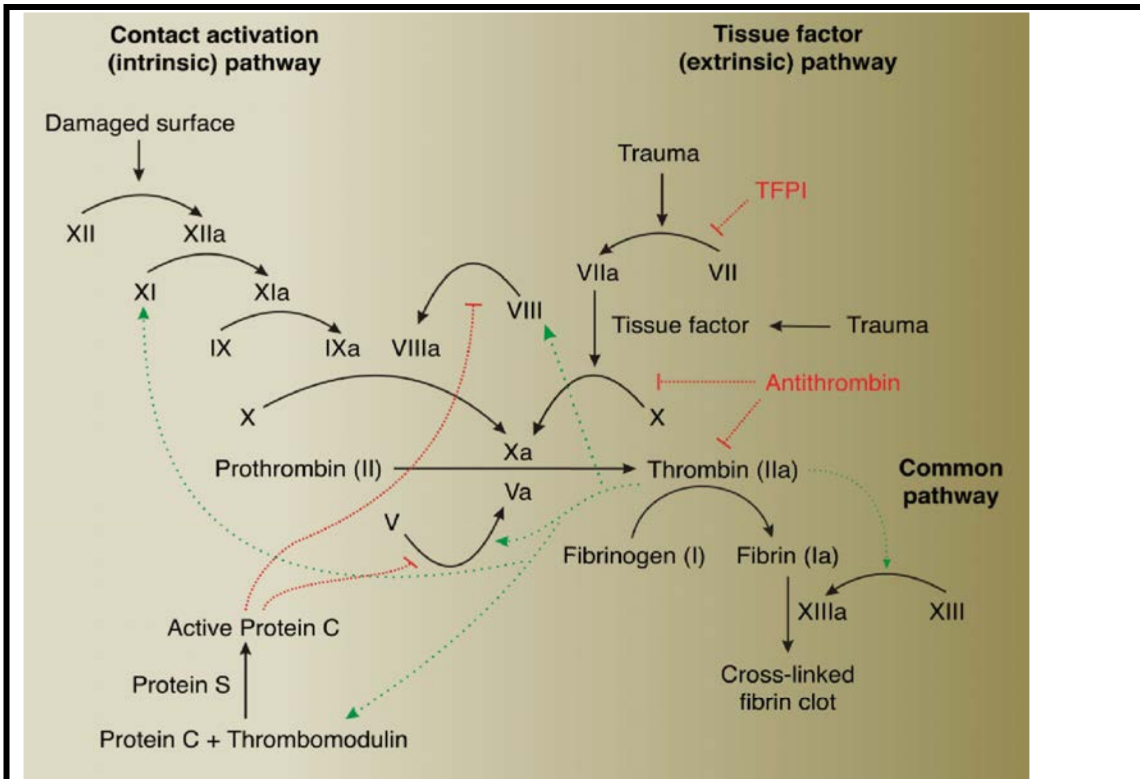

Figure 2. Representation of the blood coagulation system. *Coagulation cascade involves an extrinsic and an intrinsic system that converge into a common pathway. The interactions between the coagulation factors result in the generation of thrombin and the formation of a hemostatic plug. Anticoagulant proteins (indicated in red) that prevent excessive hemostatic plug formation tightly regulate this cascade.*

propagation, in which large amounts of thrombin are generated on the platelet surface; and 4) termination, to restrict clotting process to the damaged surface.

iv) Tissue Factor is the primary initiator of hemostasis *in vivo*

Tissue factor (TF) is the primary physiologic initiator of coagulation. TF is expressed on a variety of extravascular cells constitutively, and is also induced in blood monocytes and endothelial cells upon inflammatory stimulus [23]. Newer evidence suggests that TF is also present in blood as cellular microparticles [24] [25].

Microparticles are membrane fragments derived from various cell types (white blood cells, endothelium, platelets) and play an important role in pathological hemostasis (thrombosis) [26]. Interestingly, Hrachovinova et al. [27] had previously demonstrated that that induction of TF expression using Ig-fused P-selectin corrects hemophilia A phenotype and rescues mice from tail snip injury, clearly indicating a role for TF in monocytes in developing thrombus. It was also shown that TF microparticles localize to the site of thrombus formation. However, their role in initiating normal clotting is unclear. More recently, it was demonstrated that TF bearing neutrophils localize at the injury site prior to platelets, and initiate clot formation. This observation implicates a role for TF-bearing monocytes in initiating thrombus formation and coagulation *in vivo[28]*.

v) Coagulation occurs in a series of overlapping steps and as a "cascade"

During coagulation processes, breaks in the vessel wall allow contact of TF-bearing extravascular cells with coagulation factors that are available in plasma. Factor VII in plasma binds to cellular TF and gets rapidly activated by proteases. The exact mechanism of FVII activation is unclear but involves proteases. The FVIIa/TF complex can then activate both FX and FIX. The tissue factor pathway inhibitor (TFPI) or antithrombin III (ATIII), that also gets released rapidly, inhibits the activated FX (FXa). On the other hand, FXa that remains on the cell surface combines with FVa to produce small amounts of thrombin, amplifying the initial procoagulant signal by enhancing platelet adhesion, thus fully activating platelets as well as factors V, VIII and XI. Activated platelets contain high affinity binding sites for the coagulation factors FIXa, FXa, and FXI required for the membrane surface assembly of coagulation factor complexes. During the propagation stage of coagulation, the

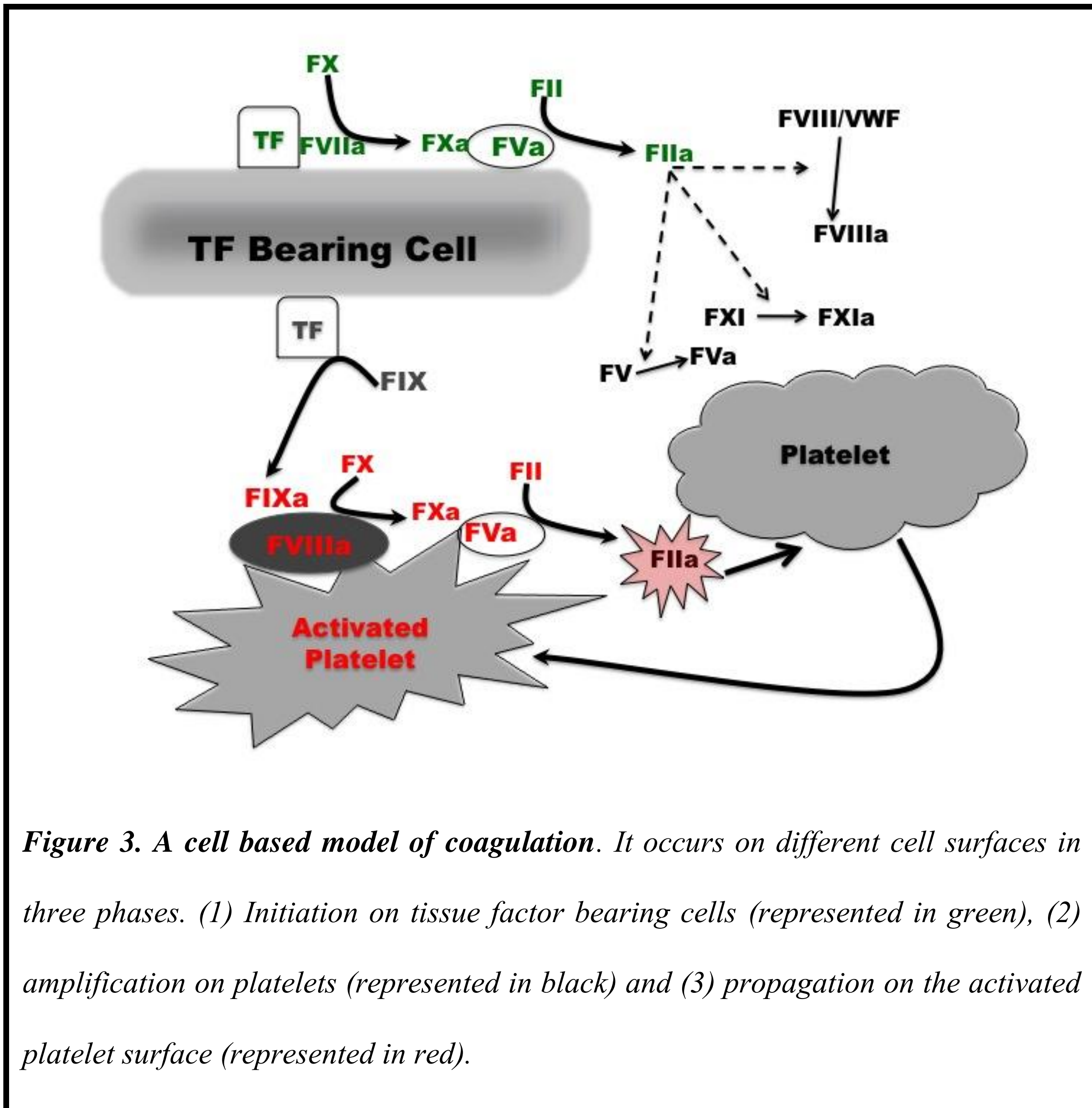

Figure 3. A cell based model of coagulation. *It occurs on different cell surfaces in three phases. (1) Initiation on tissue factor bearing cells (represented in green), (2) amplification on platelets (represented in black) and (3) propagation on the activated platelet surface (represented in red).*

intrinsic FXase complex, that includes FVIIIa, FIXa and FX, and the common prothrombinase complex, that includes FVa, FXa and FII or prothrombin, assemble on platelet surface to generate a localized "thrombin burst" necessary to form the hemostatic fibrin clot. However, recent *in vivo* imaging data indicate that the prothrombinase complex assembly occurs on the injured endothelium and/or other cell types near to the platelet plug, rather than directly on the activated platelets as had been previously proposed [10]. As more evidence accumulates, it seems likely that a role for other cellular factors may also add-up to the existing "cell-based hemostasis" model.

Another important aspect of this current cell-based model is that it requires localization of coagulation proteases close to the site of clot formation. As a correlate, the model involves/implicates two major mechanisms: 1) plasma protease inhibitors are not efficient in inactivating coagulation proteases at the surface of cells, they however rapidly inhibit the activated factors that diffuse away from the cell surface, and 2) the simultaneous activation of antithrombotic mechanisms prevents propagation of coagulation onto healthy intact endothelium. Inhibitors of coagulation include the endothelial thrombomodulin (TM)/protein C/protein S system that can potently inactivate factors Va and VIIIa, ecto-ADPase, thus suppressing amplification of platelet activation by ADP release, and endothelial surface heparanoids that bind and enhance the activity of plasma ATIII. All these different mechanisms coordinate to maintain and regulate hemostasis (figure 1).

vi) Hemostasis in Hemophilia A

In hemophilia A patients, platelet adhesion at the site of injury can occur normally as does production of FXa and small amounts of thrombin on TF-bearing cells during the initiation stage of coagulation. Indeed, patients with hemophilia A, as well as the FVIII-deficient mice, a model of severe hemophilia A, do not exhibit altered platelet activation status. Additionally, hemophilic patients also exhibit normal prothrombin time, indicating that they have normal functional extrinsic pathway as well. The prolonged bleeding is due to the fact that the propagation phase and probable platelet surface FX activation by FIXa/FVIIIa remains inefficient due to deficiency in functional FVIII and, hence, platelet surface thrombin generation does not occur (figure 2). Indeed, histological examination of clots in hemophilic patients reveals that periphery of the initial platelet plug is stabilized by a fibrin meshwork, whereas the

inner portion of the platelet plug shows little or no fibrin formation. It has also been demonstrated that deficiency in functional FVIII leads to clot instability that would be predicted to be associated with lack of thrombin generation at the site of injury. This indicates that the portions of the clot in proximity of tissues that express TF show evidence of thrombin generation. Hence, it is important to understand that a person with hemophilia A does not bleed harder or faster but bleeds longer.

Section 1.03 FVIII structure and function

Endothelial cells are the primary source for circulating FVIII [29] [30]. FVIII consists of a heavy chain (A1-a1-A2-a2-B domain) and a light chain (a3-A3-C1-C2) held together by non-covalent interactions [31]. As it is secreted in blood, FVIII rapidly associates with VWF and this interaction is necessary for maintaining its circulatory half-life [32]. Interaction of FVIII with VWF is predominantly due to its light chain [33] and seemingly spans several of the domains [34] [35] [36] [37] as mutations in several of the residues have been reported to result in hemophilia A phenotype [HTTP://WWW.VWF.GROUP.SHEF.AC.UK/]. Similarly, mutations within the D'' D3 region of VWF, that is known to interact with the FVIII, are also associated with hemophilia A phenotype [38] [39] [40] [41]. This highlights the necessity of this interaction to maintain normal FVIII levels in circulation.

FVIII is an essential component of the intrinsic pathway of blood coagulation and its cofactor activity is required for the generation of a "thrombin burst" during the propagation phase of coagulation. FVIII gets activated at the site of bleeding by its physiological activator thrombin. Thrombin cleaves FVIIII at Arg372, between the A_1 and A_2 domains; at Arg740, between the A_2 and B domains; and at Arg1689, which releases the a_3 peptide from the light chain. Thrombin-activated FVIII (FVIIIa)

dissociates from VWF and exhibits high affinity binding to phospholipid surface. Activated FVIII then serves as a cofactor for activated FIX (FIXa), increasing its

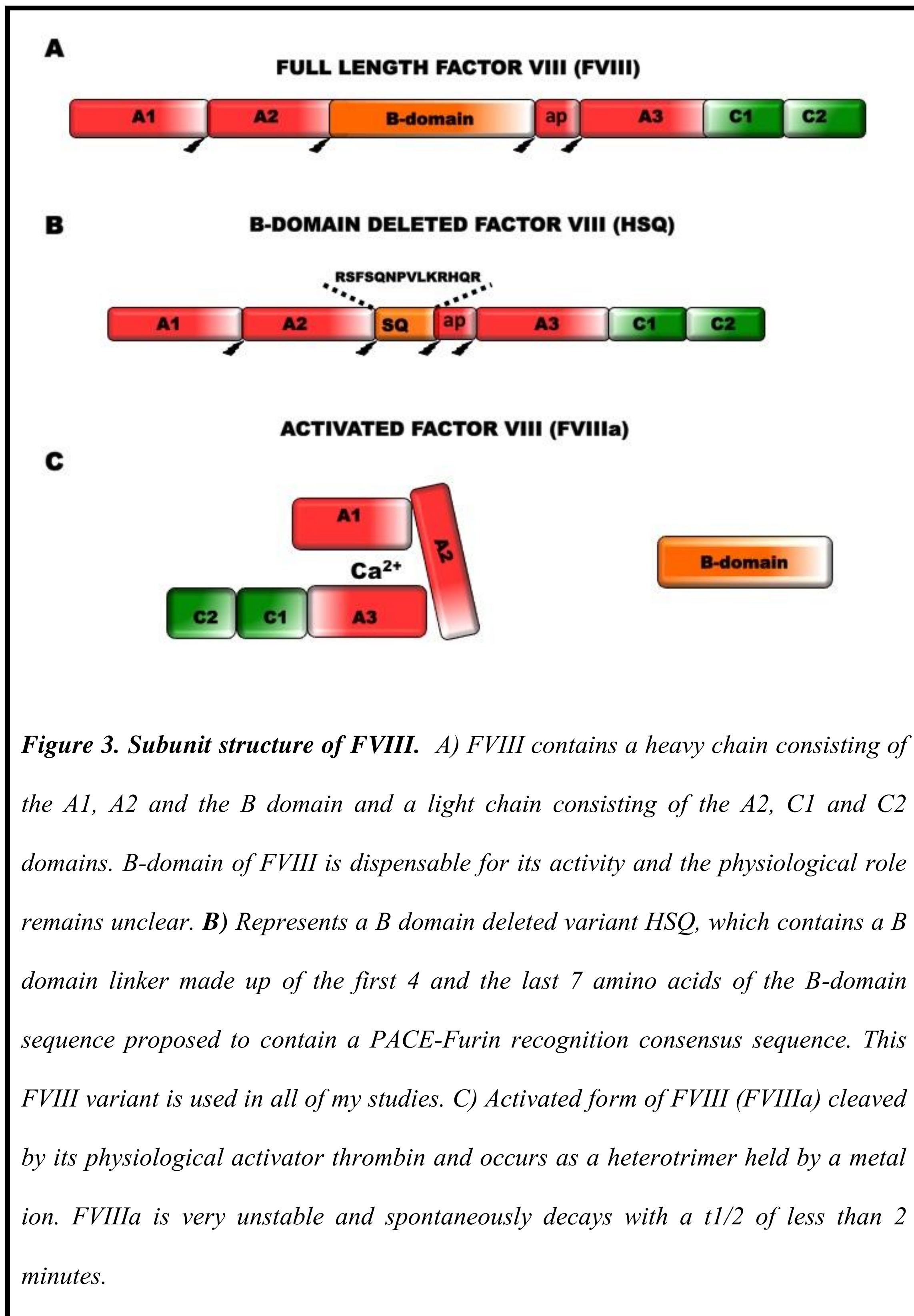

Figure 3. Subunit structure of FVIII. *A) FVIII contains a heavy chain consisting of the A1, A2 and the B domain and a light chain consisting of the A2, C1 and C2 domains. B-domain of FVIII is dispensable for its activity and the physiological role remains unclear.* ***B)*** *Represents a B domain deleted variant HSQ, which contains a B domain linker made up of the first 4 and the last 7 amino acids of the B-domain sequence proposed to contain a PACE-Furin recognition consensus sequence. This FVIII variant is used in all of my studies. C) Activated form of FVIII (FVIIIa) cleaved by its physiological activator thrombin and occurs as a heterotrimer held by a metal ion. FVIIIa is very unstable and spontaneously decays with a t1/2 of less than 2 minutes.*

catalytic activity by several orders of magnitude, and its membrane-bound complex (FXase complex) activates FX [31] [42]. The role of the phospholipid surface is to preconcentrate components of FXase complex, reduce interactions between the components from a three- to a two-dimensional space, and possibly to provide their optimal mutual orientation. [43].

The A domains are homologous with FV and ceruloplasmin and contain interactive sites for FX and FIXa. The A2 domain residues between 558-565 include residues that interact with FIXa required for its cofactor activity [44]*)*. The A1 acidic domain has been shown to contain interactive residues for FX [45] in the absence of phospholipids and the A3 domain, [46-48]involving residues 1810-1818, provides additional contact site for FIXa interaction [46] [47] [48]. Inhibitory antibodies have been reported directed against all of the FVIII domains implicating that each of these domains may play an important role in FVIII cofactor activity. It is interesting to note that FVIII has a similar binding affinity to FIXa as compared to FVIIIa [49]; it however lacks co-factor activity. This suggests that conformational changes occur during the activation process that expose several cryptic residues within the A2 domain needed for the cofactor function of FVIII. Crystal structure of FVIIIa in complex with FIXa or employing novel techniques such as deuterium-exchange mass spectrometry can provide additional insights into the interactive residues that are implicated.

The B domain of the FVIII is highly glycosylated but is the least conserved of the FVIII domains. The B domain that spans > 700 amino acids can be deleted from the FVIII molecules without losing pro-coagulant activity. The physiological role for the B domain remains unclear: but it may play a role in intracellular processing and may contribute to the catabolism of coagulation FVIII *in vivo [50]*.

The C1 and C2 domains of FVIII are rich in several basic and hydrophobic residues that are critical for its ability to interact with phospholipid membrane surfaces [51] [52] [53] [54] [55]. C domain has an isoelectric point (pI) of 9.2, making it highly positively charged at neutral pH. Structural studies by Pratt et al. [56] implicated the C2 domain as a predominant contributor to membrane binding through a combination of electrostatic and hydrophobic interactions [57] [54].

The C1 domain aligns closely to C2 but contains neither β-strands nor the proposed lipid-binding site observed in the C2 domain. Until recently, a role for the C1 domain in membrane binding remained unclear [58] [59]. However, previous studies on the homologous FV suggested a role for the C1 domain in binding membrane surfaces. Recent crystal structure of FVIII positioned the C1 domain in close proximity to membrane surfaces. Subsequent mutagenesis studies by Lu et al. *[60]* showed that the residues within the C1 domain mediate membrane interaction, and that this phenomenon is indeed important for complete cofactor function of FVIII. Particularly, mutagenesis of the residues 2092 and 2093 within the C1 domain indicated that membrane-binding contribution of the factor VIII C1 domain is large for membranes with low phosphotidyl-L-Serine (Ptd-L-Ser) content (i.e., which is the case for non-activated platelets or endothelium), but modest for membranes with 15% Ptd-L-Ser [58]. Interestingly enough, the same residues were also implicated in FVIII uptake by LRP-expressing cells [61], monocyte-derived dendritic cells (MODCs) and shown to modulate the immunogenicity of FVIII in mice [62]. Additionally, both of the C domains of FVIII also contain residues that interact with VWF, FIXa [63] [63] and FX *[64]*. Thus, one might agree that the structure of FVIII is intrinsically associated with its co-factor function; further explaining why over 2000 reported mutations result in hemophilia A phenotype.

Section 1.04 Immunogenicity of therapeutic FVIII

(i) Mechanism of immune responses to FVIII

The immunogenicity of FVIII is rather unique for a protein that is administered intravenously in the absence of any adjuvants [65]. The generation of antibodies to soluble antigens such as FVIII involves cellular interactions of antigen-presenting cells (APCs) with T-lymphocytes and with B-lymphocytes. This process requires an active uptake of FVIII involving cellular receptors and presentation of FVIII-derived peptides bound to MHC molecules to T-cell receptors (TCRs) on the surface of CD4 lymphocytes (Signal 1). Proper presentation of antigen to the TCR also requires a second interaction between the APCs and the T cells, typically through CD80/86 on the APCs and CD28 on the T cells referred to as co-stimulation (Signal 2). In the presence of both signals, the T cells are activated, which triggers secretion of cytokines (Signal 3 or cellular microenvironment) and up-regulation of a number of proteins on the surface of the CD4 T-lymphocytes, including CD40 ligand (CD40L, CD154) and CD28. These events promote the interaction of the T cells with B-lymphocytes, leading to B cell proliferation, immunoglobulin isotype switching and differentiation into antibody-secreting plasma cells.

FVIII is the least abundant of all coagulation proteins *in vivo.* Three mechanistic variables can potentially influence the development of antibody against FVIII and include (i) the type of mutation in FVIII and its functional effect on the protein[66], (ii) recognition, uptake and processing of FVIII by antigen-presenting cells and (iii) the haplotype of the major histocompatibility complex (MHC) molecules which bind specific peptide sequences and that are required for antigen presentation (figure 4). The simplest explanation for the occurrence of an anti-FVIII immune response is that patients with hemophilia A develop inhibitors since therapeutic FVIII is seen as a

foreign. However, only 30% of severe hemophiliacs develop an anti-FVIII immune response [67]. This indicates that other yet unknown factors may be involved in recognition of FVIII as "non-self". Interestingly, a large majority of patients with hemophilia A also achieve functional immune tolerance to FVIII, either following initial infusions or after eradication of an inhibitor response [68]. Endogenous FVIII is also the most commonly targeted of the coagulation proteins to develop

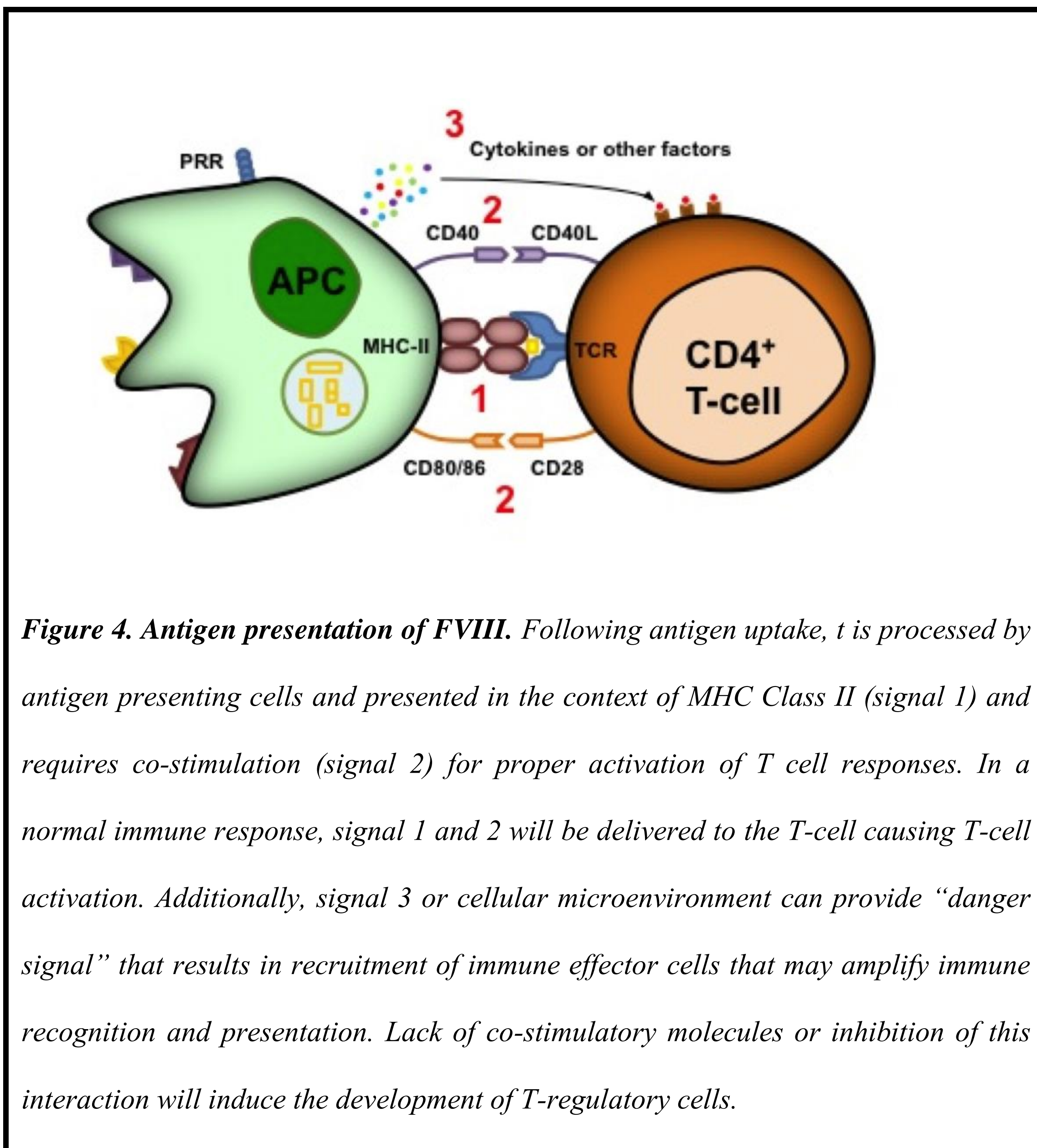

Figure 4. Antigen presentation of FVIII. *Following antigen uptake, t is processed by antigen presenting cells and presented in the context of MHC Class II (signal 1) and requires co-stimulation (signal 2) for proper activation of T cell responses. In a normal immune response, signal 1 and 2 will be delivered to the T-cell causing T-cell activation. Additionally, signal 3 or cellular microenvironment can provide "danger signal" that results in recruitment of immune effector cells that may amplify immune recognition and presentation. Lack of co-stimulatory molecules or inhibition of this interaction will induce the development of T-regulatory cells.*

autoantibodies resulting in “acquired hemophilia A” [69]. All these reflect upon an intriguing facet regarding the development of anti-FVIII immune responses and suggest that there exist genetic factors [70] that pre-dispose some patients with hemophilia A to develop an immune response to FVIII. The first evidence that inhibitor formation is a CD4+ T-cell dependent process came from the observation of a disappearance of the inhibitors in hemophilia A patients infected with HIV upon reduction of CD4+ T cells [71]*)*. Subsequently, it was shown using the mouse model of hemophilia A that blocking co- stimulation experimentally using monoclonal antibodies that block CD86 (anti-B7-2) achieved non-responsiveness. Likewise, blockade of interactions between CD40 on APCs and CD40L[72] on T-cells also diminished inhibitor formation in hemophilia A patients and indicated the occurrence of class-switched antibodies and[66, 70-74]affinity maturation, thus implicating a requirement for T-cell help[75] [76, 77].

(ii) Allo-immune responses to therapeutic FVIII

Inhibitors to FVIII are a major and costly complication associated to FVIII replacement therapy in hemophilia. Inhibitors are characterized as antibodies that inactivate FVIII in a time-, temperature- and pH-dependent manner. Together, all the above observations confirm that the immune response to FVIII is a T-cell dependent process that requires active antigen uptake and presentation. The importance of antigen uptake and its modulation on FVIII immunogenicity is discussed in a subsequent paragraph.[67-70, 72, 75-77].

Inhibitor development is due to a complex, multifactorial immune response, involving both patient-specific and treatment-related factors (figure 6). The greatest risk for

patients to develop FVIII inhibitors occurs during the first 20 exposure days (EDs)[78]. Anti-FVIII antibody responses are polyclonal and FVIII inhibitors are

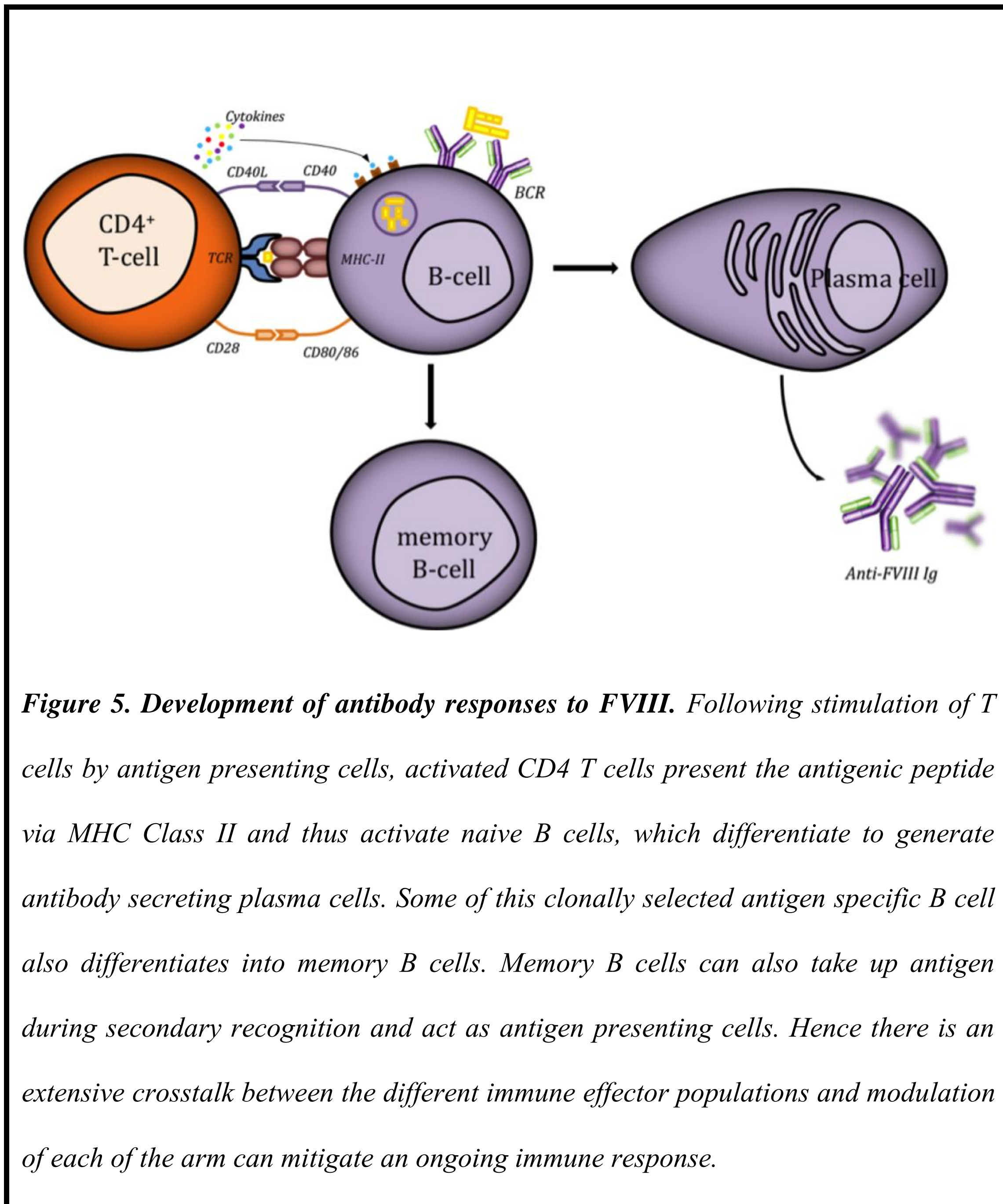

Figure 5. Development of antibody responses to FVIII. *Following stimulation of T cells by antigen presenting cells, activated CD4 T cells present the antigenic peptide via MHC Class II and thus activate naive B cells, which differentiate to generate antibody secreting plasma cells. Some of this clonally selected antigen specific B cell also differentiates into memory B cells. Memory B cells can also take up antigen during secondary recognition and act as antigen presenting cells. Hence there is an extensive crosstalk between the different immune effector populations and modulation of each of the arm can mitigate an ongoing immune response.*

usually composed of IgG1 and IgG4 antibody subtypes and do not fix complement [79] [80]. Epitopes located within the A2, C2 or A3-C1 domains of FVIII can elicit antibody formation, but most inhibitors generally target epitopes within the A2 and C2 domain of FVIII [81] [82] [83]. These inhibitory antibodies tend to interfere with

cofactor activity by inhibiting FVIIIa-FIXa interaction, thrombin cleavage or FVIII or FVIII-VWF/membrane interaction[84] [85] [86] [87]. Hence for this reason, the A2 and C2 domains of FVIII are considered to be immunodominant domains. Anti-FVIII immune responses also result in non-inhibitory antibodies, many of them targeting the B domain of the FVIII. Since B domain of FVIII is dispensable for its function, very little is known regarding the role of these antibodies in pathogenesis. However, based on few anecdotal evidences from patients reported to exhibit enhanced clearance, it has been proposed that these antibodies are probably involved and partly also play a role in clearance of FVIII by formation of antibody complexes [88].

The reasons why inhibitors develop in only 30% of patients with hemophilia A remain unclear, but studies of genetically related subjects have indicated that the immunological response is to a large extent determined by patient-related risk factors [89]. The risk of developing anti-FVIII antibodies has been associated with the severity of the disease, and the highest incidence (20–30%) occurs severe hemophilia A patients. Thus, there may exist a link between the type of mutation and the risk for inhibitor development [90]. Likewise, several class II alleles have been suggested to influence the risk for inhibitor development, [91] but associations identified to date are weak and determined on small patient cohorts; the overall impact of the MHC thus remains to be established [92]. Additional genetic risk factors have been suggested and include polymorphisms within the promoters of the genes encoding interleukin 10 (IL-10) [93], tumor necrosis factor (TNF)-alfa(α) [94], cytotoxic T-lymphocyte antigen-4 (CTLA-4) [95] [92] and more recently that of hemoxygenase -1 (HO-1) [96]. However a more recent elaborate study of larger patient cohorts for single nucleotide polymorphism (SNPs) within the immunoregulatory genes were found not to be consistently associated with inhibitor development [97, 98]. The

discrepancy between previously reported populations and the latest study supports the complexity of the immune response and

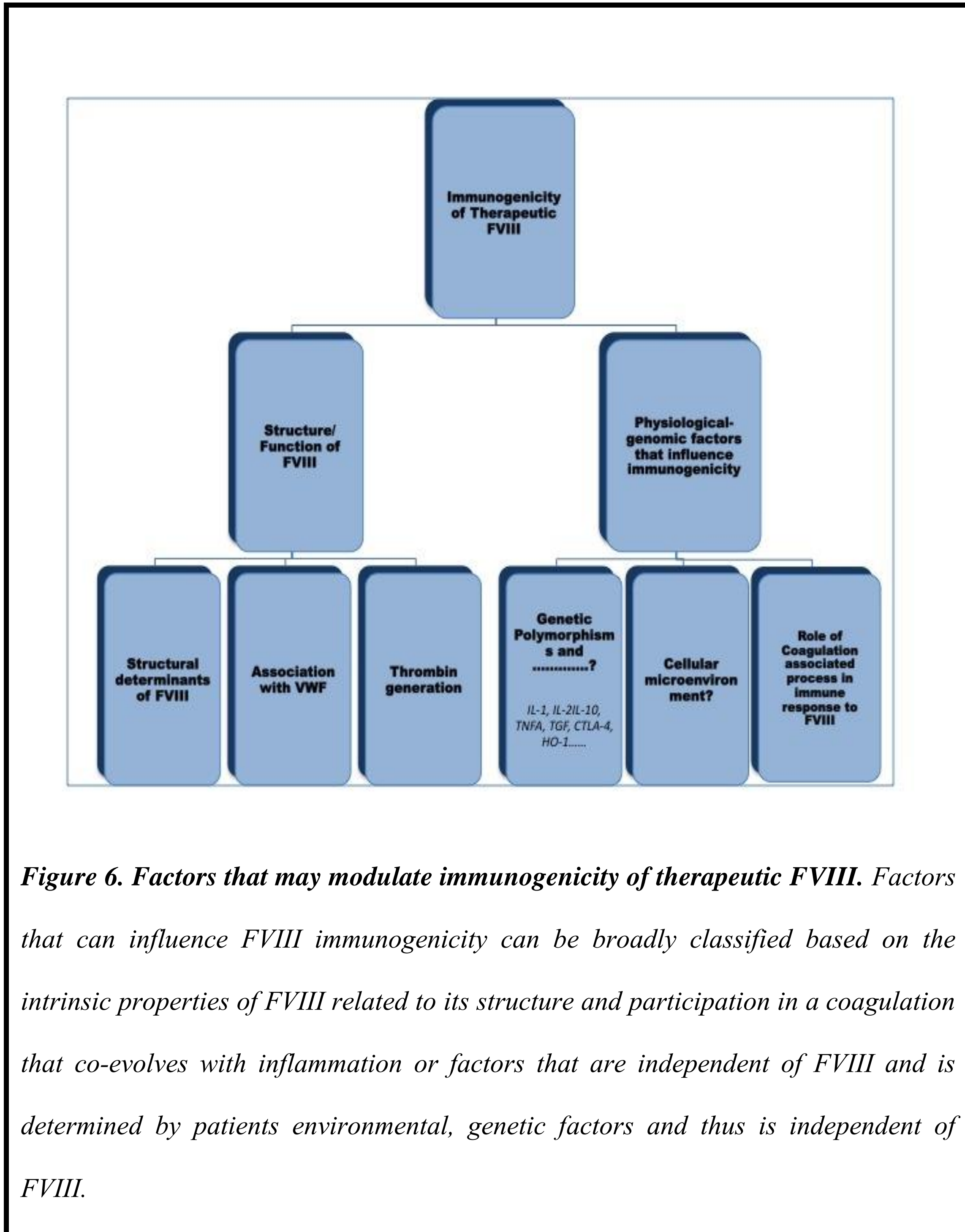

Figure 6. Factors that may modulate immunogenicity of therapeutic FVIII. *Factors that can influence FVIII immunogenicity can be broadly classified based on the intrinsic properties of FVIII related to its structure and participation in a coagulation that co-evolves with inflammation or factors that are independent of FVIII and is determined by patients environmental, genetic factors and thus is independent of FVIII.*

also emphasizes the need for understanding the immune pathways involved. Non-genetic risk factors for the occurrence of inhibitory antibodies are largely related to FVIII treatment. Several of the proposed risk factors, such as age of first treatment,

intensity of treatment, type of factor concentrate used for treatments, mode of administration, all yielded conflicting information as most of the [96] reported studies lack controlled evaluation of study population and concomitant assessment of the genetic risk profiles. It is also important from an immunological standpoint to note that the incidence of non-inhibitory anti-FVIII immune response in patients is relatively unknown, but for one recent study [67]. Given that this study provides information that the occurrence of anti-FVIII antibodies to be about 35%, it is likely that yet unidentified genetic factors may play an important role in development of anti-FVIII immune responses [67].

Non-genetic associated risks for inhibitor development can be explained partly by the "immunologic danger theory" proposed by P Matzinger a decade ago [99]. The model proposes that "alarm signals" are required to trigger the maturation of APCs. In the case of hemophilia A however, FVIII, activated FVIIIa or FVIII bound to VWF do not provide "danger signal" that result in maturation of monocyte-derived dendritic cells *in vitro* [100]. Thus, it is more likely that during a bleeding episode, a combination of tissue damage-associated signals and inflammation provide the signals that may enhance presentation of FVIII. In support of this notion, the administration of high-dose FVIII both subsequent to large bleeding episodes and at the time of surgery has been associated with an increased prevalence of inhibitor development [78].

Conversely, one could hypothesize patients that receive prophylactic doses of FVIII will provide substantially lesser signals required for allo-immune responses. However, a recent study by Gouw et al., [101] [78], investigating inhibitor development in relation to on-demand intensive FVIII treatment versus prophylactic FVIII treatment, found that during the first 20 exposure days (a determinant strongly

associated with the incidence of inhibitors), patients receiving prophylaxis had exactly the same inhibitor incidence as patients treated on-demand. Additionally, even though the study showed increased risk for inhibitor development in patients with high intensive treatment at greater than 50 exposure days, neither the initial frequency nor doses of prophylaxis were associated with the risk for inhibitor development. Thus, though there have been significant progress in our understanding of FVIII immunogenicity and inhibitor development, there still exists a lack of clarity regarding the factors dictating the increased incidence of inhibitor in hemophilia A patients.

(iii) Can coagulation *per se* provide „danger signal" adjuvanting the anti-FVIII immune response?

Inflammation and coagulation play an important role in the pathogenesis of vascular diseases and are also important for host defense. As it has been described in the above paragraphs, coagulation processes involve the sequential activation of several enzymes and cofactors required for stable clot formation. These proteases, such as Factor Xa, thrombin and the TF–FVIIa complex, have each been shown to elicit pro-inflammatory activities. The most important mechanism by which coagulation proteases influence inflammation is by activating protease-activated receptors (PARs). There are 4 types of PARs (PAR 1 to 4, summarized in Table 2) that have been identified, all belonging to the family of transmembrane domain, G-protein-coupled receptors. In the mice, PARs 1, 3, and 4 are thrombin receptors. Unlike PAR-1, PAR-2 does not bind thrombin but can be activated by the tissue factor-factor VIIa complex, factor Xa, and trypsin. PAR-1 may also serve as a receptor for the tissue factor-factor VIIa complex and factor Xa. Activation of PAR receptor have been linked to several inflammatory disorders such as sickle cell disease. As explained

Protease activated receptors	Activator	Tissue expression
PAR-1	**Thrombin/FXa**	Platelets, endothelial cells, smooth muscle cells, neutrophils, leukocytes, neurons, glial cells
PAR-2	**Trypsin, TF/FXa**	Activation of T cells and neutrophils, enterocytes, pancreas
PAR-3	**Thrombin/FXa**	In humans, heart, bone marrow, small intestine; endothelium and **not on platelets.** **In mice, highly expressed on platelets and required for complete activation of PAR-4**
PAR-4	**Thrombin**	Lung, Pancreas, thyroid, testes and platelets. Has low affinity for the peptide

Table 2. Protease activated receptors tissue expression and activators. Coagulation factors are major activators of the protease-activated pathway. However, other proteases such as trypsin can also efficiently activate PAR receptors.

previously, the physiological role of FVIII during coagulation is the generation of a „thrombin burst" required for stable clot formation at the site of injury. Excess thrombin generated during coagulation process also plays an important role in other coagulation-related events such as 1) remodeling clot structure, 2) activating TAFI, a component required to enhance clot resistance to fibrinolysis, 3) activating PAR-4.

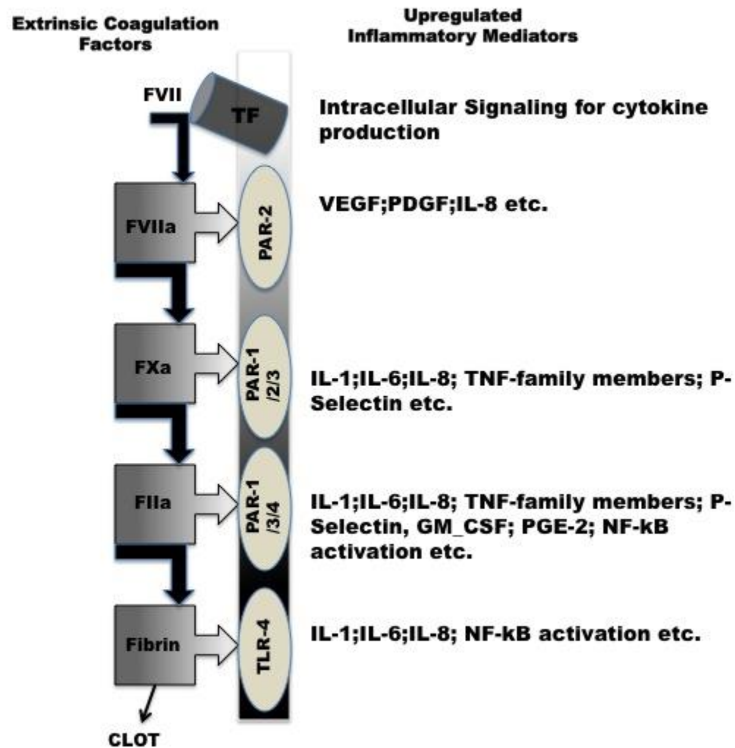

Figure 7. Coagulation activation can lead to pathological inflammation. *Several of the coagulation proteases activate PAR receptors. Activation of PAR receptor can upregulate of several inflammatory signals that are necessary for recruitment of cellular mediators. This can result in downstream activation of various inflammatory pathways and also immune activation.*

PAR-4 activation requires higher levels of thrombin and it seems thus likely other coagulation-related events such as that PAR-4 is activated during the propagation phase of coagulation (perhaps requiring FVIII cofactor activity), and 4) performing a cytokine or growth factor-like activities that play a role in inflammation and wound healing [102].

Likewise, inflammation also can result in coagulation activation. The major physiological initiator of inflammation-induced thrombin generation is TF as blocking TF activity completely abrogates the activation of inflammation-induced coagulation

in models of experimental endotoxemia or bacteremia [103, 104]. Additionally, platelets also play a pivotal role in the pathogenesis of inflammation-induced pathological thrombosis, in particular in case of acute arterial thrombosis on plaque rupture, such as in acute coronary syndromes. The expression of P-selectin on the platelet membrane not only mediates the adherence of platelets to leukocytes and endothelial cells but also enhances the expression of TF on monocytes [26] [105]. Thus, one can deduce that there exists an extensive bi-directional cross talk between coagulation and inflammation, whereby inflammation leads to activation of coagulation, and coagulation considerably affects inflammatory activity. Thus, it can be hypothesized that coagulation-associated events and coagulation factor activation may also provide "danger signals" and establish a pro-inflammatory milieu that may be mandatory for initiation of anti-FVIII immune responses.

Indeed, Skupsky et al.[106] previously demonstrated a role for thrombin in initiation of anti-FVIII immune responses in hemophilic mice. They demonstrated that inhibition of thrombin generation using hirudin (a thrombin-specific inhibitor) decreased the intensity of the anti-FVIII immune response. Hence, it was proposed that thrombin generated upon FVIII injection plays a role in the initiation of anti-FVIII immune responses. However, Meeks et al.[107] recently demonstrated that FVIII that does not possess cofactor function is equally immunogenic in the mouse model of hemophilia A. As it will be discussed more in detail in the first chapter of my thesis, I provide evidence that, neither coagulation nor thrombin generation may be involved in FVIII immunogenicity, at least in a mouse model of hemophilia A.

(iv) Importance of FVIII Structure in its uptake for the initiation allo-immune responses

Several candidate receptors have been shown to interact with FVIII *in vitro*. Several of these receptors are also proposed to play a role in FVIII catabolism (table 2). Hence, it is important to differentiate endocytic receptors that may play a role in antigen presentation versus catabolism. *In vivo*, FVIII rapidly associates with VWF and this association dictates its half-life [112]. Recently, it was demonstrated that macrophage LRP (LDL receptor-related protein, CD91) is the most relevant receptor involved in VWF clearance, thus implicating its role for clearance of the FVIII/VWF complex as well [113] [114]. However, FVIII catabolism in the absence of VWF remains unclear and is relatively unknown.

FVIII contains 25 potential consensus sites for asparagine (Asn)-linked glycosylations, of which 19 are located within the B domains that may be target for C-type lectin receptors. Thus, several original studies addressed the role of these glycans in FVIII uptake and presentation. Indeed, it was shown that deglycosyated FVIII was endocytosed to lower extent [108] than wild-type FVIII. Additionally, blockade of mannose-mediated interactions with mannan also resulted in a partial reduction of FVIII endocytosis by DCs and decreased T-cell activation [115]. Since B domain-deleted FVIII is endocytosed at similar levels as full length FVIII, a role for mannose-ending glycans at Asn^{239} and Asn^{2118} located within the heavy and light chains of the FVIII protein was proposed; a role for the macrophage mannose receptor (MR, CD206) was also suggested [115].

Members of the LDL receptor family have been demonstrated to be involved in FVIII clearance *in vivo* [116, 117]. However, in contrast to the proposed role for MR and LRP, a more recent study demonstrated that siRNA-mediated knockdown of MR or LRP on DCs does not influence FVIII endocytosis. This dismissed the potential role for these receptors in FVIII uptake and presentation at least *in vitro*.[118] However,

one potential inference could be that catabolic receptors proposed to play a role in FVIII clearance may not necessarily be implicated in endocytosis by APCs at least *in vitro*. The other explanation may lie in the fact that, as FVIII exhibits lower binding affinities to LRP and MR, it requires other co-receptors that play a role in this process. Thus, a two-receptor model for FVIII endocytosis has been proposed recently and may involve members of heparan sulfate proteoglycans (HSPG) [119]. However, treatment of monocyte-derived dendritic cells *in vitro* using heparinase III does not inhibit FVIII uptake, implicating other cellular receptors in FVIII uptake [120]. Interestingly, a recent study demonstrated that an anti-C1 domain antibody prevents FVIII endocytosis by APCs *in vitro* and is protective *in vivo* [118]. This observation was confirmed by subsequent mutagenesis of the residues within the antibody-binding region. Thus it is becoming evident that membrane-binding domains of FVIII, in particular the residues that are proposed to interact with membrane surface, play an important role in FVIII uptake and perhaps in its clearance as well.

It is important to highlight that all of the above experiments performed in the absence of VWF. When FVIII is bound to VWF, its uptake and presentation are blocked completely *in vitro*. Thus caution is warranted and further studies understanding the nature of FVIII/VWF interaction may provide better insights on the role of this interaction in FVIII catabolism and antigen presentation.

(v) Immunomodulation of the anti-FVIII immune response

The primary immune response to FVIII in mice and humans more than likely occurs in the spleen and to a lesser extent in the liver [121]. As it has been explained earlier, it requires three signals for its initiation. Thus, protocols directed to inhibit each of these signals likely result in down-modulating immune responses to therapeutic

FVIII. However, the most successful strategy for eradicating FVIII inhibitors clinically is immune tolerance induction protocol (ITI)[122, 123]. Several ITI registries indicate that this strategy is effective in approximately 75% of treated patients [124, 125]. The success rate of FVIII-mediated ITI is influenced by factors such as the pre-treatment Bethesda titer and the peak Bethesda titer on ITI. Whether the type of FVIII concentrate (recombinant or plasma-derived) is influential in the ITI process is controversial and currently unresolved [126]. Most ITI protocols take months to several years of daily FVIII infusions to achieve tolerance and is coupled with exorbitant costs. Besides, the protocols are practically challenging and require patient compliance. ITI results in eradication of inhibitors, however 30-40% of patients have circulating anti-FVIII antibody that are not inhibitory [67]. Despite its clinical success, little is known on the mechanism by which ITI works [127] [68].

Development of animal models that can recapitulate the effect of ITI observed in humans has proven to be difficult. Inability to develop a model for evaluating ITI may partly relate to the presence of long-lived plasma cells and lack of tools available to follow FVIII-specific T and B-lymphocytes. However, it has been suggested that T-cell immune exhaustion (over stimulation and subsequent T-cell anergy or apoptosis) may play a role in tolerance induction [128], but there is lack of experimental evidence that supports this hypothesis. It is important to emphasize that successful ITI does not necessarily eliminate all anti-FVIII antibodies. Additionally in certain cases on acquired hemophilia A, the inhibitory antibodies are also known to regress spontaneously. Thus exhaustion though might sound to be a simpler explanation, it can only partly explain the mechanism of ITI success.

More recent studies investigating the mechanisms of ITI have shown that high FVIII levels inhibit memory B-cell differentiation both *in vitro* and *in vivo*. Hausl etal. [129]

showed that supra-physiological concentrations of FVIII potentially mirroring the FVIII levels in some high-dose ITI patients, diminished memory B-cell populations to differentiate into anti-FVIII IgG-secreting plasma cells both *in vitro* and *in vivo*. The authors also demonstrated that this inhibition was irreversible, and caspase-dependent[130]. However, whether the effect observed *in vivo* is due to the inhibition of B-cell differentiation *in vivo* or to the effect of high dose FVIII on other cellular fractions that were co-injected ~~is~~ unclear. Interestingly, several groups have reported successful ITI using low dose FVIII infusions as well [131].

Plasma from hemophilia A patients that undergo ITI successfully, have been also shown to contain anti-idiotypic IgG [132] [127, 133]. Presence of anti-idiotypic IgG can sterically inhibit the binding of anti-FVIII antibodies to FVIII, or may occupy the antigen recognition site on FVIII-specific BCRs without inducing B-cell activation or Ab secretion. Another potential inhibitory mechanism of anti-idiotypic antibodies may involve their binding to the antigen recognition site on a FVIII-specific BCR, while simultaneously interacting with the inhibitory Fc receptor, FcγRIIB (CD32), on B cells through the Fc region of the anti-idiotypic antibody. However, though these may provide plausible explanation for the observed effect of successful ITI, it still does not address why only inhibitory antibodies are specifically targeted.

Several approaches have been tried to inhibit the development of anti-FVIII immune responses in animal models of hemophilia A. The most successful protocols exploited monoclonal antibodies that can disrupt the interaction between T cells and APCs. As would be expected, inhibition of co-stimulation using anti-CD40L antibody or CD80-CD28 interaction using CTLA4 Ig resulted in non-responsiveness to administered FVIII. However, a lack of specificity of the protocol towards FVIII, combined with the generalized immunosuppression, has hampered the applicability of these protocols

in humans, but has indeed expanded our understanding of the requirements for generating an immune response to FVIII. Similar strategies using low dose anti-CD3 Ab [134], IL-2/IL-2 Ab complexes [135] or anti-CD4 [136] non-depleting Ab have all shown success in animal models. However, translational values of all these studies are challenging thus warranting the development of other strategies.

One such strategy that has shown moderate success is the use of Rituximab. Rituximab is a chimeric mouse monoclonal antibody that engages CD20 thus resulting in rapid and prolonged *in vivo* depletion of all CD20-expressing cells. In a recent clinical trial in patients that failed ITI, Rituximab showed 67% eradication of inhibitors, but 21% of the patients relapsed within 14 months of the therapy. Interestingly, recurrence of anti-FVIII antibodies showed epitope specificity identical of FVIII inhibitor present before rituximab treatment. These observation highlight that eradication of FVIII inhibitors by immune intervention requires careful design and application for achieving long-term benefit for patients refractory to conventional ITI[137, 138]. Antigen-specific B-cell depletion therapy may provide a potent way to expedite the ITI induction and the development of newer targeted therapies may provide both mechanistic insight and also provide impetus for translational studies in patients refractory to ITI, acquired hemophilia A and probably as a means to eradicate inhibitory memory cells *in vivo*.

Section II. Scope of my thesis

Though our current understanding of risk factors involved in development of an anti-FVIII immune response continues to expand, very little is known on what are the critical determinants that result in immune recognition of FVIII and the factors that provide signals that may contribute to its "unique" immunogenicity. Studies using hemophilic mouse models have provided mechanistic information regarding steps involved in development of immune responses to FVIII, cellular types that maybe implicated in FVIII recognition, presentation and experimental approaches that can modulate immune responses to FVIII.

The aim of my thesis is to

1) Address the proposed contentious role of coagulation related events during the initiation process of FVIII immune response in a mouse model of hemophilia A

   and

2) Provide additional insight into the role of membrane binding C2 domain of FVIII in its uptake and presentation both *in vitro* and *in vivo*.

RESULTS

Article 1: Role of coagulation-associated processes on FVIII immunogenicity in a mouse model of severe hemophilia A

Section III. RESULTS

Section 3.01 Immune response to FVIII is independent of its co-factor function and coagulation events do not provide danger signal.

Development of anti-FVIII is a major complication that occurs in about 30% of severe hemophilia A patients. Anti-FVIII antibody response is unique for a protein administered intravenously at very low concentrations (approx. 0.2-1µg). Why FVIII is immunogenic at such low concentrations remains an intriguing question that haunts researchers. One potential explanation is the localization of FVIII at the site of clot formation or active bleeding, where there is an active recruitment of immune cells and an extensive cross talk between inflammation and coagulation. Given the only known function of FVIII cofactor activity is generation of thrombin, it has been tempting to speculate the role of thrombin generation in anti-FVIII immune responses. Indeed, this has been a paradigm that has not been completely explored and is very controversial. Recent work by Skupsky et al. implicated a role for thrombin in initiation of an anti-FVIII immune responses while, Meeks et al. had demonstrated that FVIII that is structurally intact but does not possess cofactor activity is equally immunogenic in a mouse model of hemophilia A.

Thrombin generation can occur via TF pathway independent of FVIII cofactor activity and is the trigger for initiating coagulation. FVIII functions as a major amplifier of FXa generation and thereby thrombin. The study by Meeks et al. has a potential weakness as the study did not address the evolution of an anti-FVIII antibody response and their protocol for FVIIII administration could provide a snapshot of the overall immune response, thus never addressed whether coagulation events are implicated.

I have addressed this contentious hypothesis of the role of coagulation in the initiation of anti-FVIII immune response by 1) following the kinetics of antibody generation to wild type or inactive FVIII and 2) by generating two different models of TF inhibition and show that absence of TF activity is not affect immune responses FVIII. In the first model, I blocked TF activity using a monolonal antibody and show that TF antibody inhibits thrombin generatin in hemophilic mice. Next, I demonstrated that pre-treament with anti-TF antibody did not modulate anti-FVIII immune response. In the second model, I generated a chimeric mouse by bone marrow transplantation using either wild-type or bone marrow cells from mice conditionally knocked out for TF expression in hematopoetic cells. After 8 weeks of engraftment, we administered inactive FVIII. We did not observe any differences in the kinetics of immune responses between the two groups implying a lack of role for TF expression in monocytes on development of anti-FVIII immune responses. Additionally i also demonstrated that warfarin, a potent anticoagulant that functions by inhibition of vitamin K dependent coagulation factors, also did not modulate immune responses to inactive FVIII in a mouse model of hemophilia A. Thus my observation provides novel information that argues against a role for coagulation events *per se* in development of anti-FVIII immune responses. The implications of my observations are discussed in detail in my later chapters.

(a) Role of coagulation-associated processes on FVIII immunogenicity in a mouse model of severe hemophilia A

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Keywords: Hemophilia A, Factor VIII, Immunogenicity, Coagulation, Tissue factor

Word count in abstract: 191

Word count in text: 1582

B&W figures: 4

Number of references: 16

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*Manuscript under revision

Abstract

Background. Immune responses to therapeutic factor VIII (FVIII) remain a major hurdle affecting 30% of patients with severe hemophilia A. The primary factors that drive immune responses in these patients remain elusive. There have been conflicting reports for a role of coagulation (or thrombin) in anti-FVIII immune responses.

Objective. To assess the importance of coagulation-associated processes on the onset of the anti-FVIII immune response.

Methods. Using FVIII-deficient mice, we compared the immunogenicity of recombinant FVIII or the inactive $FVIII^{V634M}$ mutant. In parallel, the implication of tissue factor (TF) activity on the anti-FVIII immune response was investigated upon injection of a neutralizing anti-TF antibody or using chimeric mice that lack TF expression in myeloid cells. The development of the anti-FVIII immune response was also monitored after treatment with warfarin.

Results. Kinetics of development of antibody responses to $FVIII^{V634M}$ was indistinguishable from that of wild-type FVIII. Inhibition of TF activity did not modulate immune responses to exogenous FVIII. Additionally, global inhibition of coagulation using warfarin failed to reduce the anti-FVIII immune response.

Conclusions. Thrombin generation or coagulation-associated processes do not modulate the anti-FVIII antibody response in mouse model of severe hemophilia A.

Introduction

Current treatment for factor VIII (FVIII) deficiency requires prophylactic infusions of plasma-derived or recombinant FVIII. However, about 30% of severe hemophilia A patients develop an immune response to the exogenous FVIII, that renders this treatment ineffective[66]. The immunogenicity of FVIII is rather unique for a protein that is injected intravenously and in the absence of adjuvant [64]. The factors that determine the initiation of anti-FVIII immune responses remain unclear.

The physiological role of FVIII (in its activated form FVIIIa) is its participation in the coagulation cascade as a cofactor to activated factor IX (FIXa). FVIIIa cofactor activity at the hemorrhagic sites is essential to generate a „thrombin burst" required for stable clot formation. There exists an extensive cross-talk between coagulation and inflammation [138]. Hence events involving coagulation may be sufficient to drive FVIII immunogenicity. Indeed, Skupsky *et al.*[105]showed that inhibition of thrombin generation either using warfarin (an anticoagulant that depletes vitamin K required for carboxylation of vitamin K-dependent clotting factors such as factor VII, factor X, prothrombin, and regulators such as protein C, protein S, and protein Z) or using hirudin (a direct thrombin inhibitor), dampens anti-FVIII immune responses in a mouse model of severe hemophilia A. Conversely, Meeks *et al.*[106] have demonstrated that FVIII immunogenicity is independent of its cofactor function.

In this study, we investigated a role for coagulation-associated events in FVIII immunogenicity. First, we confirm that the immunogenicity of FVIII is independent of its cofactor function. Next, we show that the immune response to FVIII is independent of TF activity using chimeric mice and neutralizing antibodies. We also show that pretreatment with warfarin does not modulate antibody responses to FVIII.

Together, the data indicate that initiation of immune responses to FVIII in a mouse model of hemophilia A does not involve coagulation-associated process.

Material and methods

Plasmid encoding B-domain deleted recombinant FVIII (HSQ-ReNeo) and BHK-M cells were a kind gift from Dr Pete Lollar (Emory University, Atlanta, GA, USA). Splicing by overlap extension PCR was used to generate the inactive FVIII mutant: $FVIII^{V634M}$. Stable cell lines producing recombinant FVIII were generated as previously described[139]. Specific activities of wild-type FVIII ranged between 7500-10000 IU/mg and were less than 100 IU/mg for $FVIII^{V634M}$, using either one-stage clotting or chromogenic assays (data not shown).

All experiments used 6-10 week-old exon 16 (E16) FVIII-deficient mice on a C57BL/6 background (a gift from Prof H.H. Kazazian, University of Pennsylvania School of Medicine, Philadelphia, USA). Animals were handled in agreement with local ethical authorities (Comité Régional d'Éthique p3/2005/002). The rat anti-mouse TF IgG2a antibody (clone 1H1)[140] was a kind gift from Dr. Daniel Kirchhofer (Genentech, Inc, San Francisco, CA, USA). The neutralizing activity of 1H1 to TF *in vivo* was determined *ex vivo* by calibrated automated thrombogram (CAT) using citrated plasma from mice injected with 100 µg of 1H1 or isotype control. 1H1 has also been shown to inhibit TF-dependent coagulation *in vivo* in mouse models of thrombosis[141]. Chimeric mice were generated by transplantation of bone marrow cells from LysM TF-knockout (LYSM-TF KO)[142] or wild-type mice into completely ablated (11 Gy) receiver FVIII-deficient mice. Engraftment was confirmed 8 weeks post-transplantation by PCR analysis [142] on circulating

peripheral blood cells, prior to FVIII administration. Replacement therapy was performed using 1 µg of FVIII intravenously once a week for four to five weeks. The anti-FVIII immune response was evaluated by measuring anti-FVIII IgG by ELISA and FVIII inhibitors using chromogenic assay (Siemens) as described elsewhere [143]. Warfarin (Sigma) was administered subcutaneously in peanut oil (5mg/kg or approx 125 µg), as previously described [105], on day -3 and day-1 prior to the first and third FVIII administrations. The effect of warfarin on coagulation was measured 2 days after the second injection by measuring the prothrombin time using Sysmex-CA500 (Siemens). All statistical analyses were performed using the two-tailed non-parametric Mann-Whitney U test unless mentioned otherwise (Prism, GraphPad Software, Inc, La Jolla, CA, USA).

Results and discussion

Coagulation processes involve the sequential activation of several enzymes and cofactors required for stable clot formation. Several of these proteases are also potent activators of inflammation [23, 138]. In order to decipher whether coagulation-associated signaling events bring about activation signals for the initiation of the anti-FVIII immune response, we compared the kinetics of FVIII antibody responses to wild-type FVIII and to an inactive FVIII mutant. The $FVIII^{V634M}$ mutant retains fundamental structural elements required for FVIII procoagulant function, such as capacity to bind von Willebrand factor, activated factor IX and phospholipids, and being cleaved by thrombin ([106]and data not shown). We did not observe significant differences in the kinetics of anti-FVIII IgG development induced by FVIII or $FVIII^{V634M}$ (Figure 1 A and B).

We next addressed the role for coagulation-associated processes in the immune response to FVIII by inhibiting TF activity. In order to avoid potential bias due to the procoagulant activity of FVIII, all subsequent experiments were performed using inactive $FVIII^{V634M}$. TF is the key initiator of the coagulation cascade *in vivo*[21]. TF provides an important link between coagulation and inflammation: monocyte-derived TF participates in coagulation-associated events [27] and has been shown to play a role in inflammation [142]. We evaluated the immunogenicity of $FVIII^{V634M}$, in a context where the expression of TF is lacking in hematopoietic cells. Bone marrow cells from LysM-TF KO mice were transplanted into irradiated hemophilic mice. Bone marrow from congenic C57BL/6 mice was used as control. The engraftment was verified 8 weeks post transplantation by PCR on the genomic DNA from blood cells from the recipient mice (Figure 2A). Mice then experienced replacement therapy with exogenous FVIII. Mice reconstituted with bone marrow from either TF-deficient donors or wild-type donors demonstrated similar immune responses to $FVIII^{V634M}$, as assessed by ELISA and Bethesda assay (Figures 2B and 2C).

We also investigated whether systemic inhibition of TF activity using a TF-specific monoclonal antibody (1H1) modulates the immune response to $FVIII^{V634M}$. We first validated the impaired TF-dependent thrombin generation in the plasma of mice treated with 1H1, a neutralizing anti-TF antibody, as compared to mice treated with an isotype control, with a significant reduction in both peak of thrombin and endogenous thrombin potential (Figure 3A). The treatment of hemophilic mice with 1H1 did not prevent the onset of an immune response to $FVIII^{V634M}$, as compared to control mice (Figure 3B and 3C).

We next evaluated the immune response to $FVIII^{V634M}$ after inhibition of coagulation using warfarin. Mice treated with warfarin exhibited a prolonged prothrombin time as compared to control mice (8.1±0.3 sec vs 26.31±21.6 sec, $P<0.05$, Figure 4A), thus validating the anticoagulant effect of warfarin. Although the median titers were lower in the cohort of mice treated with warfarin as compared to the control group (anti-FVIII IgG titers: 431 vs 222 AU), we did not observe significant reduction in the immune response to $FVIII^{V634M}$ (P= 0.1299 and Fig. 4B).

Our results are in contrast with the observations made by Skupsky*et al.*[105] who had shown that pretreatment with warfarin or with a thrombin-specific inhibitor (hirudin) resulted in a significant reduction of anti-FVIII immune responses to B domain-containing wild-type FVIII (Advate®). Whether the discrepancy between the two studies relate to differences in levels of anticoagulation achieved remains difficult to assess. Alternatively, it may be speculated that the observed differences partly relate to the nature of the FVIII molecules used. The $FVIII^{V634M}$ mutant was originally identified in a cross-reactive material-positive patient with severe hemophilia A: the patient exhibited 125% FVIII:Ag in circulation [144]. The B domain-deleted $FVIII^{V634M}$ mutant binds VWF with an affinity similar to that of wild-type FVIII (data not shown and[106]), and is recognized by several monoclonal antibodies directed against different domains of wild-type FVIII[106]. Importantly, the mutation of V634 into M634 is not predicted to generate or abolish immune-dominant T-cell epitopes in C57BL/6 mice, that have the H2-IAb allele (data not shown). Accordingly, the levels of inhibitory anti-FVIII IgG achieved in FVIII-deficient mice were identical whether B domain-deleted FVIII or $FVIII^{V634M}$ were used (Figure 1).

Factors that predict the onset of inhibitor development in hemophilia A patients can be both genetic or non-genetic [145]. One such non-genetic factor that has been proposed to be associated with the development of inhibitors is the severity of bleeding and the intensity of treatment associated with the bleeding episodes[100]. As bleeding invariably is related to coagulation processes, it is tempting to propose a role for the coagulation events in providing signals that initiate an anti-FVIII immune response. However, the data presented in this study do not support a role for coagulation processes, that result in thrombin generation, in the initiation of anti-FVIII antibody responses, at least in the mouse model of hemophilia A. Whether hemorrhages or bleeding-associated inflammation augment FVIII immune response warrants further investigation.

Acknowledgements

The authors thank Dr. Daniel Kirchhofer(Genentech, Inc, San Francisco, CA, USA) for providing us with the rat anti-TF antibody, and Ying Zhang for her technical assistance. This workwassupported by Institut national de la santé et de la recherche médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), and Université Pierre et Marie Curie (UPMC) Paris 6, and by an unrestricted grant from Grifols (Barcelona, Spain).

Contributions

BG, SVK, and SLD planned the work; BG, SD, NG, and VO performed experiments and edited the manuscript; BG, SLD analyzed the data; NM contributed essential reagents and critically editing the manuscript; BG, SVK, and SLD wrote the report. All authors discussed the results and commented on the manuscript.

The authors declare no conflict of interest

Legends to Figures

Figure 1. The cofactor function of FVIII is dispensable for its immunogenicity. E16 FVIII-deficient mice (n=8 per group) were treated intravenously with 1 μg of either wild-type FVIII (open circles) or inactive FVIII ($FVIII^{V634M}$, closed circles) once a week for 5 weeks. Anti-FVIII IgG responses were monitored after the third FVIII administration and were followed until the fifth administration. Anti-FVIII IgG titers are defined as arbitrary units based on standard curves generated using a purified mouse monoclonal anti-FVIII IgG (mAb6) serially diluted on FVIII coated ELISA plates. Inhibitory titers were measured using chromogenic assay and are expressed in Bethesda Units/ml (BU/ml). The data is expressed as median±SD (A) or Box and Whiskers plot (B). Statistical significance was performed using the two-tailed non-parametric Mann-Whitney U test (ns: not significant).

Figure 2.Monocyte-derived TF is not a requisite for developing an anti-FVIII immune response. E16 FVIII-deficient mice were transplanted with bone marrow cells from C57Bl/6 or LYSM-TF knockout mice (n=8-10/group) under complete myeloablation (11 Gy). Eight weeks post-transplantation, genomic DNA from blood cells was purified using QiagenDNEasy kit. PCR amplification was performed as described previously [142]. These primers generate a fragment of 559 base pairs for wild type (WT) allele and 611 base pairs for the floxed allele. Panel A is representative gel migrations of PCR fragments from either LYSM-TF (n=5) or WT (n=4) bone marrow transplanted cohorts. Subsequently, mice were administered with 1 μg of $FVIII^{V634M}$ intravenously once a week for four consecutive weeks. Anti-FVIII IgG responses (panel B) and inhibitory titers (panel C) were evaluated as described

previously. In both panels, open boxes and filled boxes represent wild-type (WT) and LYSM-TF bone marrow recipient cohorts, respectively.Data are represented as box and whisker plots and statistical significance was assessed using the two-tailed non-parametric Mann-Whitney U test (ns: not significant).

Figure 3: Administration of anti-TF antibody does not modulate immune responses to FVIII^{V634M}: A) Effect of TF antibody on thrombin generation in FVIII-deficient mice To evaluate the inhibitory effect of TF activity, mice (n=4) were injectedintraperitoneally with 100 µg of 1H1 or isotype control 2 hr prior to collection of plasma. Thrombin generation was then measured using a calibrated automated thombogram by initiating the reaction using either human tissue factor (0.5 pM, panel A) or without addition of exogenous human TF (panel B). Plasma samples from mice treated with 1H1 displayed significantly decreased peak of thrombin. Statistical analysis was performed using two-tailed Student T test.

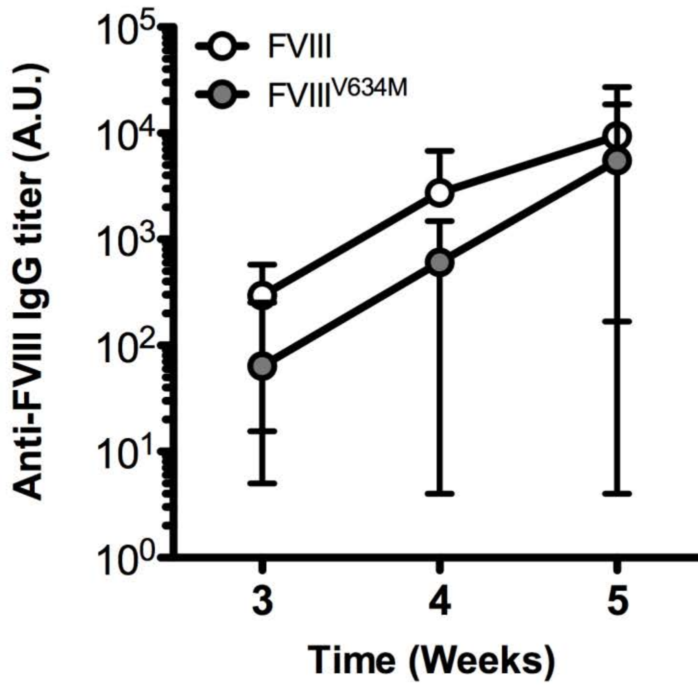
Next, E16 FVIII-deficient mice were first administered intraperitoneally with 100 µg of rat-anti mouse TF antibody (1H1) or isotype control (n = 8/group) each week for four weeks at least 90 min prior to each FVIII^{V634M} administration. Anti-FVIII IgG responses (panel C) and inhibitory titers (panel D) for were evaluated as described previously. Data are represented as box and whisker plots and statistical significance was assessed using the two-tailed non-parametric Mann-Whitney U test (ns: not significant).

Figure 4: Warfarin does not modulate initiation of immune responses to FVIII^{V634M}. (**A**) Effect of warfarin on clotting time in FVIII-deficient mice. FVIII knockout mice were administered subcutaneously with 5mg/kg warfarin in peanut oil or vehicle alone as control on day -3 and day-1. Two days after the second treatment with warfarin, mice

were bled retro-orbitaly and PT times measured using Sysmex CA-500 automated coagulometer (Siemens). Depicted data indicate clot time measured in seconds (n= 6 mice/group). (**B**) Effect of warfarin on $FVIII^{V634M}$ immunogenicity. E16 FVIII-deficient mice were administered with 1 μg of $FVIII^{V634M}$ intravenously once a week for four consecutive weeks. Mice were also administered subcutaneously with 5mg/kg warfarin in peanut oil or vehicle alone control (n=10 mice/group) on day -3 and day -1 prior to the 1st and 3rd FVIII administration. The anti-FVIII IgG responses were evaluated as described previously. Data are depicted as box and whiskers plots, and are the pool of two independent experiments. Statistical significance was assessed using the two-tailed non-parametric Mann-Whitney U test (*: P<0.05, ns: not significant).

Figure 1

A



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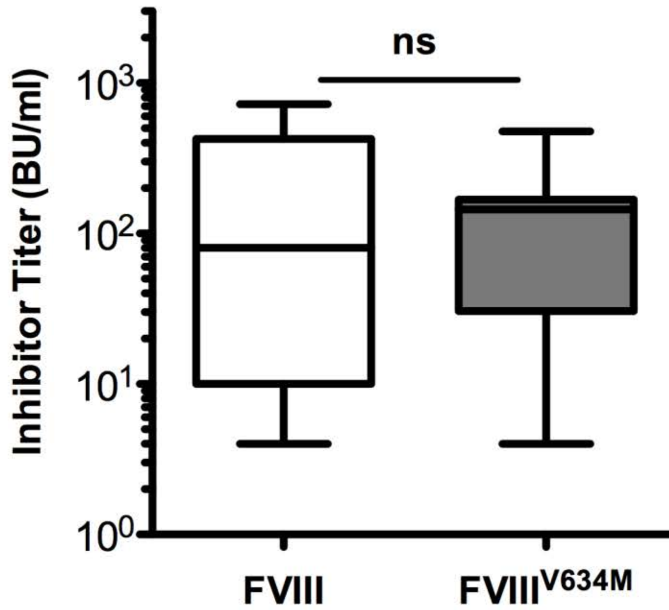
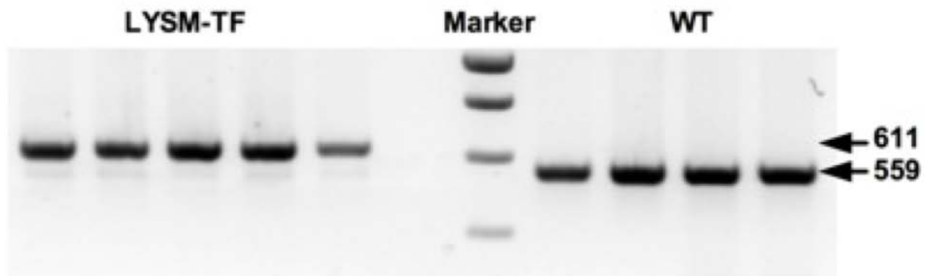


Figure 2

A



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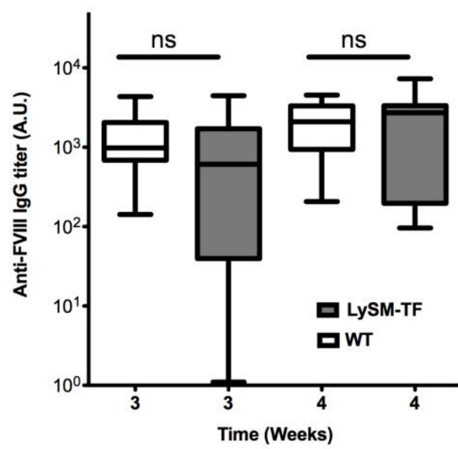

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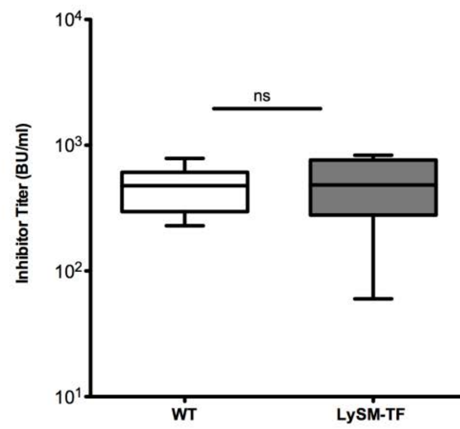
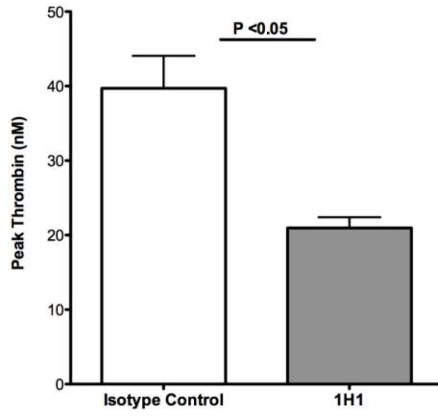


Figure 3

A



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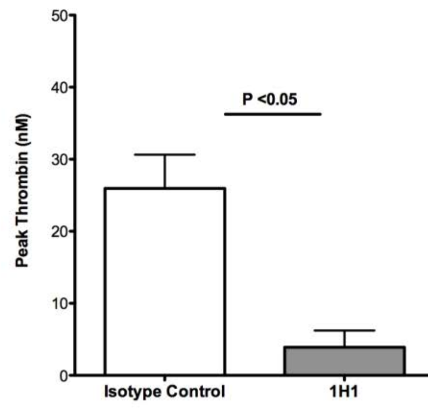

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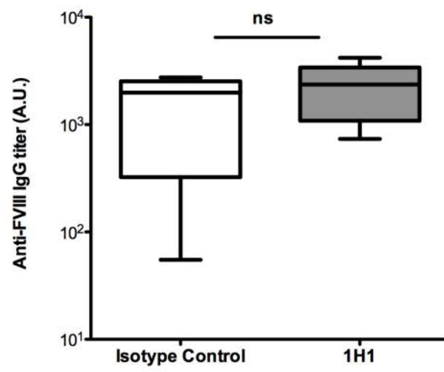

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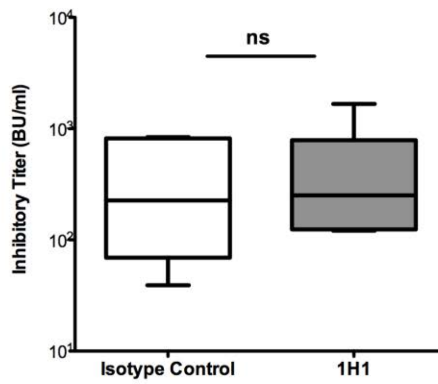
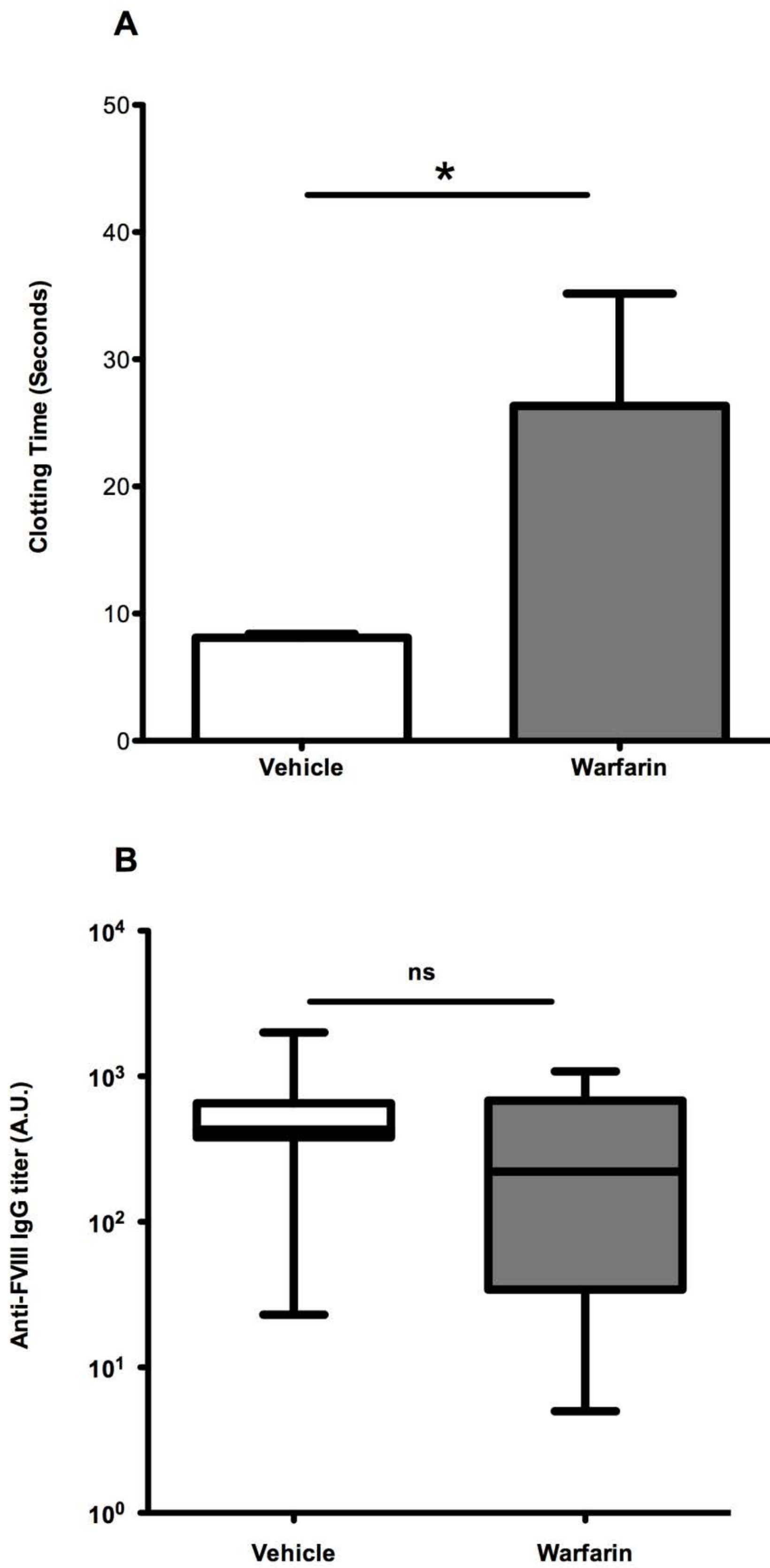


Figure: 4



Article 2: A role for phospholipid-binding residues in the C2 domain of factor VIII for endocytosis

Section 3.02

Section 3.03 A role for phospholipid-binding residues in the C2 domain of factor VIII for endocytosis

In the first chapter, I established that the immunogenicity of FVIII is independent of its co-factor function. In the second chapter, I have investigated the role of the C2 domain of FVIII in its uptake *in vitro* and immunogenicity *in vivo*.

Several groups, including ours, have investigated the endocytic receptors involved in FVIII uptake by antigen-presenting cells (APCs) or in FVIII catabolism. Candidate receptors such as macrophage mannose receptor (MMR, CD206), low-density lipoprotein receptor-related protein (LRP, CD91), or other receptor-associated protein (RAP)-sensitive receptors have been proposed [Dasgupta, 2008 #519;Repesse, 2012 #505]. Equally, residues within FVIII domain(s) that contribute to these interactions are also being investigated. Albeit these efforts, the *in vivo* relevance of these receptors and the nature of the FVIII residues involved in FVIII uptake and immunogenicity *in vivo* remains difficult to assess and requires further examination.

Recent work by Herczenik *et al*. [Herczenik, 2012 #791] implicated a role for membrane-binding residues within the C1 domain of FVIII in this process. In the present chapter, I demonstrate that FVIII bound to an anti-C2 antibody (BO2C11) dampens the anti-FVIII immune response *in vivo*. Next, I establish that this antibody inhibits FVIII uptake and presentation by APCs *in vitro*. Additionally, I show that this inhbition is restircted to membranebinding residues within the C2 domain, while ESH-8, another anti-C2 monoclonal antibody that does not compete for membrane

binding, has little effect on FVIII uptake by APCs *in vitro*. Functionally, a reduction in endocytosis also dimished antigen presentation to a CD4+ T-cell hybridoma.

Next, by generating FVIII mutants that alter binding to BO2C11, I ascertain that arginine at position 2215 is implicated in this process. Substitution of R2215 to an alanine diminished FVIII uptake by APCs. However, this substitution did not affect its ability to bind to VWF and to antibodies that recognize epitopes in other domains of FVIII, and retained a specific activity similar to that of wild-type FVIII in both a one-stage or two-stage coagulation assay

Together, my data argues for a role of the C2 domain in FVIII uptake by antigen presenting cells. Together with previous publications, the data suggest a synergy between membrane-binding residues in both the C1 and C2 domains of FVIII in mediating FVIII uptake by APCs and thus conferring immunogenicity to therapeutic FVIII. However, it remains unclear whether these mutations shall substantially influence FVIII immunogenicity *in vivo* when VWF is present and binds FVIII. The ramifications of these findings are extensively discussed in the discussion and a model is proposed.

A role for phospholipid-binding residues in the C2 domain of factor VIII for endocytosis

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Keywords: Hemophilia A, Factor VIII, Immunogenicity, Coagulation, Antigen presentation

*** Manuscript under preparation**

Introduction

Hemophilia A is a monogenic disorder associated with mutations causing reductions in functional levels of coagulation factor VIII (FVIII). FVIII consists of a heavy chain (A1-a1-A2-a2-B domain) and a light chain (a3-A3-C1-C2) held together by non-covalent interactions [1]. It rapidly associates with von Willebrand factor (VWF) in plasma and this interaction is necessary for maintaining its circulatory half-life.[2] Current treatment for FVIII deficiency requires prophylactic infusions of plasma-derived or recombinant FVIII. However, up to 30% of patients with hemophilia A develop anti-FVIII immune responses rendering treatment ineffective.[3] Development of anti-FVIII antibody responses is dependent on T helper cells, requiring antigen uptake and presentation [4]. Hence understanding initial steps involved in FVIII presentation may provide novel strategies to prevent the onset of immune responses.

Several groups including ours have investigated the endocytic pathways involved in FVIII uptake. Candidate receptors such as macrophage mannose receptor (MMR, CD206), low-density lipoprotein receptor-related protein (LRP, CD91), or other receptor-associated protein (RAP)-sensitive receptors have been proposed. [5, 6] Equally, residues within FVIII domain(s) that contribute to these interactions are also an area of active investigation. Albeit these efforts, the *in vivo* relevance of these receptors and the nature of the FVIII residues involved in FVIII uptake require further examination.

Recently Herczenik *et al*.[7] demonstrated that KM33, a mouse C1 domain-specific monoclonal IgG, inhibits FVIII endocytosis by monocyte-derived dendritic cells (MoDCs) or mouse bone marrow-derived dendritic cells (BMDCs). KM33 engages residues K2092, F2093 and R2090 that are involved in interactions with phospholipid membrane surfaces.[8] Additionally, KM33 inhibits interactions of C1 domain with

membrane surfaces, Von willebrand factor (VWF) and lipoprotein receptor-related protein 1 (LRP)[5, 9]. FVIII uptake by LRP in MoDCs used as model antigen-presenting cells (APCs) has been ruled out, while a role for CD206 has been controversially documented [7] [5]. This suggests that FVIII uptake by APCs may involve other endocytic receptor(s). Importantly, FVIII mutants containing alanine substitutions of the K2092, F2093 and R2090 C1 residues exhibit diminished uptake *in vitro* and reduced immunogenicity in a mouse model of severe hemophilia A [8]. Together, these results point to the significance of phospholipid-binding residues within the C1 domain for FVIII uptake both *in vitro* and *in vivo*.

Similar to the C1 domain, the C2 domain of FVIII interacts with phospholipid membrane surface, a binding that involves several basic residues [10] [11] [12] [13] [14] . In addition, the C2 domain contains a low affinity-binding site for LRP [15]. This interaction is inhibited in the presence of VWF or ESH4, a murine monoclonal anti-C2 IgG that competes for VWF/phospholipid interaction [15]. In this study, we investigated whether membrane-binding residues in the C2 domain of FVIII are involved in FVIII uptake. We first show that BO2C11, a well-characterized human monoclonal IgG that engages membrane-binding residues in C2,[16] inhibits FVIII uptake and presentation *in vitro*, and reduces FVIII immunogenicity *in vivo*. Additionally, by site directed mutagenesis, we demonstrate that R2215 residue plays an important role in the uptake of FVIII by APCs. Together with the published data, our results suggest a synergy between membrane-binding residues in both the C1 and C2 domains of FVIII in mediating FVIII uptake by APCs and conferring immunogenicity to therapeutic FVIII.

Materials and Methods

Reagents

Recombinant human FVIII (Advate) was from Baxter Bioscience (Vienna, Austria). DMEM-F12, IMDM, AIMV and RPMI were from Gibco® (Life technologies). Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), CD14 microbeads (MACS, Miltenyi Biotech, Auburn, CA, USA), and human recombinant GM-CSF and IL-4 (Cellgenix Technology Transfer, Freiburg, Germany) were used for human monocyte-derived dendritic cells (MoDCs) preparation. Murine cytokines GM-CSF and IL-4 were from (Cellgenix Technology Transfer, Freiburg, Germany). The mouse monoclonal antibody Mab6 and human anti-C2 antibody BO2C11 (JM Saint-Remy, KUL, Leuven, Belgium) were produced in the laboratory and purified using protein G affinity purification (Pierce). The anti-A2 antibody GMA-8015 was purchased from Green Mountain Antibodies (Burlington, VT, USA). Fab or $F(ab')_2$ fragments from the antibodies were generated by papain or pepsin digestion following manufacturer's instructions (Pierce).

Production and purification of recombinant mutated or wild-type FVIII

Recombinant FVIII variants were generated by splicing by overlap extension PCR (SOE) using B domain-deleted recombinant HSQ-Reneo plasmid [17] (as the template. The presence of the mutation was confirmed by standard sequencing analysis. BHK-M cells were transfected and selected for neomycin resistant clones using Geneticin® G418 (500 μg/ml). The highest expressing stable clone was scaled-up for FVIII production in serum free AIM-V medium. Medium was collected and stored at -80°C until purification. FVIII purification was performed as previously described. [17]Specific activity of purified Y1680C was similar to that of HSQ and ranged between 7000-9000 IU/mg by one-stage clotting assay and molar extinction

coefficient of 256,300 $M^{-1}cm^{-1}$. Interaction with VWF was verified using both ELISA and Surface Plasmon resonance (SPR) as described elsewhere. All C2 mutants expressed and their specific activities ranged between IU/mg.

FVIII binding to VWF, BO2C11 and LRP

ELISA plates (Maxisorb, Nunc) were coated with human plasma-derived VWF (Wilfactin, LFB, Les Ulis, France) at 1 µg/ml, BO2C11 at 1 µg/ml or recombinant human CD91/LRP (R&D systems) at 0.5 µg/ml in bicarbonate buffer, pH 9.5. Wells were blocked in 20 mM HEPES, 150 mM NaCl, 0.05% tween 20 and 5% BSA. FVIII mutants were then diluted in blocking buffer and incubated in coated wells. Bound FVIII was revealed after incubation with a biotinylated GMA-8015 at 1 µg/ml in blocking buffer, followed by incubation with streptavidin coupled to horseradish peroxidase (R&D systems) and ortho-phenylenediamine dihydrochloride (Sigma-Aldrich). Absorbance was read at 492 nm.

Generation of human MoDCs and murine BMDCs

Human MoDCs were prepared as previously described (ref). Briefly, monocytes were isolated from healthy donors using CD14 microbeads and differentiated into immature dendritic cells in RPMI complete medium containing GM-CSF and IL-4 for 5-6 days. Murine BMDCs were generated from E16 FVIII-deficient mice and differentiated into immature DCs [18] by culturing bone marrow cells in RPMI complete medium supplemented with murine recombinant GM-CSF and 50 mM 2-mercaptoethanol. After 5-6 days of culture MoDCs or BMDCs contained greater than 90% differentiated cells as analyzed by FACS..

***In vitro* FVIII endocytosis**

FVIII endocytosis was measured as described ([7]) with the use of biotinylated GMA-8015 followed by streptavidin PE (BD Biosciences, San Jose, CA, USA). Fixation was done using BD Cytofix and eBioscience Perm buffer to permeabilize the cells and visualize internalized FVIII. FACS plots were analyzed with FlowJo Version 7.6 software (TreeStar Inc) or BD FACS Diva Software.

FVIII antigen presentation assay *in vitro* using FVIII-specific CD4+ T-cell hybridoma

For T-cell presentation assays, MoDCs from a healthy donor with the HLA class II locus DRB1*0101/0301 were generated and cultured for 24 hr in 96 round-bottom plates with the 1G8-A2 FVIII-specific HLA-DRB1*0101-restricted CD4+ T-cell hybridoma (ratio 1:10) in the presence of FVIII. In case of splenocytes, total splenocytes from HLA-DRB1*0101 transgenic SURE-L1 mice were used as the source of APCs [19]. Activation of T cells was measured by IL-2 secretion in the supernatant by ELISA (BD Biosciences).

FVIII administration and characterization of anti-FVIII immune responses

FVIII-deficient E16 mice (8-12 week-old) were administered retro-orbitally using 10 nM FVIII alone or pre-incubated with 20 nM Fab fragments in a final volume of 100 µl at weekly intervals. Blood was drawn in heparinized tubes by retro-orbital bleeding 4 days after the 3rd and 4th administration of FVIII. Plasma was collected and kept at -80°C until use. For the studies using $F(ab'')_2$, 1 IU/ml of FVIII was pre-incubated with $F(ab'')_2$ IgG4 isotype control or BO2C11 at 1:12 molar ratio prior to administration.

For determining anti-FVIII antibody responses, ELISA plates were coated with 1 µg FVIII in bicarbonate buffer, pH 9.5 overnight at 4°C. Wells were blocked with PBS, 0.05% tween 20 and 2% BSA. Serial dilutions of the samples were incubated for 1 hr at 37°C and bound IgG were revealed using anti-mouse IgG conjugated with horseradish peroxidase. The mouse monoclonal anti-FVIII antibody Mab6 was used as a standard. Titers are expressed as µg/ml mAb6-equivalent. Inhibitory titers were estimated by incubating FVIII with different dilutions of plasma for 2 hr at 37°C followed by the estimation of FVIII residual activity using Siemens Chromogenic Assay following manufacturers instruction. T cell proliferation assay were performed as described [19].

Statistical analysis

Statistical significance was assessed using the double-sided Mann–Whitney non-parametric test (StatView 5.0.1 software).

Results:

We first evaluated whether BO2C11 inhibits FVIII uptake by monocyte-derived dendritic cells (MoDCs) or bone marrow-derived dendritic cells (BMDCs) *in vitro*. We hypothesized that the uptake is restricted within the BO2C11 binding region and thus as control employed another C2 antibody ESH-8. ESH-8 does not compete for BO2C11 epitope and is known not to inhibit FVIII binding to VWF or phosphatidylserine (PS) membrane surfaces [20]. We incubated 20 nM FVIII or FVIII pre-incubated with equimolar concentrations of anti-C2 antibodies prior to incubation with MoDCs or BMDCs. Additionally, we evaluated the inhibition by incubating with a 2 molar excess of the Fab fragments of the antibodies. Following the addition of BO2C11 or the corresponding Fab fragments, we observed over 70% reduction in

FVIII internalization (Fig 1A, 1C). This was similar to the effect observed using an anti-C1 antibody KM33 (data not shown and [7]). ESH-8 moderately but significantly inhibited FVIII uptake by MoDCs but did not have any effect on BMDCs. We observed similar inhibitory effects with BO2C11 or Fab of BO2C11 (B-Fab) in the antigen presentation assay used as a functional read-out for efficient uptake and processing (Fig 1B, 1D). Together, these results implicate the essential role of the C2 membrane binding residues in FVIII uptake by APCs.

FVIII bound to BO2C11 induces diminished immune responses *in vivo*

We next investigated the effect of BO2C11 on FVIII uptake and presentation *in vivo*. We administered FVIII pre-incubated with $F(ab')_2$ of an isotype control or of BO2C11 for four weeks at weekly intervals. After 4 weeks, FVIII bound to BO2C11 exhibited diminished immunogenicity compared to the isotype control-treated mice (Fig 1A). Since BO2C11 inhibits FVIII interaction with VWF, we generated a FVIII variant, $FVIII^{Y1680C}$ that cannot associate with VWF and re-evaluated FVIII immunogenicity in the presence of BO2C11. $FVIII^{Y1680C}$ had a specific activity similar to that of wild-type FVIII (approximately 7000 IU/mg). We confirmed its lack of interaction with VWF using ELISA and SPR (data not shown). When administered to FVIII-deficient mice, $FVIII^{Y1680C}$ exhibited rapid clearance (T1/2 18 minutes versus 405 minutes for wild-type FVIII, Fig 2B), confirming its inability to interact with VWF *in vivo*.

$FVIII^{Y1680C}$ alone or pre-incubated with the 2 molar excess of BO2C11 Fab fragments were administered to FVIII-deficient mice at weekly intervals for four weeks. Five days after the last FVIII administration, the anti-FVIII immune response was measured. We observed a reduction both in the anti-FVIII immune response and T

cell proliferation in the case of FVIII pre-incubated with BO2C11 Fab fragments as compared to the FVIII alone (Fig 2C, 2D, P<0.001). T cells responded to the non-specific activation stimulus concanavalin A (not shown). Thus, our results implicate a role for the C2 domain of FVIII in FVIII recognition and presentation *in vivo*.

Characterization of FVIII containing alanine substitutions of the residues known to interact BO2C11 binding

We generated FVIII mutants wherein the two arginine residues located at position 2215 and 2220 or the phenylalanine residue located at position 2196 were substituted to alanine residues. The FVIII mutants were purified, were functional and exhibited specific activities between 4800-8000 IU/mg (Supplemental figure 1, data not shown). Substitution at R2215 resulted in diminished binding to BO2C11. SPR analysis indicated a faster dissociation and markedly reduced binding to BO2C11 paratope (Supplemental Table 1). Substitution at R2220 resulted in near complete inhibition of FVIII interaction to BO2C11 (Fig 3A). Double mutation of these residues provided little additional benefit, confirming that R2220 contributes to most of the binding to BO2C11. Importantly, these FVIII variants retained binding to antibodies targeting other domains of FVIII. In particular, the mutations did not have significant effects on the ability of the different FVIII mutants to interact with VWF, ESH-8, KM33 or BO2BII (Fig 3B, 3C, 3D, 3E).

Recently, arginine residues within the C1 domain were implicated in FVIII uptake[8]. Though as a single mutation R2090A only had minimal effect on FVIII uptake, it was shown the alanine substitution provided substantial additive effect to the F2092A/K2093A on FVIII uptake by APCs. Hence as control, we also performed the assays using the C1 mutant (R2090A/F2092A/K2093A). First we characterized FVIII

mutants binding to KM33. C1 mutant showed diminished binding to KM33 but retained substantial binding to VWF (data not shown).

C2 domain residues are implicated in FVIII uptake by antigen presenting cells

We next evaluated the role of these residues in FVIII uptake and presentation. Human MoDCs and murine BMDCs were incubated with 20 nM FVIII, fixed, permeabilized and FVIII was revealed with the anti-A2 antibody, GMA-8015. FVIII uptake was significantly reduced for R2215A and the double mutation R2215A-R2220A (Fig 4A). Similarly, we observed concentration dependent increase in MFI for HSQ, Y1680C but not much increase for R2215A and R2215-20A (Supplement Figure 2). We did not perform the latter assay with R2220A since previous experiments did not show any alteration in FVIII uptake.

We also confirmed our observation in an antigen presentation assay to an HLA-DR1 restricted CD4+ T-cell hybridoma using MoDCs derived from a HLA-DR1 donor or using splenocytes from SURE-L1 mice, that contains human MHC class II (Fig 4C and D). We observed significant decrease in the uptake and presentation as measured by IL-2 secretion for both R2215A and R2215-R2220A. Our present data indicate that the C2 domain participates in FVIII uptake and involves at least R2215.

Discussion

Factors that influence an immune response to FVIII remain elusive and controversial. However, several mouse studies have indicated that steps involved in generating an antibody response follows a classical pathway [21], requiring active antigen uptake and presentation by professional APCs[4]. Though the circumstances under which

FVIII gets presented and the trigger that may provide „danger signal" remain controversial [22, 23] (and manuscript under review), it is likely that modulating the uptake and the presentation of FVIII can moderate the development of anti-FVIII immune responses.

In this study, we demonstrated that an anti-C2 monoclonal antibody, BO2C11, that competes for binding to VWF and phospholipids, inhibits FVIII uptake by APCs *in vitro* and that FVIII bound to the Fab or F(ab')$_2$ fragment of BO2C11 exhibits a reduced immunogenicity *in vivo*. Importantly, FVIII endocytosis by BMDCs was not inhibited by ESH-8, an anti-C2 antibody that does not compete for VWF binding nor membrane binding and had only partial effect on uptake by MoDCs. Thus, our results suggested a role for membrane binding residues within the FVIII C2 domain in the uptake of FVIII by APCs. One possible bias in the observed protective effect could be that BO2C11 inhibits FVIII interaction with VWF[24] leading to a rapid clearance of FVIII, thus affecting its immunogenic potential *in vivo*. However, since we observed a similar protective effect (Fig 2C and 2D) for FVIII^{Y1680C}, a mutant that does not associate with VWF *in vivo* (Fig 2B), we propose that residues within the antibody-binding region are implicated in FVIII recognition and uptake by APCs independent of FVIII half-life and capacity to interact with VWF.

Our results are similar to a previous work by Wroblewska et al. [8] that implicated a role for C1 membrane binding residues in the antigen uptake both *in vitro* and *in vivo*. We find it interesting to note that two distinct antibodies targeting the C domains of FVIII can confer a protective effect, and both also involve membrane interacting residues. Whether the two domains are involved in a co-operative manner or are targeting multiple receptors is unclear.

We also demonstrated by site directed mutagenesis of surface exposed phospho-L-serine moieties within the BO2C11 binding pocket to play a role in the FVIII uptake by APCs. In this study, we did not target residues at 2199/2200 and 2251/2252 [14] that form the hydrophobic feet, as they were previously shown to be essential for FVIII cofactor activity and to also be involved in interaction with VWF. We hypothesized that the arginine residues that are buried deep in the BO2C11 binding region may be implicated in FVIII recognition and uptake [16]. Additionally, we also mutated F2196, a hydrophobic residue that interacts with BO2C11 and is also surface exposed. F2196 is predicted to be an HLA-DR1 epitope and was recently shown to exhibit a diminished presentation to a T-cell hybridoma [25]. The residues F2196 and R2215 are not conserved within the predicted orthologous FVIII sequences.

As expected, the mutations displayed varying effects on FVIII binding to BO2C11. All of them exhibited faster dissociation kinetics, as recently reported by Nguyen et al.[26] who employed purified C2 domain mutants. Thus, our data utilizing intact FVIII mutants substantiates their previous observations using the purified C2 mutants. Additionally, our data implicate that R2220 provides significant binding energy (Fig 3B) for this interaction since the single substitution to an alanine by itself was able to abrogate binding to BO2C11. More importantly, single mutation to an alanine at R2215 and R2220 or double mutation did not exhibit significant decrease in binding to VWF or antibodies directed against other domains of FVIII, including ESH-8, an anti-C2 antibody with its epitope distant from that of BO2C11[3, 20].

When we evaluated the effect of these substitutions on FVIII uptake, each of the substitutions displayed distinct endocytosis. R2220A displayed little effect on FVIII uptake by BMDCs or MoDCs, while single substitution at R2215A was sufficient to diminish uptake by APCs by over 60%. The double mutation did not display any

additional reduction in endocytosis, indicating that only the surface exposed R2215 is implicated in this process. This inhibition in FVIII uptake is similar to previously reported substitutions of surface exposed membrane binding arginine residues within the C1 domain of FVIII. It is of interest to note that modification of surface exposed R2090 provided additive effect and diminished FVIII uptake by APCs when combined with the two other surface exposed residues at 2092/2093 [8]. Our data together with previous reported role for the membrane binding residues in the C1 indicate that FVIII uptake by APCs may partially involve surface exposed hydrophobic or positively charged basic residues.

It is tempting to speculate that independent substitutions of surface exposed arginine residues may yield FVIII mutants that display-reduced uptake or binding to antibodies, probably by inhibiting charge-based interactions. Further studies employing single or multiple substitutions of the surface exposed residues within the C domains are warranted to substantiate these observations and their role in antigen recognition and uptake *in vivo*. Whether the observed inhibition of FVIII uptake *in vitro* can translate *in vivo* remains to be determined. One complicating factor would be the role of VWF, as it is known to effectively block FVIII uptake by APCs and also by catabolic receptors such as LRP or MMR. Thus, further studies *in vivo* are required to replicate the previous findings demonstrated by the C1 mutants and whether addition of the C2 mutation alone or in combination with the C1 mutations can provide additional benefit. Future studies focusing on these aspects may provide interesting observations that can develop newer FVIII products that can exhibit longer half-life and/or reduced immunogenicity profiles.

Acknowledgements

This work was supported by Institut national de la santé et de la recherche médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), and Université Pierre et Marie Curie (UPMC) Paris 6, and by an unrestricted grant from Grifols (Barcelona, Spain).

Contributions

BG, MI, and SLD planned the work; BG, MI, SD, IP, OC performed experiments and edited the manuscript; BG, MI, SLD analyzed the data; BG, SVK, MI, OC and SLD wrote the report. All authors discussed the results and commented on the manuscript.

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Legends to Figures:

Figure 1. Anti-C2 antibody BO2C11 inhibits FVIII uptake and presentation *in vitro*. FVIII (20 nM) was pre-incubated with equimolar concentrations of ESH-8, BO2C11 or with a 2 molar excess BO2C11 Fab fragments. Uptake by h**uman monocyte-derived dendritic cells** (**A) or murine bone marrow-derived dendritic cells (C)** was analyzed by fluorescence-activated cell sorting (FACS)**.** Results are expressed as the percentage of median fluorescence intensity (MFI), whereby 100% corresponds to MFI obtained with wild-type FVIII (HSQ). Graphs are representative of 4 independent donors in the case of immature MoDCs (B) and 5 individual mice in that of BMDCs (D). Panels B and D represent activation of FVIII-specific HLA-DRB1*0101-restricted T cells by FVIII-loaded HLA-matched human MoDCs (10 nM) or by splenocytes (10 nM) from SURE L1 mice. Supernatant was collected after 24 hr and the IL-2 production by activated T cells was measured. Representative of three experiments (means ± SEM).

Figure 2. BO2C11 modulates the anti-FVIII immune response in a mouse model of hemophilia A. (A) Hemophilic E16 KO mice (n=6) were injected once a week for 4 weeks with 1 IU of B-domain containing FVIII pre-incubated with 6 µM $F(ab'')_2$ of BO2C11 (closed square) or IgG4 isotype control (closed circle). After four weeks, anti-FVIII antibody response was measured. Data are represented as serum dilution versus mean±SEM of absorbance (492 nm). (B) B-domain deleted FVIII (10 nM, HSQ, closed circles) or $FVIII^{Y1680C}$ (closed square) in 100 µl was administered into FVIII-deficient mice and the residual FVIII activity was measured at different time

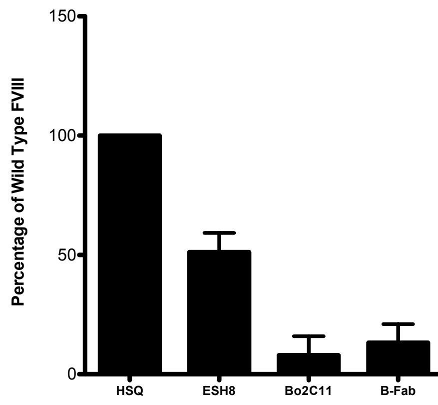
points (n=3 mice per timepoint) using a chromogenic assay. The data is plotted as % of initial FVIII activity (measured 5 minutes after administration) versus time. (C) FVIII-deficient mice were administered $FVIII^{Y1680C}$ (10 nM, closed circle) or $FVIII^{Y1680C}$ pre-incubated with 20 nM of BO2C11 Fab (closed square), intravenously for five weeks at weekly intervals (n=17; pool of two independent experiments). Anti-FVIII IgG responses were monitored after the third FVIII administration and were followed until the fifth administration. Anti-FVIII IgG titers are defined as arbitrary units based on standard curves generated using a purified mouse monoclonal anti-FVIII IgG (Mab6) serially diluted on FVIII coated ELISA plates. (D) Splenocyte proliferation assay was performed as described in the methods. Data are expressed as mean±SEM. Statistical significance was performed using the two-tailed non-parametric Mann-Whitney U test (ns: not significant, **: P<0.001).

Figure 3. Characterization of FVIII containing alanine substitutions of the residues predicted to interact BO2C11 binding. VWF (A), BO2C11 (human anti-C2 domain antibody) (B), ESH-8 (mouse anti-C2 domain antibody that does not overlap with BO2C11) (C), BO2BII (human anti-A2 domain antibody) (D) and KM33 (mouse anti-C1 domain antibody) (E) were immobilized on microtiter plates. After blocking, wild-type FVIII (HSQ) or C2 mutants were serially diluted across the plate and bound FVIII was revealed using the biotinylated anti-A2 antibody, GMA-8015 (Green mountain antibodies, USA). FVIII bound to BO2BII was revealed using ESH8, followed by anti-mouse HRP. The graphs are represented as absorbance measured at 492 nm (mean±SD).

Figure 4. C2 domain mutations alter FVIII endocytosis and antigen presentation by APCs. Wild-type FVIII (HSQ) or mutants FVIII-Y1680C, FVIII-F2196A, FVIII-R2215A, FVIII-R2215-20A, or FVIII-R2090A/K2092A/F2093A were added at 20 nM to MoDCs (A) or BMDCs (C) for 30 min at 37°C. Internalized FVIII was detected as described above. Results are expressed as the percentage of median fluorescence intensity (MFI), whereby 100% corresponds to MFI obtained with wild-type FVIII. Panels B and D represent activation of FVIII-specific HLA-DRB1*0101-restricted T cells by FVIII-loaded (10 nM) HLA-matched human MoDCs orsplenocytesfrom SURE L1 mice, respectively. Supernatant was collected after 24 hr and the IL-2 production by activated T cells was measured. Representative of three experiments (means ± SEM)

Figure: 1

A



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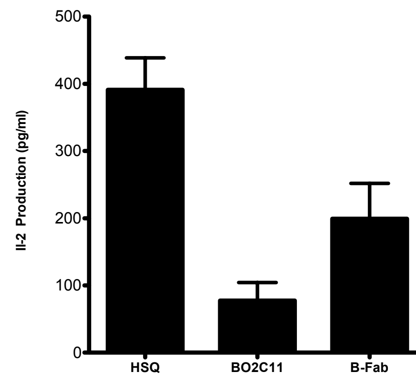

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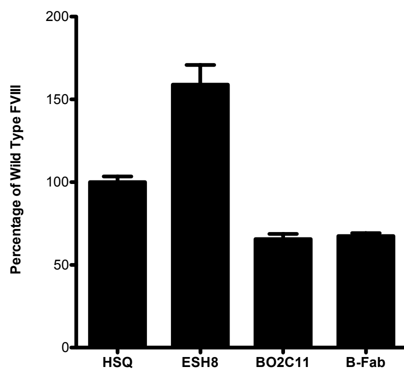

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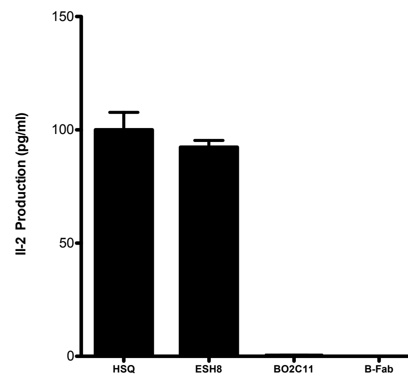
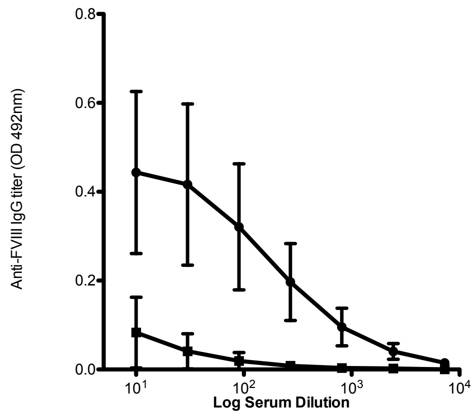


Figure:2

A



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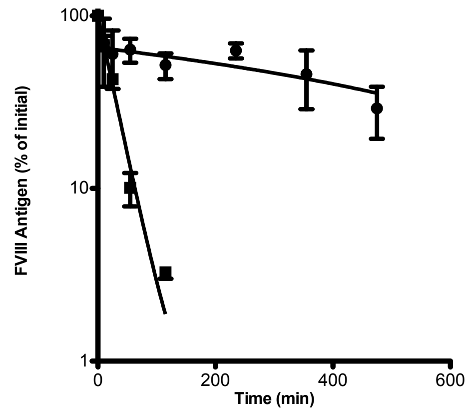

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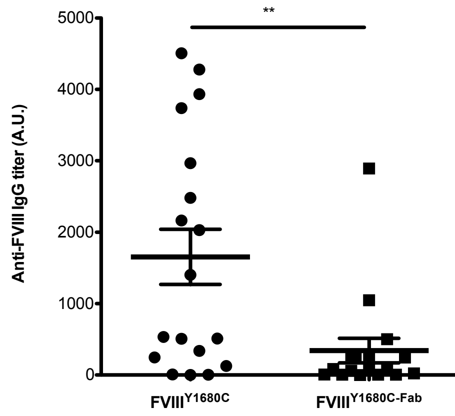

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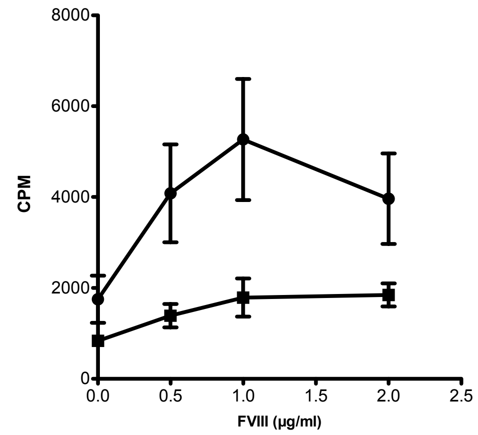


Figure:3

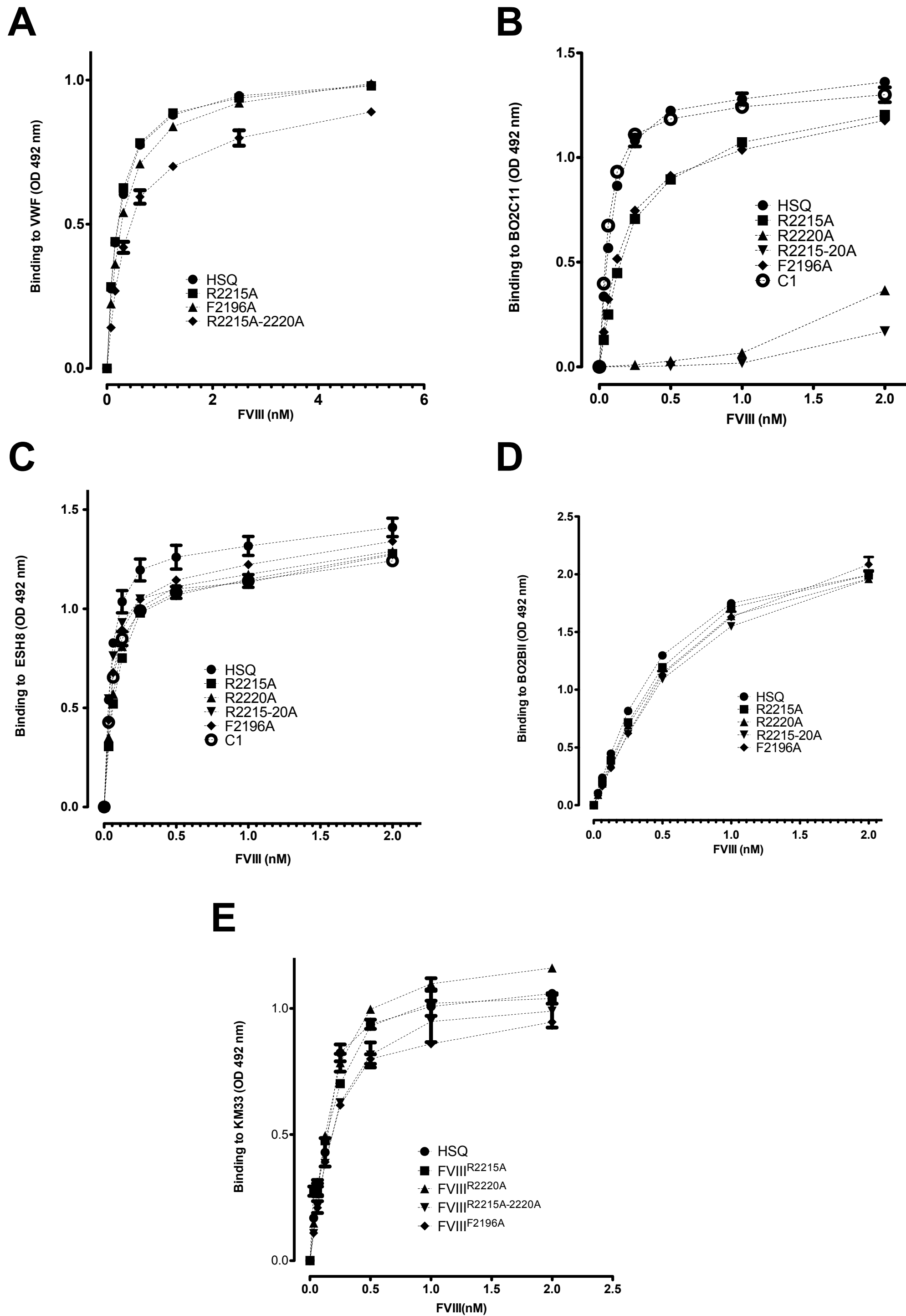
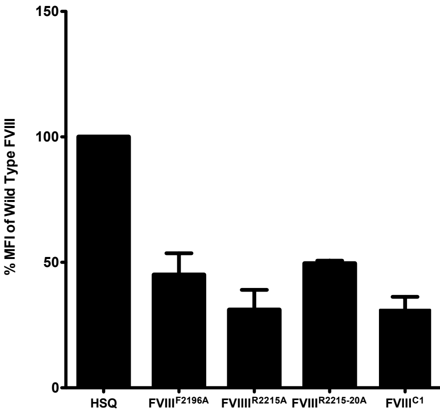


Figure: 4

A



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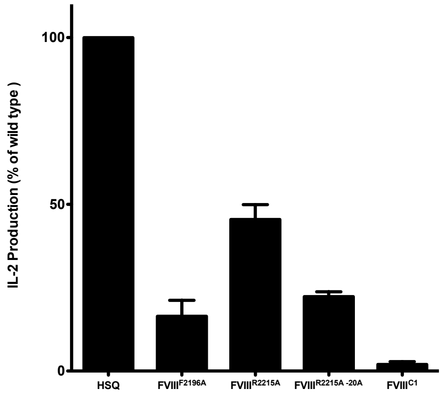

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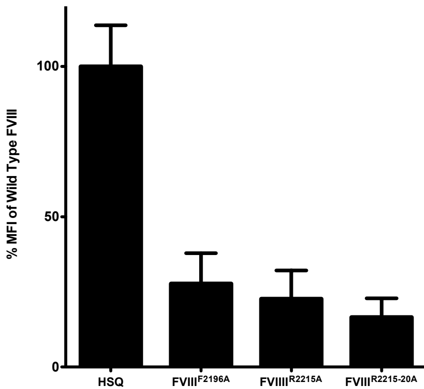

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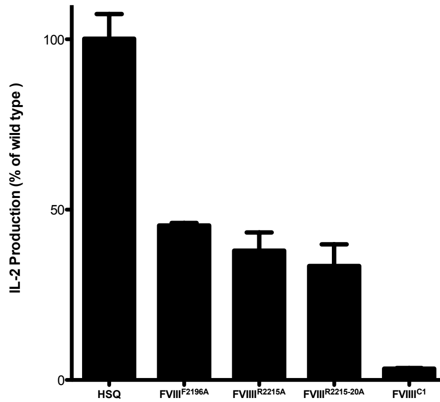
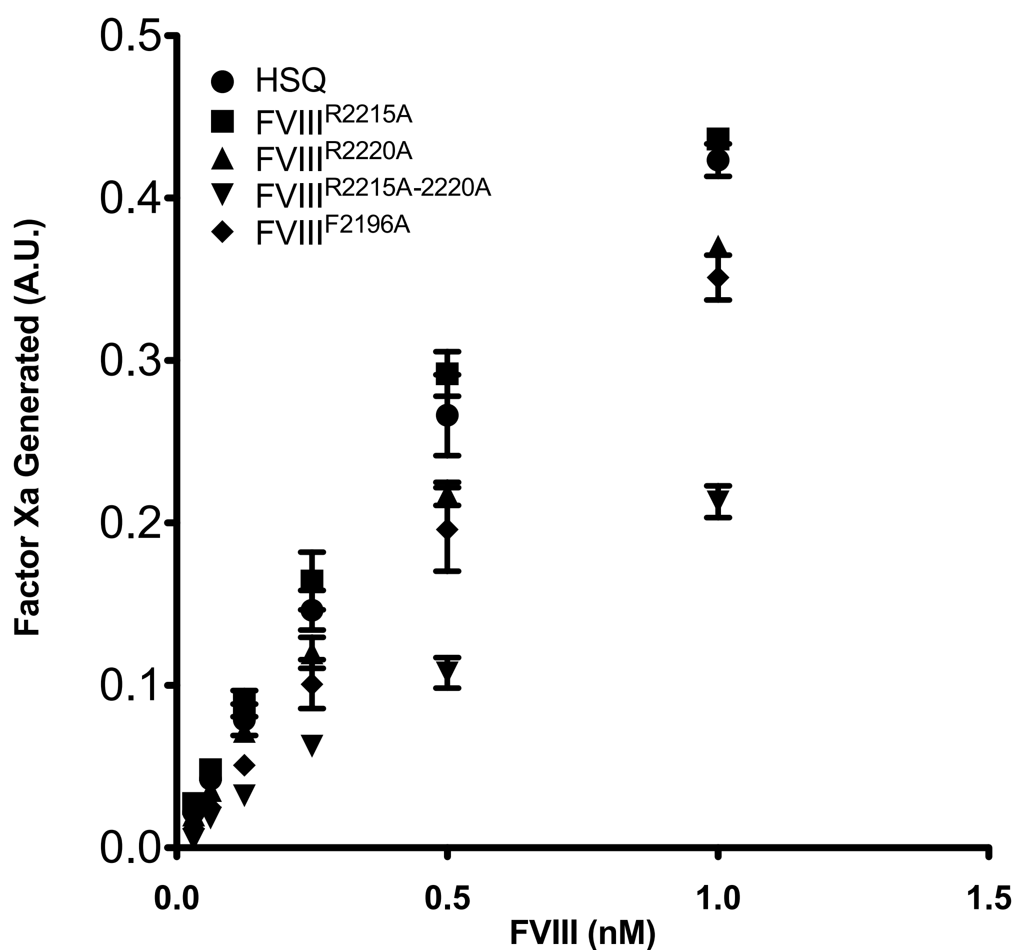
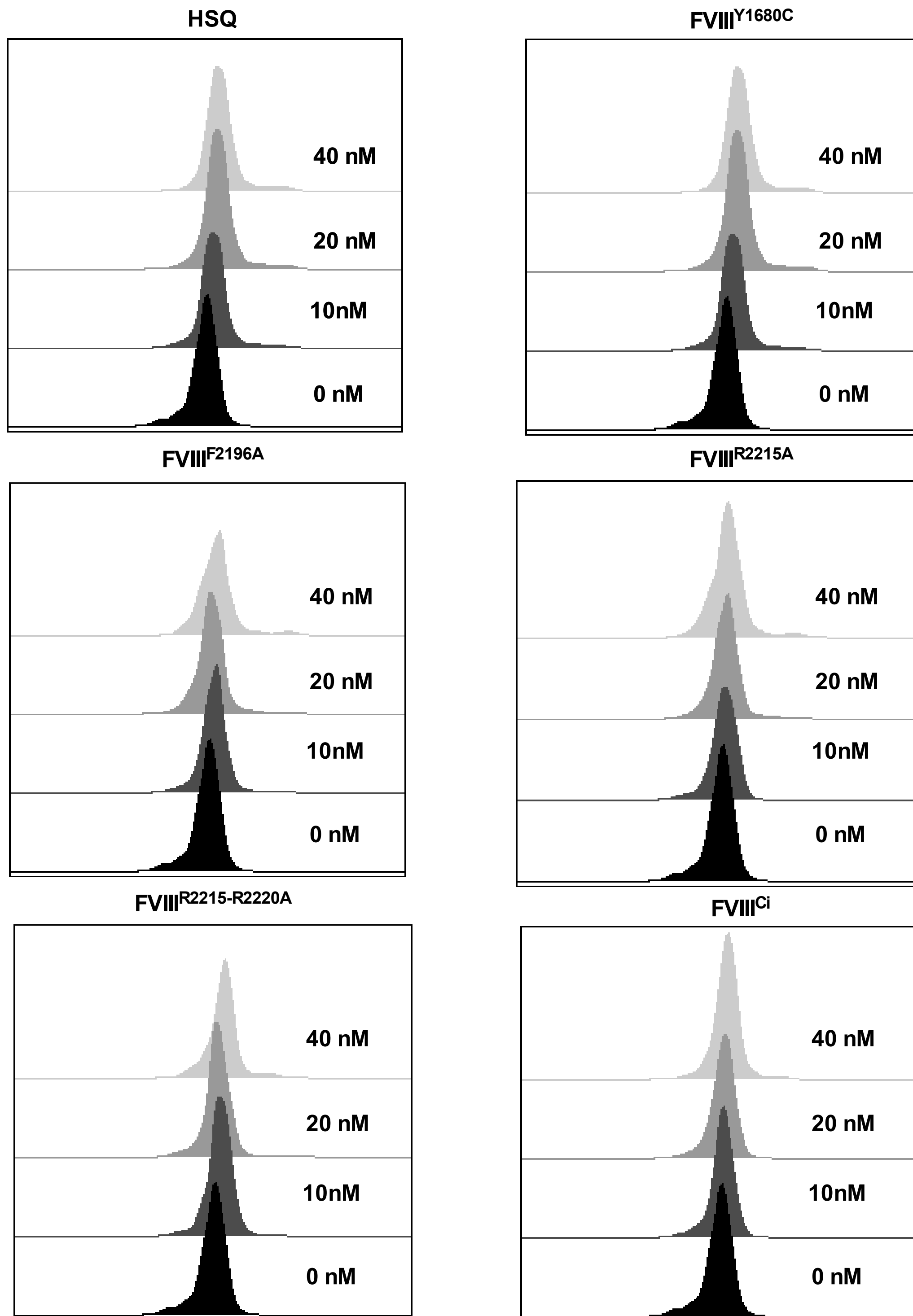


Supplemental data



Supplemental Figure 1. FVIII C2 variants are functional and generate factor Xa in chromogenic assay. FVIII or FVIII variants were serially diluted 2-fold starting at 1 nM. Factor Xa generation was measured using FVIII chromogenic assay. The reaction was stopped at 10 min using 20% acetic acid and the final absorbance measured at 405 nm. The data is represented as factor Xa generated in arbitrary units and is equivalent to the absorbance at 405 nm. The data observed were compatible to the observed specific activities. The R2215-20A has lower specific activity and probably relates to the differential binding to the PS membrane surface.



Supplementary Figure 2. FVIII uptake by MoDCs. Increasing concentrations of wild-type FVIII (HSQ) or mutants FVIII-Y1680C, FVIII-F2196A, FVIII-R2215A, FVIII-R2215-20A, or FVIII-R2090A/K2092A/F2093A were added to MoDCs for

30 min at 37°C. FVIII endocytosis was assessed as described earlier and data analyzed using FlowJo.

Supplemental Table 1. Kinetic and functional data of each FVIII C2 variant with BO2C11 antibody as determined in SPR (Biacore)

FVIII	**Ka ($M^{-1}S^{-1}$)**	**Kd (S^{-1})**	**K_D (M)**
HSQ	4.14e5±1.9e+03	1.74e-4± 3.58e-05	4.413e-10
F2196A	2.85e5± 1.73e+07	1.18e-3± 1.53e-07	3.13e-9
R2215A	2.32e5± 63	7.26e-4± 4.14 e-05	3.13e-9

For determination of association, various concentrations (10 nM-0.3 nM) of FVIII variants were injected for 300 sec at 20 µl/min. After this time, dissociation started by replacing the FVIII solution with buffer (20 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween 20) for a further 900 sec. Signal detected on uncoated channel was subtracted from the signal measured on BO2C11 coated channel. Biosensor data were fitted to 1:1 models and binding parameters determined.

DISCUSSION

Section IV. Discussion.

Development of an anti-FVIII immune response is a major complication associated with treatment of hemophilia A patients. There exists extensive information that associate several genetic and non-genetic risk factors that can influence development of inhibitory antibodies. However, the primary factor that drives FVIII immune response is not known. During my PhD research, I have attempted to recognize the relationship between the structural and functional aspects of FVIII in its immunogenicity using a mouse model of hemophilia A. In the present chapter, the major conclusions and potential implications thereof are discussed with particular reference to the role of the FVIII membrane binding residues in this process.

Section 4.01 The role of coagulation associated processes in development of an anti-FVIII immune response.

In Results section 1, we demonstrated that immune response to therapeutic FVIII in a mouse model of hemophilia A does not involve coagulation-associated process.
Severity of bleeding is a risk factor for developing inhibitors in hemophilia A patients [139]. Active bleeding is inherently associated with inflammation and coagulation [23] . There exists an extensive overlap between coagulation-associated inflammation and inflammation-associated coagulation. Thus, factors that are implicated in either of these processes can provide danger signals. Clotting is the primary source for thrombin and thus generation of thrombin requires activation of coagulation processes *in vivo*. The primary physiological function of FVIII is to participate in coagulation events and generate a “thrombin burst”. Lack of this thrombin burst results in uncontrolled bleeding and thus the hemophilic phenotype. Importantly, thrombin at

normal physiological concentrations possesses varied functions which are summarized in (Figure 8.) [140]. Thus it is plausible that thrombin generation upon FVIII administration may provides danger signals required for the initiation of an anti-FVIII immune response.

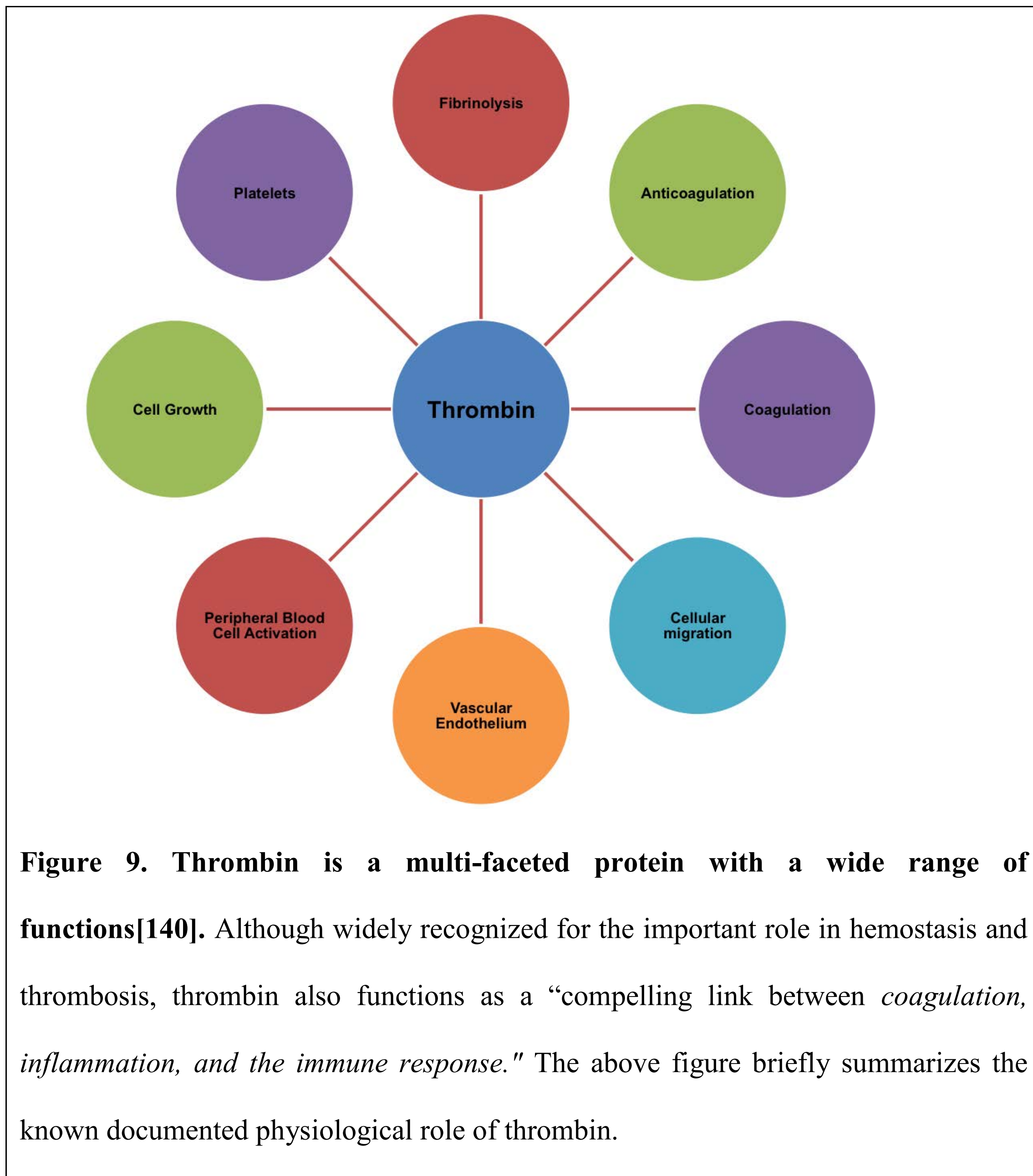


Figure 9. Thrombin is a multi-faceted protein with a wide range of functions[140]. Although widely recognized for the important role in hemostasis and thrombosis, thrombin also functions as a "compelling link between *coagulation, inflammation, and the immune response."* The above figure briefly summarizes the known documented physiological role of thrombin.

Indeed, Skupsky et al. demonstrated a role of thrombin in the initiation of anti-FVIII immune responses. In contrast, Meeks et al. [107], using a recombinant FVIII mutant model of hemophilia A. However, the study by Meeks et al. could not provide conclusive evidence for the role of thrombin and engaged a FVIII administration that

lacks cofactor activity, demonstrated that FVIII-dependent thrombin generation was dispensable for the initiation of the anti-FVIII immune response in a mouse schedule that was intense (i.e. 5 injections followed by two booster injections at twice the initial dose of FVIII). However, it provided a snapshot, and observations that were restricted to FVIII-dependent thrombin generation. In my first chapter, I have systematically delineated the role of coagulation events in providing "danger signal" necessary for the initiation of anti-FVIII immune responses.

Thrombin generation occurs via both intrinsic and extrinsic pathways of clotting (Figure 2, Section 1). We first asked whether thrombin generation by FVIII-dependent pathway is implicated by following the kinetics of anti-FVIII immune responses. If thrombin is implicated in the anti-FVIII immune response, it is more likely that we observe differences in the kinetics of the antibody development. However, we observed that $FVIII^{V634M}$, a FVIII mutant that lacks cofactor activity, was equally immunogenic and the kinetics of antibody generation was not distinguishable from that of wt-FVIII (Results section 3.01 1 figure 1). It is important to bear in mind that FVIII exhibits rapid clearance compared to other coagulation proteins and is administered at weekly intervals. However, it is plausible that the introduction of a mutation may introduce a novel epitope that influenced its immunogenicity. Using IEDB we verified that $FVIII^{V634}$ is not a predicted epitope and that introduction of the 634M mutation does not alter the epitope (Table 1). Thus we have demonstrated that immune response to FVIII is not dependent on its ability to generate thrombin.

Section 4.02 $FVIII^{V634M}$ - a tool to understand FVIII cofactor activity?

From a structural perspective, $FVIII^{V634M}$ retains all known FVIII structural properties but lacks cofactor function. Interestingly, $FVIII^{V634M}$ binds FIXa (data not shown) similar to wt-FVIII and yet lacks cofactor activity. This observation thus may not be surprising since most of FVIII binding affinity to FIXa occurs from the light chain of FVIII [46] [47] [141]. Interestingly, the valine at 634 is conserved throughout all predicted FVIII sequences and is partially exposed to the solvent (data not shown). This implies that there probably occurs a conformational change upon activation by thrombin. This conformational change then exposes residues contiguous to V634, thus allowing for it to participate in its interaction with FIXa. This proposition is also in agreement with Duffy et al. [49] who demonstrated that FVIII binds FIXa with similar binding affinities as that of activated FVIII (FVIIIa) but seemingly undergoes a dramatic conformational change upon activation of thrombin and in the presence of phospholipids. Further studies are necessary to characterize the role of the residues surrounding this region in FVIII cofactor function and thus shedding light on what makes FVIIIa a good cofactor.

Section 4.03 TF activity is dispensable for the initiation of anti-FVIII antibody response

TF is the only physiological activator of coagulation [21]. We specifically addressed the role of TF in the initiation of immune responses to $FVIII^{V634M}$. Importantly, TF is considered to be the major link between inflammation and coagulation and increased TF activity is related to pathological thrombosis. We addressed the role of TF by employing two complementary approaches. Unlike the use of anticoagulants, such as warfarin, that may possess anti-inflammatory effects ([142-144]), these targeted

Table 1. Computation of the predicted binding affinity for mouse H2-IAb of the peptides encompassing residue V634 or M634.

Start	End	Sequence	Percentile rank	
			V634	**M634**
620	634	YVFDSLQLSVCLHE**V/M**	43.5	44.6
621	635	VFDSLQLSVCLHE**V/M**A	66.4	69.8
622	636	FDSLQLSVCLHE**V/M**AY	69.8	73.3
623	637	DSLQLSVCLHE**V/M**AYW	74.9	80.3
624	638	SLQLSVCLHE**V/M**AYWY	76.2	80.6
625	639	LQLSVCLHE**V/M**AYWYI	81.2	83.2
626	640	QLSVCLHE**V/M**AYWYIL	84.9	84.6
627	641	LSVCLHE**V/M**AYWYILS	85.6	84.8
628	642	SVCLHE**V/M**AYWYILSI	65.1	70.1
629	643	VCLHE**V/M**AYWYILSIG	55.1	58.6
630	644	CLHE**V/M**AYWYILSIGA	37.0	37.0
631	645	LHE**V/M**AYWYILSIGAQ	33.9	33.8
632	646	HE**V/M**AYWYILSIGAQT	24.9	25.0
633	647	E**V/M**AYWYILSIGAQTD	21.5	21.3
634	648	**V/M**AYWYILSIGAQTDF	21.6	21.5

The binding affinity of each of the 15 15-mer peptides that include the V634 or M634 residue for the mouse H2-IAb class II molecule was predicted using the IEDB consensus method (HTTP://TOOLS.IMMUNEEPITOPE.ORG/MHCII). The "IEDB Analysis Resource" website provides a peptide binding affinity in terms of "percentile rank", ranging between 0 and 100, with a low percentile value (high rank) reflecting a high binding affinity.

approaches are directed towards the activation steps involved in coagulation. We used: 1) systemic inhibition of TF activity using an anti-TF neutralizing antibody [145] and 2) a novel chimeric mouse model that lacks TF expression in hematopoietic cells. This is probably the first reported study to have used bone marrow transplant chimera to evaluate anti-FVIII immune responses. Both strategies are complementary to each other and both approaches have inherent defects that are compensated by each other.

For example, chimeric mice lacking TF cannot address the role of TF expression or activation in endothelial cells as TF deficiency is restricted only to hematopoietic cells, in particular monocytes. However, the use of the TF-specific antibody circumvents this obstacle as it allows for a more generalized inhibition of TF activity. Likewise, the efficacy of the antibody in neutralizing TF can only be evaluated in plasma and its extent of inhibition *in vivo* can never be determined and is dependent on the levels of TF expression and nature of injury. However, the bone marrow chimera achieves complete abolishment of TF expression. More recently, it was demonstrated that hematopoietic cell-derived TF plays a role in initiation of thrombus formation *in vivo* [28]. Similarly, this strategy can also inhibit the induction of TF expression in myeloid cells upon engagement with P-selectin expressed on activated platelets [26]. Interestingly, this TF derived from monocytes can protect hemophilic mice from acute bleeding injury such as a tail snip model. Additionally, during pathological conditions such as in sickle cell disease, hematopoietic cells contribute to about 50% of the TF activity [146]. Notably, in chimeric mice, secondary activation of coagulation or coagulation dependent inflammatory signals via TF is also inhibited [23]. Therefore these two approaches provide complementarity and together allowed

us to specifically evaluate the role of TF dependent activation signals in the initiation of anti-FVIII immune responses.

Section 4.04 Coagulation dependent on PAR activation is dispensable in initiation of anti-FVIII immune responses

Protease activated receptors provides the necessary crosstalk between coagulation and inflammation. These receptors are distributed widely in various tissues (Section 1.04, table 2) and are activated predominantly by the coagulation proteases with the exception of PAR-2.

In our study, inhibition of TF activity and a lack of “thrombin burst” did not modulate the anti-FVIII immune response. Since inhibition of TF does not modulate immune responses to $FVIII^{V634M}$, it signifies that the amount of thrombin generated or FXa may not be sufficient to activate the PAR receptor signaling. Indeed, it has been proposed that the levels of thrombin required for PAR-4 activation is much higher and probably implicates the amplification phase of coagulation and thus may require a “thrombin burst”. Hence, we can postulate that coagulation-dependent PAR activation is not involved in the initiation of antibody responses to FVIII. However, during bleeding-associated inflammation, there is an active recruitment of cellular mediators that can express other proteases such as tryptase, which may activate PARs independent of coagulation [147]. Thus, we cannot completely rule out the role of PAR signaling and further studies investigating this may shed light into the role of these receptors in this process.

Section 4.05 Importance of primary hemostasis in initiation of anti-FVIII immune responses

We investigated the role of platelets in anti-FVIII immune response using a mouse model that lacks GPIIb receptor (fibrinogen receptor). These mice lack the ability to aggregate platelets, which is an important step for primary hemostasis to occur. In addition, we also evaluated FVIII immunogenicity in FVIII or FVIII/VWF double

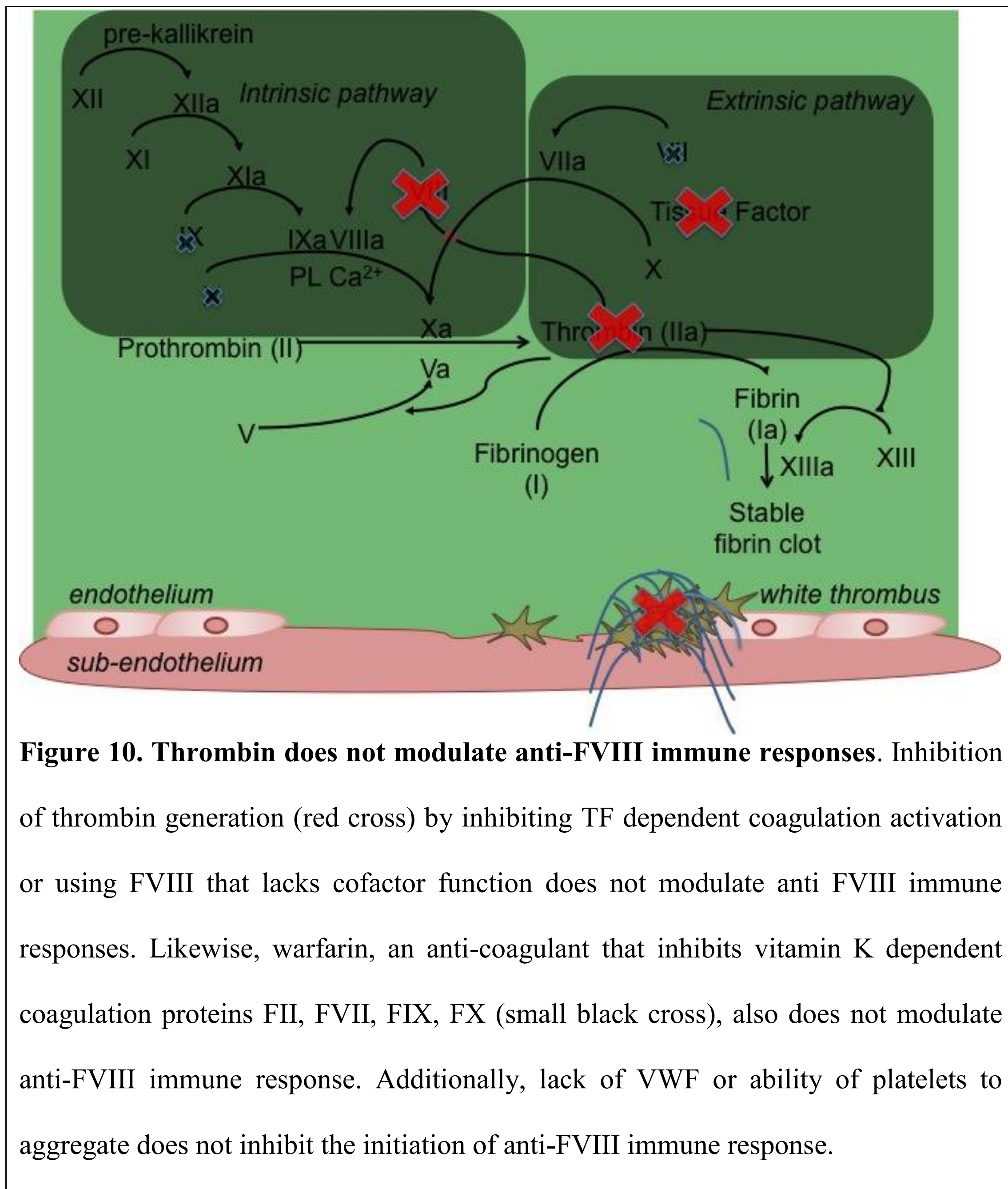


Figure 10. Thrombin does not modulate anti-FVIII immune responses. Inhibition of thrombin generation (red cross) by inhibiting TF dependent coagulation activation or using FVIII that lacks cofactor function does not modulate anti FVIII immune responses. Likewise, warfarin, an anti-coagulant that inhibits vitamin K dependent coagulation proteins FII, FVII, FIX, FX (small black cross), also does not modulate anti-FVIII immune response. Additionally, lack of VWF or ability of platelets to aggregate does not inhibit the initiation of anti-FVIII immune response.

knockout mice using wt FVIII or $FVIII^{Y1680C}$, a mutant that cannot associate with VWF *in vivo*. Interestingly, in both instances, we observed no significant difference in

the generation of anti-FVIII antibody responses. This further substantiates our conclusion that coagulation events are not implicated during the initial phases of anti-FVIII antibody responses. These conclusions are further supported by the observations during my previous work with Meeks et al., [107] where we demonstrated that immune responses to FVIII resistant to thrombin cleavage is as immunogenic as that of wt FVIII or $FVIII^{V634M}$. Together, the data presented in the first chapter demonstrate that the immunogenicity of FVIII in a mouse model of hemophilia A is not dependent on coagulation-associated events.

Section 4.06 Limitations of the current study

For over a decade, the murine model of hemophilia A has been valuable in understanding the development of antibody responses. However, one limitation of our study is the inherent weakness of using a mouse model and the presumption that there exists an active bleeding or coagulation (or lack of coagulation) in hemophilic mice. However, neither of these presumptions can be corroborated and are difficult to measure *in vivo*. Unlike humans, hemophilic mice do not exhibit spontaneous bleeding episodes when handled carefully or do not show signs of spontaneous arthropathy, a major complication seen in humans. This lack of development of spontaneous bleeding or hemarthroses may be due to the horizontal posture of mice compared to humans [148]. Indeed, mice carry body weight on four joints (rather than two). Hence, it is difficult to address experimentally the role of bleeding in the initiation of anti-FVIII immune responses. However, we recently showed that induction of an acute bleeding injury does not modulate FVIII immunogenicity. In fact, it was related to a decrease in anti-FVIII antibody responses (Peyron et al. Annex 1). Though this seemingly may not be representative of clinical observations, it

highlights the limitation of the existing mouse model and also that bleeding is perhaps not a risk factor in the mouse model of hemophilia A.

Another limitation in using a mouse model is the lack of appropriate mouse specific tools making it more difficult to dissect mechanistically the factors that govern the onset of an antibody response to FVIII. Of note, the only study that compared immunogenicity of murine versus human FVIII in a mouse model of hemophilia A demonstrated a reduced immunogenicity towards murine FVIII compared to human FVIII. None of the murine FVIII-treated mice developed inhibitory antibodies [149]. Additionally, comparative immunogenicity studies using human versus porcine FVIII demonstrated the inherent differences in the recognition of structural epitopes within orthologous FVIII molecules.

Equally, it is important to note that, in contrast to FVIII-deficient mice, not all severe hemophilia A patients develop inhibitors. Only 20% of patients who are severe hemophiliac due to inversion 22 (a mutation that is found in over 50% of the reported cases of hemophilia A) [6] develop antibodies to FVIII. This further corroborates that coagulation events can only provide secondary signals for initiation of anti-FVIII immune responses and that there exists a genetic predisposition to develop inhibitors.

Future studies employing appropriate mouse specific tools (i.e., mouse FVIII) and generation of newer FVIII mutants may provide a better understanding of the factors that determine development of anti-FVIII immune responses. In summary, my current study does not support the role of coagulation events that result in thrombin

generation to be a player in immune responses to therapeutic FVIII in a mouse model of hemophilia A.

Section 4.07 Role of C2 domain in FVIII recognition and uptake

In my first chapter, I demonstrated that the coagulation-associated processes play a limited role in the initiation of anti-FVIII immune responses in a mouse model of hemophilia A. In section 3.02, I evaluated the role for the C2 domain of FVIII in FVIII uptake and presentation.

Immune responses to soluble protein such as FVIII require active uptake of the antigen by professional APCs and these include macrophages, B lymphocytes and dendritic cells [150]. Uptake can occur via several different mechanisms and may involve receptor-dependent or independent pathways [108]. FVIII uptake by dendritic cells and macrophages has been demonstrated to occur both *in vitro* and *in vivo[108]*. Subsequent to its uptake, FVIII is efficiently processed and presented by MHC Class II both *in vitro* and *in vivo* [109] [110] [111]. However, the nature of receptors involved in the uptake and presentation of FVIII continues to be an active area of research and yet unknown. Previous work by Worbleswka et al. [62] demonstrated that the C1 domain of FVIII is involved in this process and that previously reported receptor(s) (LRP, MMR) known to be implicated in FVIII uptake by APCs or for its catabolism are not implicated. We hypothesized that the above observations are not restricted to FVIII C1 domain and may also involve the membrane interacting residues in the C2 domain.

Section 4.08 Importance of C2 domain of FVIII for its immunogenicity and cofactor function

The C2 domain is one of the most extensively studied regions within the FVIII molecule. It is important for its cofactor activity and also binding to VWF and phospholipid membrane surfaces [151]. Additionally, the C2 domain also contains a thrombin and factor Xa recognition motif [152], a factor IXa contact site[48, 153], and possibly a factor Xa contact site[64]. It also includes several of the dominant epitopes recognized by FVIII neutralizing antibodies [87]. Crystal structure of FVIII [154] in the absence of phospholipids show that the C domains are parallely aligned in the FVIII structure. This suggested that both domains of FVIII interact with membrane surfaces. This hypothesis was further substantiated by recent biochemical studies by Lu et al. [60] and Novakovic et al.[155] demonstrating that both C1 and C2 domains of FVIII cooperate to mediate high affinity binding to phospholipid membrane surfaces.

Structurally, the C2 domain membrane-binding spike involves hydrophobic residues [156] while the C1 domain encompasses the hydrophilic residues. This suggests that the residues of the C2 may interact with the hydrophobic core of membrane while the C1 residues interact with the polar phospholipid head group [60]. Additionally, the C2 domain contains several charged basic amino acids such as arginines that are proposed to provide stability by interacting with the PS head group [57].

Recent work by Meeks at al. showed that anti-human FVIII C2 domain antibodies in hemophilia A mice recognize a functionally complex continuous spectrum of epitopes that span the entirety of the C2 domain [87]. They also demonstrated that C2 domain

contains 18 distinct epitopes that can potentially be recognized by monoclonal antibodies. Whether these residues are equally recognized, as T-cell epitopes is unclear, though there exists several dominant T-cell epitopes within these regions [157]. Majority of the inhibitory antibodies targeted the ability of the C2 domain to interact with VWF or phospholipid membrane surfaces and were termed "classical" C2 antibodies[87]. However, there exists a class of antibodies that inhibit thrombin or FXa cleavage of FVIII and are termed non-classical antibodies. These antibodies have been reported to be present in hemophilia A patients as well [158].

Section 4.09 C2 domain is involved in FVIII uptake in vitro and in vivo

BO2C11 is a classical antibody and its crystal structure bound to C2 domain of FVIII was deciphered [159]. Using BO2C11, we now have reported that administration of wt FVIII pre-incubated with BO2C11 exhibits diminished immune responses *in vivo*. We confirmed that this observation was not due to the rapid clearance of FVIII by generating a FVIII mutant that cannot associate with VWF (Section 3.02 b Figure 2, panel b) and observed a similar protection. Additionally, we have shown that BO2C11 inhibits FVIII uptake by APCs *in vitro* and this inhibition is restricted to the membrane interacting residues within the C2 domain since ESH-8 (another C2 antibody that does not compete for FVIII interaction with VWF or membrane surfaces) did not have any effect on FVIII uptake or presentation. Our observations thus propose a role for the membrane interacting residues within the BO2C11 binding region in its uptake both *in vitro* and *in vivo*.

Interestingly, our observations are analogous to that observed using KM33, an anti-C1 domain specific monoclonal antibody that also inhibits FVIII interaction with

membrane surfaces, VWF and also uptake by APCs. However, one possible explanation of the observed effect might be that this inhibition could be merely steric or perhaps BO2C11 when bound to FVIII resulted in orientation of the FVIII molecule such that the C1 domain cannot interact with putative cellular receptors.

We investigated this further by generating FVIII variants that involve residues known to interact with BO2C11. We mutated three FVIII residues (F2196A, R2215A, R2220A) since these residues were previously implicated in membrane interaction [57]. Additionally, these residues are not conserved among orthologous FVIII species or between C1/C2 domains. Mutations included one hydrophobic and two polar residues that exhibit varying surface exposure and are not implicated to be within the hydrophobic feet of the C2 domain that are generally buried when bound to lipid membranes. All the mutants were functional and retained ability to interact VWF (Section 3.02b, Figure 3A) but exhibited varying levels of reduced binding to BO2C11 (Section 3.02b, Figure 3b). Importantly, all FVIII variants retained their ability to interact with antibodies directed against other domains of FVIII and also to ESH8 (Section 3.02b, Figure 3C).

Interestingly, R2220, that contributes a major binding site for BO2C11 interaction (Section 3.02b, Figure 3b), had only a moderate effect in FVIII uptake and presentation (Section 3.02b, Figure 4 C,D). Our current evidence, that R2220A substitution does not alter FVIII specific activity and retains its ability to interact with VWF and ESH-8, is different from the previous observations of Nguyen et al. [160] who found that substitution at R2220 to an alanine destabilizes the purified C2 mutant such that it has lost binding to several C2 antibodies (including BO2C11 and ESH8)

that were not part of a contiguous epitope. A reasonable explanation for the observed difference could be that purified C2 domain of FVIII exhibits a tertiary organization that is different from that of intact FVIII due to structural constraints and also due to the arrangements of other domains.

Our results are also in contrast with a previous report from the group [161]who had reported that R2215 is involved in binding to VWF. However, R2215 is not conserved. This indicates that this substitution might retain the ability to bind VWF and as shown in Chapter 2 (Section 3.02b,figure 3A) R2215A had very little effect in this interaction. Even though both serine and alanine are small amino acids, serine is polar and thus may provide additional interactions that could have stabilized FVIII differently. However, when we generated a R2215S variant, we observed similar effect: R2215A and R2215S (data not shown) both interacted with similar binding affinities to VWF and BO2C11. Thus, we currently do not have a clear explanation for these observed differences but to speculate that the use of unpurified supernatant in the earlier study, rather than affinity purified recombinant FVIII, may have partly contributed to the observations.

Section 4.10 Effect of FVIII C2 mutations on FVIII uptake and presentation

As has been shown earlier in chapter 2 (Section 3.02b, Figure 4 A,C), mutations within the C2 domain diminished FVIII uptake. Thus, we are tempted to propose that there exists a synergy between membrane binding residues in both the C1 and C2 domains of FVIII in mediating FVIII uptake and presentation by APCs.The mechanisms by which this may occur are being discussed in the subsequent paragraphs.

The cellular receptors implicated in FVIII uptake by APCs are controversial and relatively unknown [162]. Previous studies by Dasgupta et al.[115] implicated a role for mannose ending glycans within the A1 or the C1 domain of FVIII in FVIII endocytosis. However, recently published membrane bound 2D structure of FVIII position N2118 at the interface of the C1-A1 domains and along with previously published crystal structure [163] appears to be away from the membrane interacting residues of the C1 and C2 domain of FVIII. In addition, it is unclear whether this positioning can allow for the free solvent accessibility of the sugar residue to interact with MMR receptor. In support of the above hypothesis that MR may not be implicated, Herczenik[118]et al. demonstrated that siRNA knockdown of mannose receptor did not interfere with the uptake of FVIII by APCs. Additionally, they reported that treatment of monocyte derived dendritic cells with mannan also failed to inhibit antigen presentation and proliferation of human patient derived C2 specific T cell clone[118]. Likewise, Delignat et al. from our group also has reported that the mannose ending glycans are not implicated in FVIII uptake by murine BMDCs. More studies are needed to clarify these contrasting observations on the role of MR. Nevertheless it seems likely that FVIII endocytosis by MMR is dependent on the cellular type, experimental conditions and its tissue distribution. The other explanation could be that APCs from humans and mouse do not uptake FVIII by similar processes and thus involve different receptors.

One potential conflicting observation that questions the importance of MMR *in vivo* is that the binding affinity of FVIII to MMR is reported to be approximately 240 nM [164]. Thus, it is unlikely that this interaction can occur independently *in vivo*. However, one possible mechanism by which this occurs could be at sites highly

enriched for MMR expression or that MMR facilitates the uptake of FVIII by APCs but requires an initial interaction via the membrane binding residues. Further mutagenic studies of mannose-ending residues may perhaps shed light on why there is such distinct differences observed between the studies and provide novel insights on the potential mechanism by which mannan may inhibit FVIII endocytosis.

Based on the presented evidence from my work, it seems logical to hypothesize that both C1 and C2 domain residues are involved in FVIII uptake and presentation by both murine BMDCs and human MoDCs. Thus, in an uniting model, I speculate that the uptake of FVIII by APCs also involves a two-step process that requires co-operative interactions of both the C domains followed by interactions with either single or multiple receptors expressed on the cellular surface of APCs. Even though our current evidence do not support a role of mannose ending glycans or yet unidentified receptor that is important for FVIII uptake and presentation, I believe it is impulsive to rule out a role of these glycans in FVIII uptake as they may be essential but only subsequent to a pre-concentration involving the C domains of FVIII.

Section 4.11 R2215 is implicated in binding to LRP

Our observation from this study may also have other implications that are of significance. One such area of FVIII research interest is to understand the mechanism of FVIII catabolism and develop newer therapeutics that may exhibit extended half-lif e[165]. Though, my primary interest is in understanding the mechanism of FVIII uptake by APCs and identifying structural determinants that are implicated in this process, it is tempting to investigate whether the residues may also overlap with

catabolic receptors such as LRP. Hence I will discuss some of my preliminary observations further.

Catabolism of FVIII or FVIII/VWF complex has been reported to occur through LRP [112, 113, 166] [167]. *In vivo* evidence for a role of LRP was first demonstrated when infusion of LRP-antagonist RAP increased half-life of infused FVIII. In addition, conditional LRP and LRP/LDLR-deficient mice also demonstrated a direct role for these receptors in FVIII clearance [117]. Subsequent *in vitro* studies by Meems et al. using LRP-overexpressing cells (U87-MG cells) also support these observations, since blocking of LRP using antagonist RAP leads to localization of FVIII on cellular surfaces and not its endocytosis [61]. However, the binding affinity of FVIII to LRP is about 60 nM [168] and the circulating concentration of FVIII is about 0.3 nM. Hence this interaction is rather unlikely and thus, it has been proposed that FVIII catabolism occurs via a two-step binding mechanism, in which FVIII is first pre-concentrated on the cellular surface,and this probably involves heparin sulfate proteoglycans [119, 165]. Interestingly, this LRP dependent endocytosis does not seem to occur in APCs though they expressed LRP on their surface. [120].Herczenik et al.[118] further confirmed this observation and demonstrated that inhibition LRP expression on APCs by siRNA had minimal effect on FVIII endocytosis.

Previously, Lenting et al.[168] demonstrated that interaction of FVIII to LRP is inhibited by both VWF and ESH-4, another C2 antibody that has epitopes that overlap with BO2C11. Thus, we evaluated the binding of the C2 FVIII variants with LRP. Interestingly, we observed that the binding to LRP was inhibited greatly by R2215

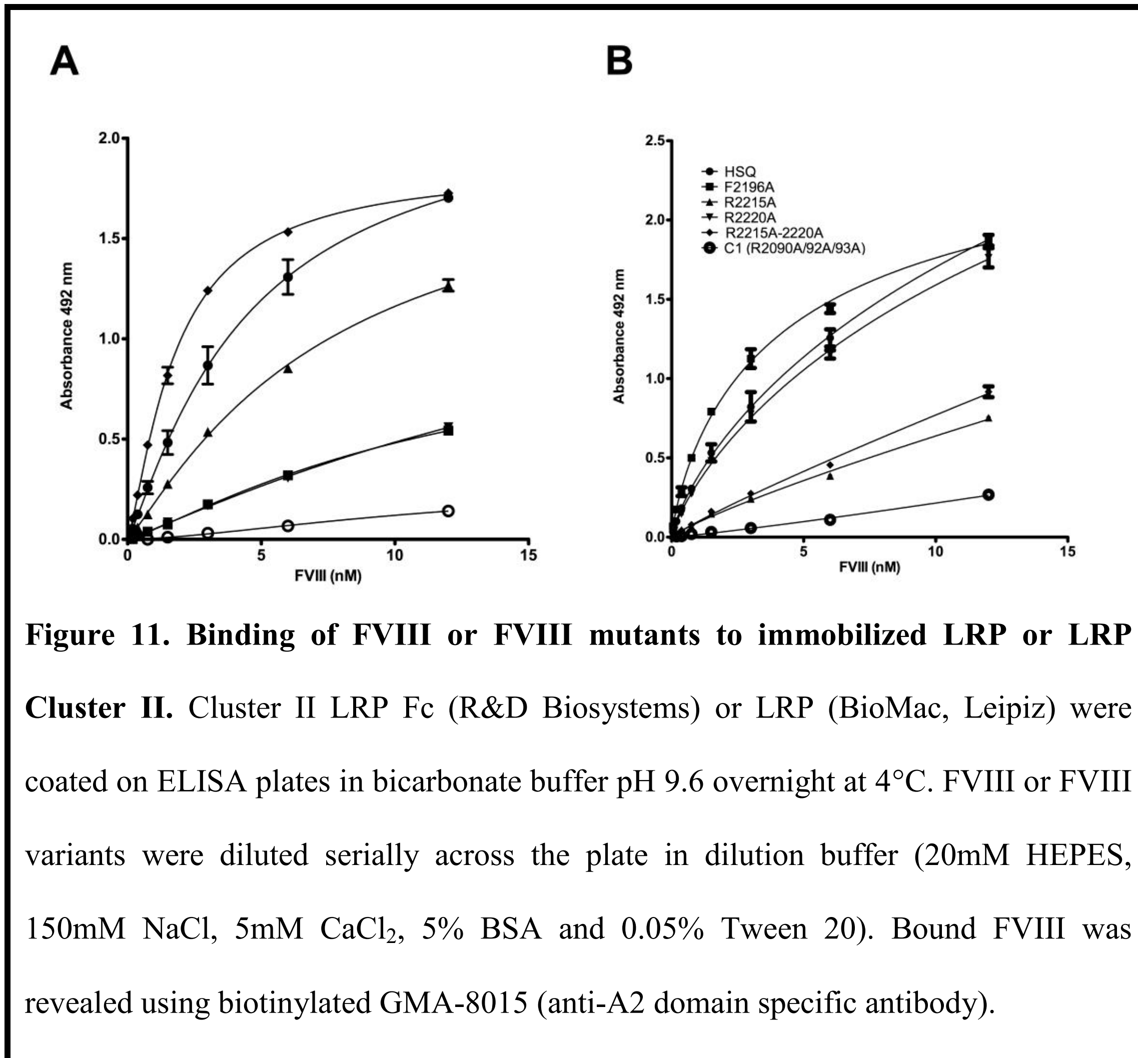


Figure 11. Binding of FVIII or FVIII mutants to immobilized LRP or LRP Cluster II. Cluster II LRP Fc (R&D Biosystems) or LRP (BioMac, Leipiz) were coated on ELISA plates in bicarbonate buffer pH 9.6 overnight at 4°C. FVIII or FVIII variants were diluted serially across the plate in dilution buffer (20mM HEPES, 150mM NaCl, 5mM $CaCl_2$, 5% BSA and 0.05% Tween 20). Bound FVIII was revealed using biotinylated GMA-8015 (anti-A2 domain specific antibody).

and not by either R2220 or F2196. However, R2215 is not implicated to be important for ESH4 binding[160], thus making us hypothesize that the inhibition by ESH-4 is probably a steric effect and this interaction may also involve other membrane binding residues within the C2 domain. On the contrary, when we immobilized cluster II of LRP, we observed modest reduction in the binding of R2220 substitution. We also confirmed a previous observation by Wroblewska et al. [62], and that the C1 mutants residues are also involved in interaction with LRP in vitro. We observed similar effect with serine substitutions of R2215. Importantly substitution of R2215S along with the C1 substitutions completely abolished its interaction with both LRP and cluster II. These observations were substantiated using SPR as well.

Our results along with the previously reported work on the C1 domain makes it appealing to propose both C1 and C2 domain membrane interacting surface exposed basic (charged) residues coordinate and interact with cellular receptors such that mutating one or more of the residues within each of these regions can impede cellular uptake of FVIII either in LRP dependent or independent manner. Interestingly, I made a serendipitous preliminary observation that the C1 mutants also lose their ability to interact with SP anion exchange column or heparin at neutral pH while wild-type FVIII and C2 mutants bind. It also makes it tempting to speculate that each of the domains maybe interacting supporting existence of a two-receptor model in its uptake. Whether this observation has physiological implication needs to be studied further.

Section 4.12 Caution is required in extrapolating observed in vitro inhibition of FVIII uptake:

As mentioned in the above paragraphs, FVIII circulates bound to VWF and this interaction is essential for maintaining its half-life [112] Previously, it was also demonstrated that VWF blocks the direct interaction between FVIII and LRP and that it could also inhibit FVIII uptake by APCs. Thus VWF exhibits a protective effect on FVIII uptake by catabolic receptors but also by yet unidentified receptors involved in uptake on APCs *in vitro*.

One probable mechanism by which this occurs could be that VWF inhibits the initial cell surface binding of FVIII, thus preventing a pre-concentration of FVIII at the cellular surface. Thus it is likely that VWF competitively blocks this initial interaction between the membrane binding residues and the cellular receptors either by direct contact with these residues or by steric inhibition. However, the mechanism by which

FVIII is cleared *in vivo* remains unclear. Though previous studies by Navarrete et al. [121] have shown that FVIII predominantly localizes in the spleen after infusion and that splenic macrophages are important for FVIII uptake an presentation *in vivo*, it still remains ambiguous whether the uptake of FVIII occurs in the spleen or in other organs or whether it occurs as FVIII/VWF complex or whether FVIII uptake precedes even when bound to VWF.

It is also important to point out that R2090 within the C1 domain of FVIII was previously reported to be involved in binding to VWF. If this were the case, one would presume that when bound to VWF it would not be capable of interacting with the cellular receptors for its uptake *in vivo*. However, as shown by the authors[62], R2090A substitution only modestly affected its ability to interact with VWF. Nonetheless, it remains unclear whether this residue is shielded in the presence of VWF *in vivo* or vice versa. Hence, I do not concur with the reported justification for the observed reduced immunogenicity of C1 mutants *in vivo*. Further studies are warranted to clarify this better. Likewise, it is also not clear whether substitutions within the C2 as demonstrated during my PhD work, remains solvent exposed when bound to VWF. Future studies to understand these processes that may help develop FVIII variants that exhibit reduced uptake and may also exhibit longer half-life *in vivo*.

It is well defined from clinical observations and substantiated by studies using animal models and *in vitro* uptake studies, that VWF is protective of FVIII uptake by catabolic receptors. It is also established that FVIII interacts with the light chain and this interactions spans several of the domains. However, the extent of this interaction and the orientation of FVIII when bound to VWF needs to be established to

understand the disappearing act of FVIII. One probable mechanism could be that under shear stress, FVIII is bound to VWF such that the C domains remain exposed for uptake *in vivo*. Supporting the above hypothesis, Castro-Nunewz et al.[169] reported that FVIII can localize and be internalized by macrophages under shear stress conditions and that VWF does not exhibit a protective effect under shear stress condition. However, this can only be partly true, as it is known that C domain contain residues that are also known to be involved in binding to VWF as mutations within these domains result in hemophilia A. Thus it remains to be established the mechanism by which FVIII presentation may occur *in viv*o. Likewise, whether the C2 or the C1 domain remains exposed for uptake also needs further investigation. Future studies using mutant FVIII C1 or C2 or combinational variants in the presence or the absence of VWF may provide newer insights into the process by which FVIII uptake occurs *in vivo*.

Section 4.13 So how can FVIII uptake occur in vivo?

Our results suggest a role for R2215 in FVIII uptake by APCs. We also have shown that this residue diminished FVIII ability to interact with its catabolic receptor LRP. We have also demonstrated that R2215S substitution along with the C1 mutants resulted in complete inhibition of its ability to interact LRP. Based on these observations, it is tempting to speculate that both the C domains, particularly membrane binding residues, are required for an initial concentration of FVIII onto cellular surfaces. We in agreement with Meems et al.[61] do not predict that this process involves a PS dependent mechanism. However, it cannot be ruled out whether FVIII bound to cellular receptors rearranges the membrane surface such that it can enhance its uptake.

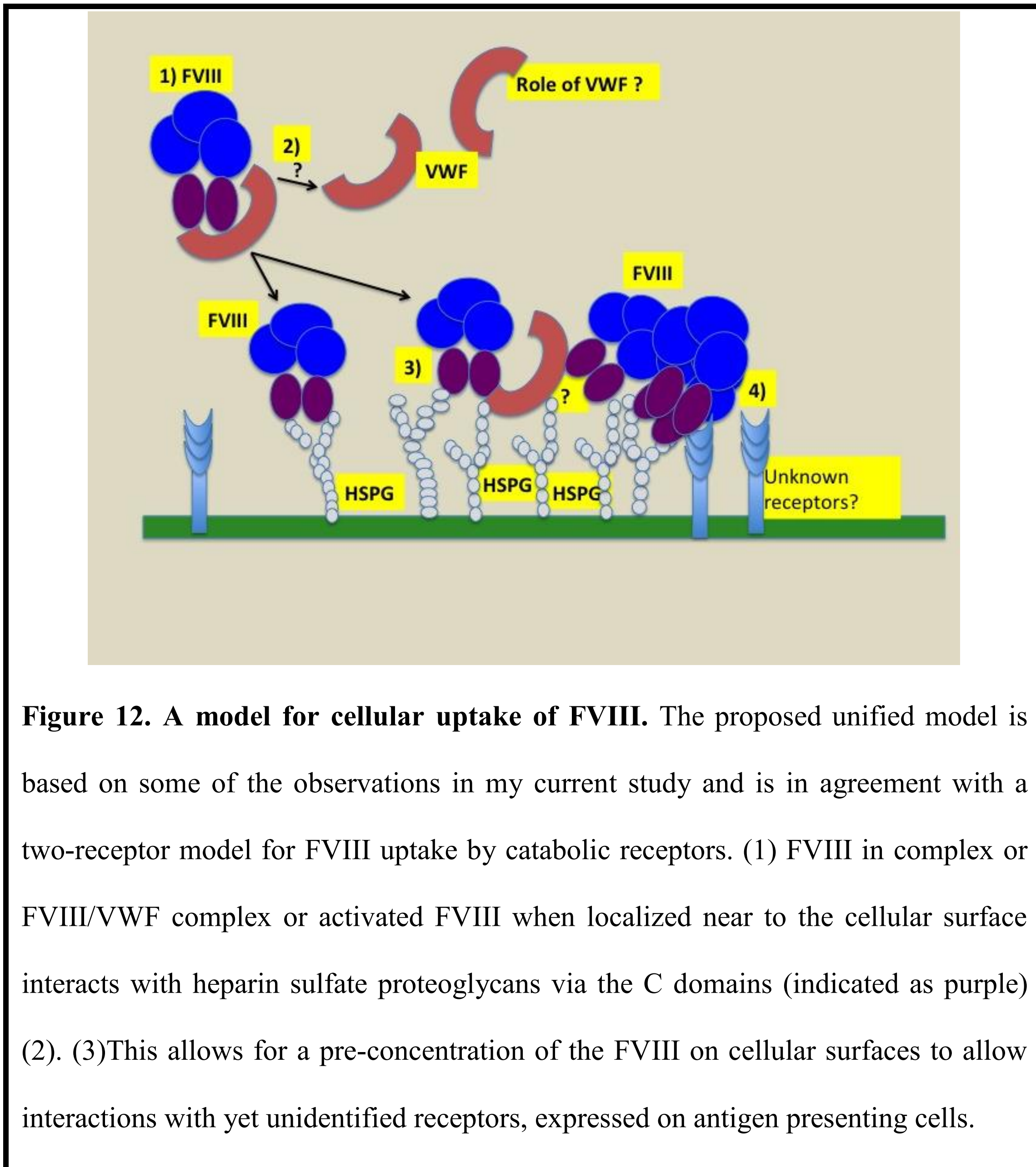


Figure 12. A model for cellular uptake of FVIII. The proposed unified model is based on some of the observations in my current study and is in agreement with a two-receptor model for FVIII uptake by catabolic receptors. (1) FVIII in complex or FVIII/VWF complex or activated FVIII when localized near to the cellular surface interacts with heparin sulfate proteoglycans via the C domains (indicated as purple) (2). (3)This allows for a pre-concentration of the FVIII on cellular surfaces to allow interactions with yet unidentified receptors, expressed on antigen presenting cells.

We further speculate that this may involve a dual receptor(s) that interact with both the domains. By this mechanism, it is possible that FVIII localizes at cellular surfaces at concentrations that permits interaction with other receptors required for FVIII uptake. We also hypothesize that this process likely involves heparin sulfate proteoglycans. Further studies using FVIII variants under shear stress conditions and employing appropriate *in vivo* mouse models (such as FVIII KO/ FVIII and VWF

double KO) to evaluate FVIII uptake either by catabolic receptors or receptors expressed by APCs may provide important insight on the processes that are involved.

Future directions and conclusions

Section V. Future directions and conclusions

A major limitation in current treatment of hemophilia A is the development of inhibitory antibodies that render treatment ineffective. Though, several of the clinical research are directed towards understanding factors that are associated with the development of anti-FVIII inhibitory antibodies, very little is known regarding factors that dictate the initiation process. In my thesis, using a mouse model of hemophilia A, I provide newer insights into the two important but contentious aspects. In my research article 1, I demonstrate that coagulation *per se* is not implicated in the initiation of anti-FVIII immune responses. This is in contrast to a previous work that proposed a role for thrombin in this process. However our results are in line with what is observed in clinics since not all hemophiliacs develop antibodies to FVIII and the risk for inhibitor development is indeed not correlated with the severity or intensity of bleeding during the first 15 exposure days. However, I believe that this work also brings to light the limitations in extrapolating such observations as the mouse model has several limitations as has been elaborately described in earlier paragraphs. One future study that is proposed is a detailed comparative immunogenicity of human versus murine FVIII in a mouse model of hemophilia A. Given that the $FVIII^{V634M}$, is conserved among all known species, it will be likely inactive and thus can be used to reconfirm the above observations and be able to make predictive statements that can subsequently be corroborated in human studies.

In the second research article, we provided evidence for a role of the C2 domain in FVIII uptake by antigen presenting cells. We demonstrated that blocking the C2 domain membrane binding residues using an antibody decreased the immune responses to FVIII *in vivo*. We also showed that R2220 is a major determinant of a

classical human antibody-binding site. Additionally, we were able to map one of the critical residues within the C2 domain (R2215) to be a player in this process. The mutational studies also provided several clues into the importance of the FVIII structure in its recognition and uptake. This allowed us to propose a model in which both C domains may coordinate in a manner that concentrates or orients FVIII and thus enhancing its uptake by APCs, in the absence of VWF. We also have showed that R2215 is implicated in FVIII interaction with LRP. Thus it is tempting to speculate that FVIII variants can be generated that can exhibit extended half-life *in vivo*. To explore this further, I believe it is important to appreciate the protective effect of VWF.

Currently I am involved in ongoing studies that are directed in providing insights into the protective role of VWF. In this aspect, we were surprised to find that $FVIII^{Y1680C}$ (FVIII variant that does not associate with VWF) was as immunogenic as that of FVIII that binds VWF in hemophilia A mice, even though it exhibited a half-life of only 18 minutes. This implicates that the above observations and hypothesis that VWF does confer a protective effect *in vivo* is likely valid. It is also tempting to propose that in the absence of VWF, FVIII is more readily endocytosed and presented by APCs *in vivo.* Further ongoing studies may provide further insights on this.

Future studies are needed to first understand on how and where FVIII uptake occurs *in vivo*? Does it occur bound to VWF? What happens to FVIII in the absence of VWF? Is it the activated form of FVIII that is more readily endocytosed than FVIII? Several basic questions revolve around the structural determinants of C domains. However they can all only be second to understanding the nature of the protective effect conferred by VWF/FVIII interactions. One potential mechanism could be that VWF induces a conformation that does not allow for FVIII C domains to be free and

thus inhibiting its interaction with cellular receptors. Hence I believe that it is important to understand the nature of this interaction and predict the domain dynamics of FVIII when bound to VWF.

One approach that could be of great value is hydrogen-deuterium (H-D) exchange mass spectrometry. This method relies on the exchange rate of hydrogen atoms of the amide backbone of polypeptides for deuterium atoms upon changing the solvent from H_2O to D_2O. The subsequent change in mass can then identified by mass spectrometry. This method is sensitive to perform interaction studies at physiological conditions and will provide information about the surface exposure of regions in FVIII and the change therein upon binding of FVIII to its ligands. Thus, this method can provide an indication regarding the FVIII peptide regions that are shielded when bound to VWF. A good understanding of FVIII/VWF interaction can further advance our knowledge regarding the determinants that are implicated in FVIII recognition *in vivo*. Additionally, it may also provide insights towards developing novel half-life extending bioengineered FVIII molecules.

In summary, I believe that my thesis research highlights the importance of the structural determinants within the C domains of FVIII for its recognition and uptake by APCs. Identifying these determinants and the cellular receptors that are implicated in this interaction will help develop novel FVIII therapeutics that may display lower immunogenicity and/or extended half-life *in vivo*. Given the recent success of gene therapy based approaches, it is also tempting to investigate the applicability of this approach using low antigenic FVIII variants in experimental model of hemophilia A with circulating inhibitors.

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Annexes

Please refer to attached files

Title: Prevention of immune responses to antigens and protein therapeutics by transplacental induction of central and peripheral T-cell tolerance

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One Sentence Summary: The transplacental modulation of the T-cell repertoire

during immune ontogeny and induction of antigen-specific regualtory T cells leads to immune tolerance to an immunogenic protein therapeutic.

Abstract:

Central tolerance plays a key role for modulating immune responses to self and exogenous antigens. The absence of self-antigen expression, as in patients with genetic deficiencies, prevents the development of antigen-specific immune tolerance. Hence, a substantial number of patients develop neutralizing antibodies to the corresponding protein therapeutics following replacement treatment. In this context, administration of missing antigens during fetal development, a key period for self-tolerance establishment should confer early and long-lasting antigen-specific tolerance. To this end, we exploited the physiological pathway of neonatal Fc receptor, by which maternal immunoglobulins are transplacentally transferred to fetuses. We demonstrate that Fc-fused antigens administered to pregnant mice reach fetal lymphoid organs, accumulate in antigen-presenting cells of myeloid origin and lead to generation of both thymic and peripheral antigen-specific regulatory T cells. This strategy was successfully transferred to a mouse model of hemophilia A, where materno-fetal transfer of the Fc-fused immunodominant domains of coagulation factor VIII conferred antigen-specific tolerance and preserved therapeutic efficacy upon factor VIII administration. Transplacental tolerance induction with Fc-fused proteins may thus prove valuable to prevent allo-immunization following replacement protein therapy of congenital deficiencies.

[Main Text:]

Introduction

Unwanted immune responses towards self-antigens develop in the case of autoimmune diseases, but also in several non-autoimmune conditions following administration of protein therapeutics, thus leading to treatment failure (*1*). This is particularly daunting for replacement therapies in the context of genetic deficiencies, as altered expression of the native self-protein results in defective immune tolerance. In this context, modulation of the T-cell repertoire and induction of antigen (Ag)-specific regulatory T cells (Tregs) appears as a strategy of choice to prevent allo- or auto-immunity (*2*).

Numerous approaches have been attempted to promote Treg-mediated tolerance, such as cytokine therapy (*3, 4*), modulation of signal transduction (*5, 6*) and intra- or extra-thymic delivery of target Ags (*7-9*). In the case of exogenous Ags, the time and route of Ag administration are critical to achieve optimal Treg induction. Since tolerance to self is first established in the thymus during immune ontogeny, the fetal period appears as a favorable time window for manipulating central tolerance employing exogenous Ags (*10, 11*). Indeed, transplacental transfer of maternal allo-Ags induces Treg-mediated Ag-specific tolerance in neonates (*12*).

Here, we exploited the physiological pathway by which maternal immunoglobulins are transferred to fetuses through the neonatal Fc receptor (FcRn) to non-invasively induce Ag-specific immune tolerance in the offspring. In a hemagglutinin (HA)-specific T-cell receptor (TcR) transgenic mouse model, we demonstrate that HA-specific Tregs are generated in the progeny following transplacental delivery of Fc-fused HA. This strategy was further translated to a preclinical model of severe

hemophilia A, where congenital deficiency of pro-coagulant factor VIII (FVIII) leads to development of inhibitory anti-FVIII antibodies upon FVIII replacement therapy (*13*). We demonstrate that transplacental delivery of Fc-fused immunodominant FVIII domains provides long-lasting tolerance upon FVIII replacement therapy.

Results

HA1Fc binds to neonatal Fc receptor and is transcytosed by syncytiotrophoblast cells

The neonatal Fc receptor **(**FcRn) is crucial for Fcγ-dependent transcytosis of maternal IgG across placenta (*14*). As a model Ag for our transplacental studies, we produced an Fcγ1-coupled HA1 (HA1Fc) (**Fig. S1-2**). HA1Fc co-localized with FcRn in syncytiotrophoblast cells (**Fig. 1A**), suggesting its interaction with FcRn. In surface plasmon resonance analysis, HA1Fc and a mouse monoclonal IgG1 (mIgG1) displayed similar binding affinities for immobilized mouse FcRn at pH 6.4 (K_D=17.9 nM and 10.1 nM, respectively), indicating that the affinity of the Fcγ1 for FcRn is not influenced by the fused protein (**Fig. 1B**) (*15*). *In vitro* transcytosis through syncytiotrophoblast cells of HA1Fc was revealed in a transwell assay: there was a time-dependent increase in basolateral levels with a decrease in apical levels (**Fig. 1C**). Absence of transcytosis at 4°C suggests an active transfer of HA1Fc (**Fig. 1C**). Altogether, HA1Fc has the biochemical characteristics required for efficient transplacental transfer.

Materno-fetal transfer of HA1Fc is Fc-dependent

In vivo imaging of pregnant mice injected intravenously with HA1Fc at embryonic day 18 (E18), revealed HA1Fc accumulation in maternal liver at 1 min and passage to

uterine horns by 4 hr (**Fig. 2A**). The transplacentally transferred HA1Fc was detectable in fetuses as early as 4 hr following injection to pregnant mice, and 24 hr later (**Fig. 2B**). By 24 hr, HA1Fc was also detected in the thymus (**Fig. 2B**).

In order to confirm the role of the Fc domain in the transplacental transfer of HA1Fc, we followed the levels of HA1Fc and of HA1 alone in mothers and fetuses following intravenous injection to pregnant mice. HA1Fc levels increased in a time-dependent manner in fetal plasma to reach 44±5% of the injected protein by 4 hr (**Fig. 2C)**. In parallel, HA1Fc levels decreased from 100 to 22±2% in mothers" plasma and concomitantly increased in the urine (*14, 16*), with 36±4% of the protein being detected by 4 hr. In contrast to HA1Fc, Fc-devoid HA1 was not detected in fetal plasma, despite a time-dependent and swift decline in mothers" plasma (down to 6±1% in 4 hr); there was a marked increase in mothers" urine with up to 46±5% of the injected protein after 4 hr (**Fig. 2D**). Taken together, these data suggest that approximately one-third of the HA1Fc is transferred to fetuses within 4 hr following administration to mothers, and that the transplacental transfer is Fc-mediated.

In order to validate the integrity of HA1Fc as a target Ag for immune effectors, we evaluated the efficacy of HA1Fc in inducing the proliferation of HA1-specific TcR-Tg $CD4^+$ T cells (**Fig. 2E**). At 60 nM, HA1Fc induced a 3-fold greater proliferation of TcR-Tg $CD4^+$ T cells than the $HA_{110\text{-}119}$ peptide (70.1±4.3% versus 19.4±0.6%), suggesting better presentation by Ag-presenting cells (APCs) (*17*).

Transplacental delivery of HA1Fc induces Tregs in an Ag-specific manner

We then addressed the potential of HA1Fc to shape the fetal immune repertoire. Based on the developmental phases of the mouse immune system, on the half-life of HA1Fc (approximately 6 hr, data not shown) and given that thymic TcR expression is

first detected around E17 (*18-20*), we administered HA1Fc at different frequencies over the E16 to E18 gestational window. The optimal delivery schedule to significantly modulate HA1-specific Tregs (Tg-Tregs) was determined by analyzing Tg-Tregs in spleens of transplacentally treated mice 2 weeks after birth (*21-23*). Transplacental delivery to fetuses of 100 µg HA1Fc daily on E16, E17 and E18 optimally induced Tg-Tregs in neonates, as compared to delivery on E16 and E18, or E16 only (**Fig. S3A**). A thorough analysis of the modulation of different T-cell subsets upon HA1Fc transfer on E16-E17-E18 was then conducted. Frequencies of TcR-Tg (6.5^+, recognized by the anti-clonotypic antibody 6.5) or non-Tg (6.5^-) total $CD4^+$ T cells were similar in the spleens of mice treated transplacentally with mIgG1 or HA1Fc (**Fig. 3A**). However, the frequency of Tg-Tregs in spleen was significantly increased by 2.6 folds in HA1Fc-treated mice as compared to mIgG1-treated mice (**Fig. 3B**, top panel and **Fig 3A**, right panel). Furthermore, we observed a marginal yet significant decrease in the frequency of transgenic effector T cells (Tg-Teff) following HA1Fc treatment as compared to mIgG1 treatment (**Fig. 3B,** middle panel). The fact that deletion of Tg-Teff was more prominent when HA1Fc was transplacentally delivered on E16 and E18 (**Fig. S3B**), may reflect differences in thymic selection thresholds compared to Tregs (*24-26*).

The frequency of Tg-Tregs also showed a more than 6-fold significant increase in the thymus of HA1Fc-treated mice as compared to mIgG1-treated mice (**Fig. 3C**), which reflects the fact that tolerance is initiated in thymus between E16-E18. The few number of cells in the thymus at the time of analysis probably results from egress of these cells to the periphery (*23*). The frequency of Tg-Teff in the thymus remained unaltered irrespective of HA1Fc or mIgG treatment (**Fig. 3C**). HA-TcR-Tg mice express only 10-15% of TCR Tg $CD4^+$ T cells (**Fig. 3A**), allowing

analysis of the effect of HA1Fc transfer on the non-specific T-cell subsets. There was no significant modulation of non-transgenic Treg or Teff subsets in both spleen (**Fig. 3B**) and thymus (**Fig. 3C**).

$CD8^+$ single positive (SP) cells in these mice also express a Tg-TcR(*27*). However, the frequency of Tg and non-Tg $CD8^+$ T cells was affected neither in spleen nor in thymus (**Fig. 3B and C,** bottom panels), thus confirming that the observed Ag-specific effects were MHC class II-restricted (*27, 28*).

Tregs may be categorized into thymic-derived or natural (nTreg) and peripherally- or adaptively induced (iTreg) subsets (*29*), based on the expression of Nrp-1 (Neuropilin-1) (*30, 31*). Moreover, the induction and expansion of iTregs by the administration of foreign Ags has already been shown (*32-34*). We found a two-fold significant increase in the frequency of Tg-nTregs in the spleen of HA1Fc-treated mice as compared to mIgG1-treated mice (**Fig. 3D**). Strikingly, the frequency of Tg-iTregs showed a significant 4.5-fold increase in the case of transplacental delivery of HA1Fc over that of mIgG1 (**Fig. 3D**). In the case of non-Tg Tregs, there was no modulation observed in both nTreg and iTreg subsets (**Fig. 3D**). We found significantly higher numbers of Tg-nTregs in the thymus of HA1Fc- (4.7-fold increase) vs. mIgG1-treated mice (**Fig. 3E**). As expected, Tg-iTregs were not detected in the thymi from mice of both the groups. Similar to spleen, non-Tg nTregs and iTregs remained unaltered in the thymus (**Fig. 3E**). Altogether, these data highlight an overall increase in HA1Fc-specific Tregs for both nTregs and iTregs. This increased number of both Treg subsets may be due to the relatively high affinity of the 6.5 TcR for the HA1 peptide/MHC complex and non-inflammatory Ag encounter in the periphery (*32-35*) as well as in the thymus (*8, 9, 24, 36*). The precise mechanism underlying this phenomenon remains, however, to be investigated.

In the presence of their cognate Ag, Tregs typically exert potent suppression of Teff proliferation (*37-39*). We thus evaluated the suppressive potential of Tregs generated upon transplacental transfer of HA1Fc. Upon stimulation with the $HA_{110-119}$ peptide, splenocytes from HA1Fc-treated mice showed a more than two-fold reduction in proliferation as compared to splenocytes from mIgG1-treated mice ($p<0.001$, **Fig. 3F**, left panel). Conversely, splenocytes from HA1Fc-treated and mIgG1-treated mice proliferated equally upon concanavalin A stimulation, thus excluding a splenocyte anergy (**Fig. 3F**, right panel). Thus, transplacental HA1Fc delivery generates Tregs in an Ag-specific manner that seem to be functional in suppressing Teff in the presence of their cognate Ag.

Transplacentally-delivered HA1Fc is endocytosed by fetal APCs of myeloid origin

Next, the nature of the APCs that contribute to the central and peripheral selection of Tregs upon transplacental Ag delivery was investigated. To this end, we identified the APC subsets that endocytose maternally delivered Alexa Fluor 647-labeled HA1Fc. SIRP-α^+ circulating dendritic cells (DCs) were the major cellular subset that endocytosed transplacentally delivered HA1Fc: HA1Fc was detected in 20, 11 and 6% of SIRP-α^+ cells in the fetal thymus, spleen and blood, respectively (**Fig. 4**). SIRP-α^+ DCs are characterized as a migratory subset capable of ferrying blood-borne Ags to the thymus (*40, 41*), thus leading to immune tolerance induction by negative selection of Ag-specific Teffs and positive selection of nTregs (*42*). HA1Fc was not detected in SIRP-α^- thymic resident DCs or SIRP-α^- DCs in the spleen (**Fig. 4**), while SIRP-α^- DCs were absent in blood. Transplacentally delivered HA1Fc was also present in fetal thymic, spleen and blood macrophages (10, 6 and 4%, respectively),

but neither in B and T cells (**Fig. 4**), nor in medullary thymic epithelial cells (data not shown). These data implicate fetal APCs of myeloid origin – mostly SIRP-α^+ migratory DCs - in the induction of Tregs specific for the administered HA1Fc.

Transplacentally-transferred Fc-fused FVIII domains induce tolerance to therapeutic FVIII in experimental hemophilia A

Finally, we exploited this strategy to impose tolerance towards self Ags not expressed in genetic deficiencies. We thus translated our approach in FVIII-deficient (HemA) mice, a model of severe hemophilia A (*43, 44*), which develop inhibitory antibodies to therapeutic FVIII upon replacement therapy, as in patients (*13, 44*). The A2 and C2 domains of FVIII are the major immunogenic determinants (*45, 46*). Therefore, we constructed two chimeric A2Fc and C2Fc proteins (**Fig. S1, B-E**), endowed with binding affinities to mouse FcRn equivalent to that of mIgG1 (**Fig. 5A** and **1B)**. *In vivo* imaging illustrated accumulation of C2Fc in uterine horns after administration to pregnant mice on E18 (**Fig. 5B**, left panel) and efficient transfer to fetal circulation by 4 hr, as documented by imaging (**Fig. 5B**, right panel) and ELISA (**Fig. 5C and 5D**). As observed for HA1Fc, transplacentally transferred C2Fc was detected in fetal thymus by 24 hr (**Fig. 5B**, right panel).

The progeny of mothers injected with A2Fc and/or C2Fc or with mIgG1 at E16-E17-E18 subsequently received replacement FVIII therapy from 6 weeks of age onwards (**Fig. S5**). As a model antigen, the immune response to C2 domain of FVIII was analysed in the progeny of mothers injected with C2Fc or with mIgG1. The Anti-C2 IgG titers were negligible in C2Fc (0.008±0.01 mg/mL, p=0.0003) transplacental treatment groups, as compared to mIgG1 treatment (0.08±0.01 mg/mL, **Fig. 6A**). Moreover, transplacental delivery of both A2Fc/C2Fc led to a remarkable reduction in

total anti-FVIII IgG titers (**Fig. 6B**): 1.5±0.5 mg/mL for A2Fc alone (p=0.07), 0.9±0.2 mg/mL for C2Fc alone (p=0.0006) and 0.4±0.2 mg/mL for A2Fc+C2Fc (p=0.002), as compared to mIgG1 (3±0.6 mg/mL). Importantly, inhibitory titers were drastically reduced in the A2Fc+C2Fc treatment group (81±31 BU) as compared to the mIgG1 treatment group (591±155 BU, p=0.004, **Fig. 6C**).

Because a role for FVIII-specific Tregs has been evoked in the establishment of tolerance to therapeutic FVIII (*47, 48*) and because transplacental delivery of HA1Fc generates HA-specific Tregs, we investigated the induction of FVIII-specific Tregs upon transplacental treatment with A2Fc+C2Fc. In the presence of FVIII, splenocytes from the offspring of A2Fc+C2Fc-treated mothers proliferated to a lesser extent (proliferation index between 2 and 3) than that of progeny from mIgG1-treated mice (proliferation index between 3.7 and 6, $p<0.05$, **Fig. 7A**, left panel). The proliferative capacity of the splenocytes to mitogen stimulation was however unaltered (**Fig. 7A**, right panel). Altogether, these results suggest the induction of Tregs upon A2Fc+C2Fc transplacental delivery.

An inherent limitation of the HemA mice, that mount polyclonal responses to FVIII, is that phenotypic identification of FVIII-specific Tregs is not feasible. We therefore relied on the functional measurement of suppressive activity by Tregs isolated from animals subjected to different treatments (*39*). In an *in vitro* assay, in the presence of FVIII, Tregs from the spleen of mice treated transplacentally with A2Fc+C2Fc significantly reduced the proliferation of $CD4^+CD25^-$ Teff from FVIII-primed mice, as compared to Tregs from mIgG1-treated mice (**Fig. 7B**). Furthermore, the adoptive transfer of Tregs from A2Fc+C2Fc transplacentally treated mice into naïve HemA mice significantly reduced the antibody response against FVIII upon replacement therapy, as compared to Tregs from mIgG1-treated mice (p=0.004) (**Fig.**

7C). Altogether, the diminished antibody response against FVIII is attributable to FVIII-specific Tregs generated upon transplacental treatment with FVIII domain-Fc fusion proteins.

Discussion

The present work provides a novel *in-utero* therapeutic strategy to manipulate the T-cell selection process during immune ontogeny and to induce Ag-specific immune tolerance. Here, we show that administration to pregnant mice of Fc-fused Ags results in effective Ag transfer to the fetal circulation. Importantly, we demonstrate that the transplacental transfer of Fc-fused Ags induces an increase of thymic and peripherally derived Tregs in an Ag-specific manner. Fc-fused Ags were taken up by fetal APCs of myeloid origin both in thymus and periphery, suggesting a role for these cells in the establishment of central and peripheral tolerance. When translated to a preclinical model of severe hemophilia A, transplacental Ag delivery induced tolerance towards therapeutic FVIII in the progeny.

The immunogenicity of protein therapeutics is a major obstacle for the management of several conditions, as development of immune responses following their administration in the patients neutralize therapeutic benefit. The unwanted immunogenicity of biologicals is linked to both extrinsic factors, such as processes associated with manufacturing, and intrinsic factors, such as recognition of few epitopes as foreign by the immune system of the patients. The latter is particularly relevant for replacement therapies in patients with genetic deficiencies, where the whole biologic agent may be recognized as foreign. In this context, T cells are central in the immune responses to these protein drugs, and therefore, strategies modulating

the T-cell repertoire towards immune tolerance may provide means to avoid such responses.

Our study provides a strategy that modulates the T-cell repertoire in an Ag-specific manner and generates Tregs that are crucial to achieve immune tolerance. The approach was successfully translated into a model of severe hemophilia A, a disease that may benefit the most from the induction of materno-fetal tolerance. Indeed, the birth of boys with hemophilia A may be anticipated based on a family history of hemophilia and on prenatal screening. Furthermore, prenatal genetic diagnosis may also predict patients with the highest risk of developing anti-FVIII antibodies upon replacement therapy (*49*). Importantly, prenatal genetic diagnosis is possible from the 12th week of pregnancy (*50*), at the same time when the first mature thymocytes are detected in the fetus (*51*). This suggests that, like in mice, there is a favorable time window for shaping central T-cell tolerance during fetal development in the human.

The potential of our approach is underscored by the fact that the sole use of immunodominant A2 and C2 domains of FVIII, which cover only 20% of the whole FVIII sequence, was sufficient to reduce immune responses in hemophilic mice by more than 80%. Ofnote, a therapeutic chimeric Fc-fused FVIII has recently been released on the market (*52*). Whether such therapeutic may be used during pregnancy for inducing tolerance to whole FVIII molecule in the progeny, remains to be established.

The present work also pave the way towards translation to autoimmune disorders for which the target antigens have been identified, such as autoimmune type 1 diabetes (S.C., N.G., S.J., S.L.D., R.M., manuscript in preparation) and to other genetic deficiencies, such as hemophilia B (*53*), for which a therapeutic factor IX-Fc

has recently been validated (*54*), or infantile Pompe disease (*55*), which becomes life-threatening upon the occurrence of neutralizing antibodies following replacement therapy.

Materials and Methods

Mice and cells

Homozygous mice that express a transgenic T-cell receptor (TcR-Tg) recognizing the $HA_{110-119}$ epitope (SFERFEIFPK) presented by I-E^d, on a Balb/c background, were bred in our animal facility. For experiments with pregnant mice, homozygous TcR-Tg males were crossed with wild-type Balb/c females purchased from Janvier Labs (St Berthevin, France). Factor VIII (FVIII)-deficient (HemA) mice were exon 16-knockout mice on a 129×C57Bl/6 (H-2Db) background (kind gift from Prof H.H. Kazazian, University of Pennsylvania School of Medicine, Philadelphia, PA, USA). Pregnant mice were obtained by homozygous crossing. Appearance of a vaginal plug was considered as day 0 of gestation. Pregnant mice were injected intravenously with 100 µg of Fc-fusion protein or mIgG1 (MOPC-21, BioLegend) on days 16, 17 and/or 18 of gestation. HA-TcR-Tg heterozygous offspring born from wild-type Balb/c females were used for analysis of Tregs at 2 weeks of age. The offspring from HemA females underwent replacement therapy with human recombinant FVIII (1 IU/mouse once a week for 4 weeks; Helixate® NexGen, CSL-Behring, Marburg, Germany) at 6 weeks of age. Plasma was kept at −20 °C until use. Animals were handled in agreement with local ethical authorities (Comité Régional d'Éthique p3/2008/024).

The mouse syncytiotrophoblast SC4235 cell line was a kind gift from Prof. C. Kanellopoulos-Langevin, Monod Institute, Paris, France. SC4235 cells were cultured

in Dulbecco's modified Eagle medium/Nutrient mixture F-12 with 10% fetal bovine serum (FBS) and 2 mM L-Alanyl-L-Glutamine, and grown in a 37°C, 5% CO_2 humidified incubator.

Antigens

Peptide. The $HA_{110\text{-}119}$ peptide (SFERFEIFPK) was custom synthesized from Polypeptide Laboratories (Strasbourg, France) and was >97% pure as assessed by HPLC and mass spectrometry.

Fc-fusion proteins. Sequences encoding the A2 and C2 FVIII domains (pSP64-VIII, ATCC, Manassas, VA), HA1 (pCI-Neo-HA, encoding HA) and Fcγ1 (B-cell hybridoma secreting mouse IgG1) were amplified by PCR employing specific oligonucleotides (Table S1). The sequences were digested with the appropriate restriction enzymes, purified and inserted at NheI/EcoRV sites by cohesive end ligation into pCDNA3.1(+) expression vector (Invitrogen). The expression was under the control of the CMV promoter and the expression cassette contains the signal peptide of IL-2, the c-myc sequence and the respective domain directly linked to the mouse Fcγ1. The different constructs were used to stably transfect HKB11 cells (ATCC) by electroporation. HKB11 cells were grown in serum free HL1 medium (Lonza). All Fcγ1-fusion proteins were expressed in cell culture medium and purified by affinity chromatography, using agarose-coupled anti-mouse IgG (Sigma-Aldrich). Fractions were dialyzed against Phosphate buffer saline (PBS) and concentrated by ultrafiltration (Amicon® Ultra 30K device, Millipore). The chimeric proteins were validated by Western blot and ELISA, using domain-specific monoclonal antibodies.

Recombinant C2 protein. A C-terminal His tag was fused to the C2 domain of FVIII by cloning the coding sequence into the pET-22b(+) vector (Novagen). The C2 domain was produced into *Escherichia coli* Rosetta-gami2(DE3)pLysS upon induction by IPTG overnight at 15°C. Bacterial raw extract was obtained by sonication in lysis buffer (20 mM HEPES pH 7.2, 400 mM NaCl, 20 mM imidazole, 2.5% triton X-100, 1 mM PMSF, 0.8 mg/mL lysozyme). The C2 protein was purified by affinity chromatography (HisTrap HP, GE) with a linear gradient of 20 to 62.5 mM imidazole. C2 was further purified by size-exclusion chromatography on a Superose 6 10/300 GL (GE Healthcare), eluted by 20 mM HEPES pH 7.2, 150 mM NaCl, 2.5% glycerol. The C2-containing fractions were pooled and concentrated by ultrafiltration (Amicon® Ultra 10K device, Millipore).

Immunofluorescence Microscopy

SC4235 cells, grown for one day on Ibidi chambers (biovalley, Marne La Vallee, France), were washed with PBS and incubated for 10 min. in PBS at 37°C. The HA1Fc -Alexa fluor (AF) 647 was added at 100 µg/mL in PBS at pH 6 and the cells were incubated for 30 min at 37°C. Cells were rinsed in PBS, fixed in 4% paraformaldehyde (Elcectorn microscopy sciences, Hatfield, PA) in PBS for 20 min at room temperature (RT) and permeabilized with 0.5% triton-X100 for 20 min. The permeabilized cells were then stained for FcRn using anti-FcRn antibody (sc-46328, SantaCruz) followed by secondary antibody conjugated with FITC (sc-2024, SantaCruz) and nuclear staining (Hoechst 33342, Molecular Probes). Images were acquired using an Axiovert M200 microscope (Zeiss) equipped with Apotome and four filters (Dapi, FITC, Rhodamine, Cy5) connected to a monochromatique CCD camera. Digital images were captured with AxioVision software.

Surface plasmon resonance analysis

The kinetic constants of the interactions between mouse FcRn (mFcRn) and Fc-fusion proteins (HA1Fc, C2Fc, A2Fc) were determined using BIAcore 2000 (GE, Uppsala, Sweden). Biotinylated mFcRn was immobilized on sensor chip SA (GE Healthcare), as described by the manufacturer. In brief, mFcRn was diluted in tris/citrate buffer (100 mM Tris, 100 mM NaCl, 0.1% tween-20 and citric acid to adjust the pH at 5.4) to finally immobilize 1000 resonance units (RUs). Experiments were performed using tris/citrate buffer. Two-fold dilutions of Fc-fusion proteins or mIgG1 (from 200 to 0.78 nM) were injected at a rate of 30 µL/min. The association and dissociation phases were monitored for 5 min. The regeneration of the chip surface was performed by injecting tris/citrate buffer at pH 8.5 with a contact time of 30 sec. The binding to the surface of the control uncoated flow cell was subtracted from the binding to the mFcRn-coated flow cells. All measurements of the interaction of mFcRn with Fc-fusion proteins were performed at 25°C. BIAevaluation version 4.1 (Biacore) was used for the estimation of the kinetic rate constants. Calculations were performed by global analysis of the experimental data using the model of Langmuir binding with a drifting baseline included in the software.

***In vitro* transcytosis assay**

SC4235 cells were grown onto 0.4 µm pore size transwell filter inserts (Corning Costar) to form a monolayer. The confluent monolayer was confirmed by staining with anti-ZO-1 antibody (Invitrogen). The HA1Fc protein was added at 5 µg/mL onto the apical chamber in 500 µl of RPMI-1640 medium supplemented with ultraglutamine (Lonza), 1% FBS, 1% non-essential amino acids, and 1% penicillin/streptomycin. The basolateral chamber was filled with the culture medium

alone. The transcytosis of HA1Fc was monitored from 1 to 24 hr at both 37°C and 4°C. Supernatants from apical and basolateral chambers were collected at indicated time points and HA1Fc levels were determined by ELISA, using a rabbit polyclonal antibody to HA1 (ab90602; Abcam) and a goat anti-mouse IgG-Horseradish Peroxidase (HRP) secondary antibody (SouthernBiotech). The starting level of HA1Fc in the culture medium loaded onto apical side was set as 100%. The levels of HA1Fc in apical and basolateral side were then estimated as relative to the percent of loaded protein.

***In vivo* imaging**

The Fc-fusion proteins were conjugated with AF680 using SAIVI Rapid Antibody AF680 labeling kit (Invitrogen), following the manufacturer‟s instructions. The pregnant HemA mice and wild-type Balb/c mice were injected intravenously with 100 μg of either C2Fc-AF680 or HA1Fc-AF680, on day 18 of gestation (E18). Control pregnant mice were injected with PBS. Fluorescence was detected using Fluobeam imaging system (Fluoptics, Grenoble, France) to follow fluorescent tracers *in vivo*. Shots were taken 1 min to 4 hr after administration of the labeled proteins. Fetuses were removed at 4 and 24 hr after injection followed by analysis with the imaging system.

Quantitative analysis of transplacental antigen transfer

To determine the role of the Fc-domain in transplacental transfer of chimeric HA1Fc, pregnant wild-type Balb/c mice were injected intravenously with 100 μg of HA1Fc or HA1 (11684-V08H1, Sino Biological Inc.) on E19 of gestation. Blood and urine from pregnant mice were collected 5 min, 1 hr and 4 hr after injection. Fetuses were then removed and blood was collected. Blood from 3 to 4 fetuses was pooled. Levels of

HA1Fc or HA1 were determined in plasma and urine from pregnant mice and corresponding fetuses by ELISA. HA1 was detected using the mouse monoclonal and rabbit polyclonal anti-HA antibodies (ab128412, ab90602, Abcam) followed by anti-rabbit IgG-HRP secondary antibody (Thermo Scientific). The optical density obtained with the mothers‟ plasma 5 min after injection was set at 100%. The HA1Fc or HA1 levels were then estimated as a % relative to the starting levels in pregnant mice.

Functional characterization of HA1Fc

$CD4^+$ T cells were isolated from the spleen of homozygous naïve HA-TcR-Tg mice using the Dynabeads® untouched mouse CD4 kit (Invitrogen). Cells were labeled with 5 μM CellTrace Violet (CTV cell proliferation kit, Invitrogen) for 15 min in PBS. Splenocytes from wild-type Balb/c mice depleted of $CD4^+$ T cells were used as a source of antigen-presenting cells (APCs). The cells were co-cultured at 1 $CD4^+$ T-cell: 2 APCs in U-bottom culture plates (Nunc) in complete proliferation medium (RPMI-1640 with ultraglutamine (Lonza) supplemented with 10 mM HEPES, 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 50 μM 2-β-mercaptoethanol and 1% penicillin/streptomycin). The cells were then incubated with equimolar concentrations of HA1Fc, $HA_{110-119}$ peptide and mIgG1 (1.66 μM to 0.06 μM). After 5 days, the percent proliferation at different Ag concentrations was determined by gating on divided 6.5 TcR-Tg $CD4^+$ T cells based on the CTV signal.

Antibodies and flow cytometry analysis

The following antibodies from BD Biosciences, e-Bioscience, BioLegend and R&D systems were used for the phenotypic analysis: peridininchlorophyll-protein (PercP)- or pacific blue (PB)-labeled anti-CD3, PB- or PercP-labeled anti-CD4, fluorescein isothiocyanate (FITC)- or PercP-labeled anti-CD8, allophycocyanin (APC)- or FITC-

labeled anti-CD25, AF700- or APC-labeled anti-Foxp3, phycoerythrin (PE)-labeled TcR Vβ8.18.2, APC-labeled anti-Nrp-1 (Neuropilin-1), PB- or PE-labeled anti-CD11c, AF-700- or PE-labeled anti-CD11b, PB-labeled anti-CD45R, FITC-labeled anti-CD172a (SIRP-α), eFluor 450-labeled CD326 (EpCAM), AF-700-labeled anti-CD45, FITC-labeled anti-F4/80, PE-labeled anti-I-A^d/I-E^d, FITC-labeled anti-NK1.1 and PE-labeled anti-LY6G. The TcR-HA was identified using the PE-labeled anti-clonotypic 6.5 antibody. Unconjugated antibody to CD16/32 (2.4G2) was used to block Fc-receptors on cells. The AF-700- or APC-labeled anti-Foxp3 staining was performed using the eBioscience kit and protocol. Dead cells were excluded using fixable viability dye eFlour 506 (eBioscience). Isotype-matched irrelevant antibodies (BD Pharmingen) were used as controls. Acquisition was performed on a LSR II cytometer and data were analyzed using FlowJo (Tree Star) software.

HA1Fc uptake by fetal immune cells

HA1Fc was conjugated with AF-647 using SAIVI AF-647 labeling kit (Invitrogen). Pregnant wild-type Balb/c mice were injected intravenously with 100 µg of HA1Fc-AF-647 on E19 of gestation. The fetuses were removed 24 hr later and the thymi, spleens and blood were collected. The tissues from 2 to 4 fetuses were pooled into one. Single-cell suspensions were obtained by enzymatic digestion in case of thymus and spleen, followed by filtration through 70 µm cell strainer (BD Falcon). Red blood cells (RBCs) were lysed using ACK lysis buffer (Lonza). The isolated cells were then stained with cell subset-specific antibodies in ice-cold buffer (1% FBS in PBS). Cells were defined as circulating dendritic cells (DCs) ($CD11b^+CD11c^+SIRP\text{-}\alpha^+$), thymic resident DCs ($CD11b^+CD11c^+SIRP\text{-}\alpha^-$), macrophages ($CD11b^+CD11c^-F4/80^+$), B cells ($CD11b^-CD45R/B220^+$), splenic T cells ($CD3^+TcRV\beta 8.1/8.2^+$), thymic CD4+ single positive (SP) T cells ($CD3^+CD4^+CD8^-$) and medullary thymic epithelial cells

($CD45^{-}CD11b^{-}EpCAM^{+}$). HA1Fc-AF-647-positive cells were identified by gating on the live cells of each defined cellular subset. Percentages of HA1Fc-AF-647-positive cells were estimated among each subset.

Splenocyte proliferation assay

Spleens were removed aseptically from 2-week-old mIgG1 or HA1Fc transplacentally treated heterozygous HA-TcR-Tg mice. Single splenocyte suspensions were prepared by mechanical dissociation, RBCs lysis and filtration through 70 μm cell strainers. Total splenocytes were stimulated with the $HA_{110-119}$ peptide (0 to 10 μg/mL) in complete proliferation medium. In the case of HemA mice, splenocytes were collected from 10-week-old mIgG1 or A2Fc+C2Fc transplacentally treated progeny, after replacement therapy with FVIII. Total splenocytes were stimulated with 0 to 10 μg/mL FVIII. Splenocytes from HemA mice were cultured in complete proliferation medium (supplemented with 2% FBS and 0.5% heat-inactivated serum from HemA mice). To define the proliferative capability of splenocytes from both treatment groups, the splenocytes were also stimulated with Concanavalin A (0 to 2 μg/mL, Sigma-Aldrich). After 48 or 72 hr of incubation, [^{3}H]-thymidine (0.5 μCi/well) was added to the cell culture media for an additional 18 hr before harvest of cells. [^{3}H]-thymidine incorporation was measured in a scintillation counter, and results of triplicates were expressed as mean counts per minute (cpm). The data are presented as proliferation index, calculated as the ratio of incorporated [^{3}H]-thymidine in stimulated vs. non-stimulated cells.

Assay for anti-FVIII IgG

ELISA plates (Maxisorp, Nunc) were coated with FVIII or recombinant C2 protein overnight at 4°C, and blocked with PBS-1% BSA for 1 hr at 37°C. Serum dilutions

were then incubated for 1 hr at 37 °C. Bound IgG was revealed using an HRP-conjugated anti-mouse IgG (SouthernBiotech) and the substrate o-Phenylenediamine dihydrochloride (Sigma-Aldrich). The mouse monoclonal anti-FVIII IgG mAb6 (a gift from Prof. J.M. Saint-Remy, KUL, Belgium) or ESH8 (American Diagnostica Inc., Stamford, CT) were used as standards. Levels of anti-FVIII IgG are presented in concentration equivalent to the mAb6 or ESH8 standards.

Titration of FVIII inhibitors

Heat-inactivated plasma was incubated with a standard pool of human plasma (Siemens, Saint-Denis, France) for 2 hr at 37 °C. The residual pro-coagulant FVIII activity was measured using the „FVIII chromogenic assay kit" following the manufacturer's recommendations (Siemens). One Bethesda unit expressed in BU/mL is defined as the reciprocal of the dilution of plasma that produces 50% residual FVIII activity.

Treg suppression assay

Spleens were dissected out from mIgG1 or A2Fc+C2Fc transplacentally treated HemA progeny. The spleens from 4-6 mice in each treatment group were pooled and mechanically dissociated. Splenic $CD4^+CD25^+$ Treg cells were isolated by magnetic selection, using mouse regulatory T-cell isolation kit (Miltenyi Biotec). For FVIII-specific suppression, untouched $CD4^+CD25^-$ responder T cells were isolated from HemA mice challenged with FVIII (5 IU/mouse, once a week for 4 consecutive weeks), following the kit protocol (Dynabeads® untouched mouse CD4, Invitrogen). Responder $CD4^+CD25^-$ T cells were labeled with CTV-proliferation dye, as described

above. Splenocytes from FVIII-challenged mice depleted of $CD4^+$ T cells, were treated with mitomycin (Sigma-Aldrich) and used as APCs. The $CD4^+CD25^+$ Tregs were co-cultured with CTV-labeled responder $CD4^+CD25^-$ T cells (Teffs) at 1:2 and 1:1 Tregs: Teffs ratios, with similar numbers of APCs. The co-cultured cells were stimulated with FVIII at 1 μg/mL in complete proliferation medium. After 72 hr, percentage proliferation was determined by gating on $CD4^+$ T cells based on CTV dilution. The percent proliferation of Teffs in the absence of $CD4^+CD25^+$ Tregs was set as 100%. The relative suppression in proliferation of Teffs in the presence of Tregs from both the treatment groups was estimated as percent suppression.

Adoptive transfer of Tregs

Spleens were removed from 3-week-old mIgG1 or A2Fc+C2Fc transplacentally treated HemA progeny. Spleens were pooled from each treatment group and $CD4^+CD25^+$ Tregs were isolated as described above. A total of 1×10^6 cells suspended in 200 μL of PBS was injected into the tail vein of naïve 6-week-old HemA recipients. As an additional control, naïve HemA recipients were injected with PBS. Twenty-four hours after adoptive transfer, all the groups experienced replacement therapy with FVIII (1 IU/mouse/week) for 4 weeks. Plasma collected one week after the last FVIII injection was analyzed for anti-FVIII IgG titer by ELISA, as described above.

Statistical analysis

In all experiments, data are expressed as means±SEM. The statistical significance of differences between groups were evaluated using the two-tailed student t-test, two-sided Mann-Whitney U test or by two-way ANOVA with Bonferroni post-hoc test

when indicated. Statistical analyses were performed using the GraphPad Prism 5.0b software (GraphPad Software, San Diego, CA, USA).

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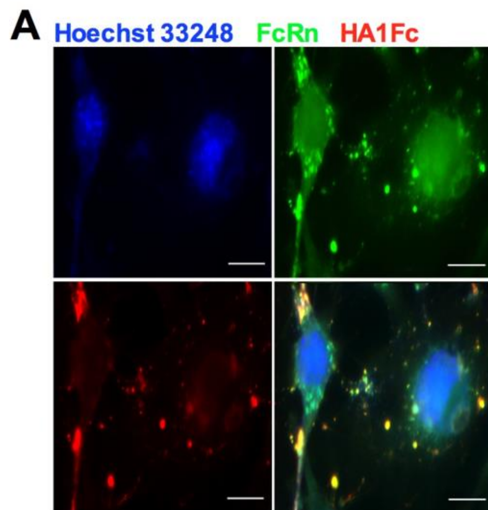
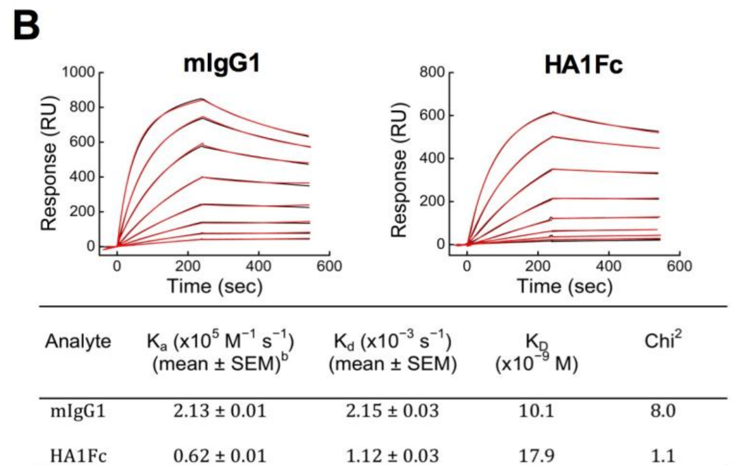
Acknowledgements: We thank Prof MV Ragni (University of Pittsburgh, PA) and Dr GF Pierce (Biogen Idec, Weston, MA) for HA TcR-Tg mice and Dr S André (CRC, UPMC-Paris6, Paris, France) for assistance. **Funding:** This work was supported by INSERM, CNRS, UPMC-Paris6, Département Hospitalo-Universitaire (DHU) authors and by grants from ANR (ANR-10-BLAN-1118), EFSD/JDRF/Novo Nordisk European Programme in Type 1 Diabetes Research 2009 and Association pour la Recherche sur le Diabète 2012. YM was the recipient of a fellowship from Fondation Recherche Médicale (PLP20071010956, Paris, France). **Author contributions:** NG, SC, SVK, BS, DWS, RM and SLD planned the work. NG, SC, YM, JDD, SD, BG and ML performed experiments. SJ and BS contributed essential material. NG, SC, YM, JDD, RM and SLD analyzed the data. NG, SVK, SLD wrote the manuscript and RM, BS and DWS critically reviewed the manuscript. **Competing interests:** The authors declare no conflict of interest. **Data and materials**

availability: Materials are available and will be provided under the material transfer policies.

Figures:





Analyte	K_a ($\times 10^5$ M^{-1} s^{-1}) (mean ± SEM)[b]	K_d ($\times 10^{-3}$ s^{-1}) (mean ± SEM)	K_D ($\times 10^{-9}$ M)	Chi[2]
mIgG1	2.13 ± 0.01	2.15 ± 0.03	10.1	8.0
HA1Fc	0.62 ± 0.01	1.12 ± 0.03	17.9	1.1

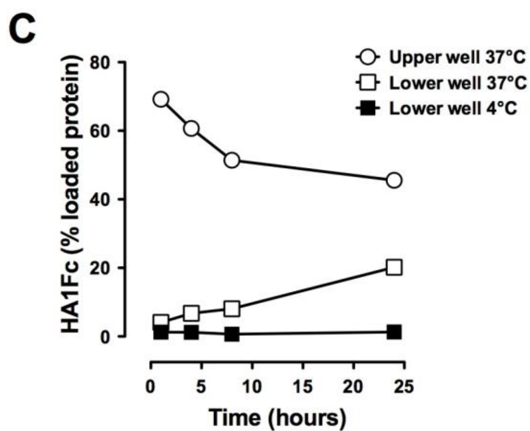


Fig. 1. HA1Fc is transcytosed via FcRn. (A) Co-localization of FcRn and HA1Fc on syncytiotrophoblast cells (63X magnification). Panels depict nuclear staining with Hoechst 33248 (blue), FcRn (green) and HA1Fc (red). Merging of FcRn-FITC with HA1Fc-Alexa Fluor 647 fluorescence in syncytiotrophoblast cells is in yellow color. Scale bar, 20 μm. The images are from one of two independent experiments. **(B)** Affinity determination by surface plasmon resonance (SPR) analysis. Real-time interaction profiles of the binding of increasing concentrations (0.78 to 200 nM, two-fold dilutions) of mIgG1 (left) and HA1Fc (right) to immobilized recombinant mouse FcRn. The experimental curves (in black) are presented along with curves generated by fitting data to the model of Langmuir binding with a drifting baseline by BIAevaluation (in red). [b]The standard error of the mean (SEM) represents the accuracy of the fit of the experimental data. Binding intensities are expressed in resonance units (RU). The lower panel shows the kinetics of the interactions. Representative SPR sensorgrams from one of two independent experiments are shown. **(C)** Transcytosis of HA1Fc by syncytiotrophoblast cells was determined using transwell assay. The cell monolayer on the transwell filter was apically exposed to HA1Fc (2.5 μg) at different temperatures. Levels of HA1Fc were determined by ELISA in the supernatant from apical (upper well) and basolateral sides (lower well), at the indicated time points. The y-axis represents the levels of HA1Fc as percent of loaded protein. Results are representative of three independent experiments.

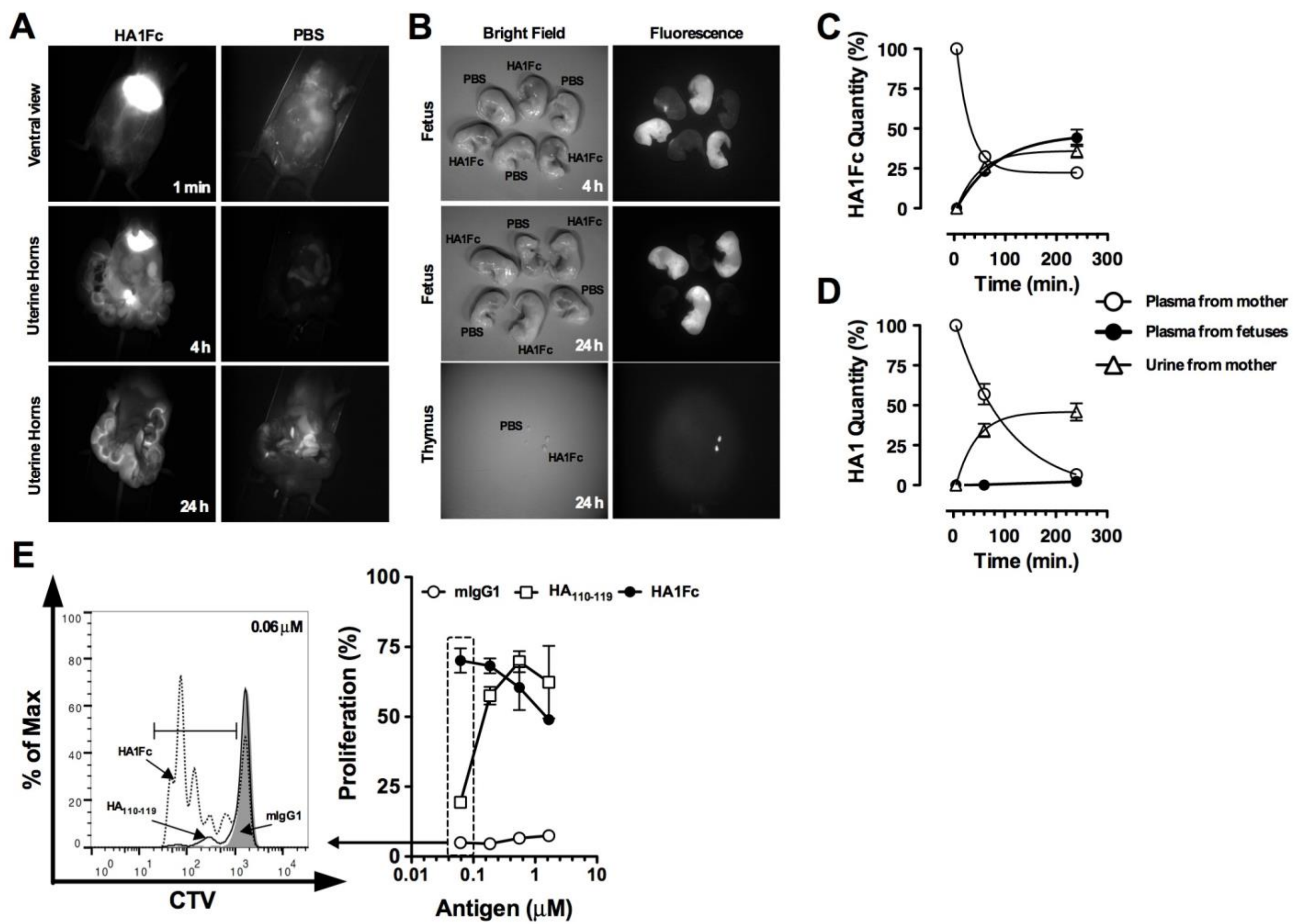
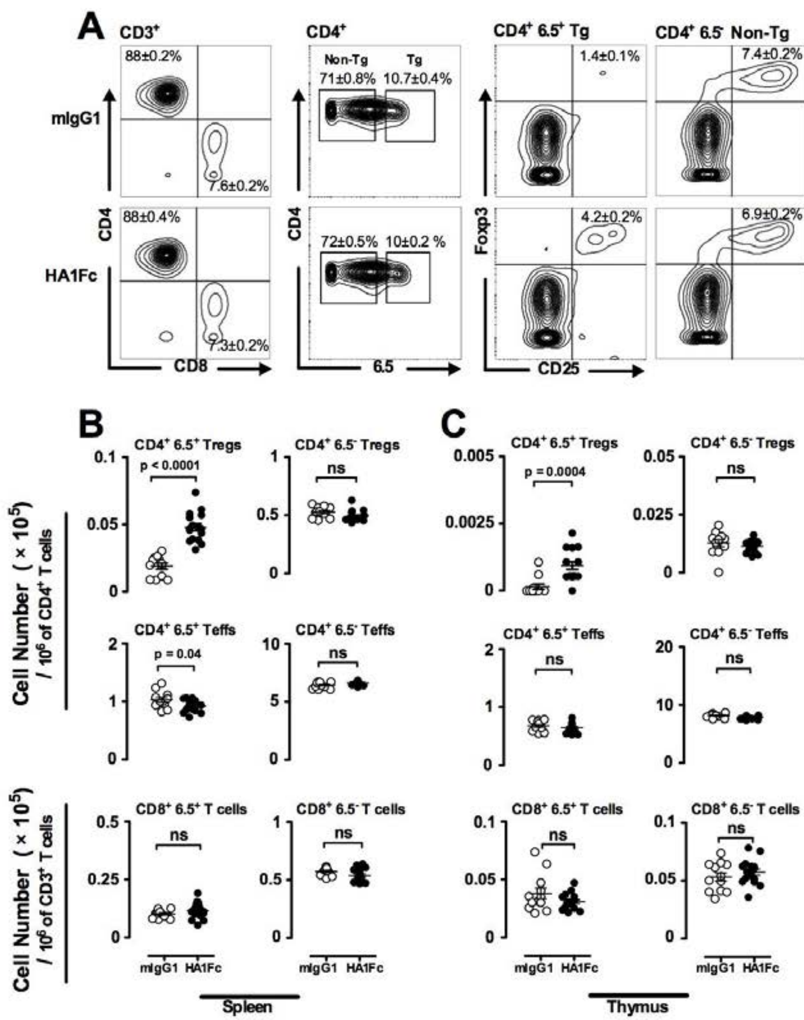

A
HA1Fc
PBS
Ventral view
Uterine Horns
Uterine Horns
1 min
4 h
24 h
B
Bright Field
Fluorescence
Fetus
Fetus
Thymus
PBS
HA1Fc
4 h
24 h
C
HA1Fc Quantity (%)
Time (min.)
D
HA1 Quantity (%)
Time (min.)
Plasma from mother
Plasma from fetuses
Urine from mother
E
% of Max
CTV
0.06 μM
HA1Fc
$HA_{110-119}$
mIgG1
Proliferation (%)
Antigen (μM)
mIgG1
$HA_{110-119}$
HA1Fc

Fig. 2. Transplacental transfer of antigenic HA1Fc from pregnant mice to fetuses. (A) *In vivo* imaging of pregnant female BALB/c mice after intravenous injection of HA1Fc-Alexa Fluor 680 protein (100 µg) or PBS at day 18 of gestation (E18). The panel shows the fluorescence at the indicated time points after injection. **(B)** The bright field images are optical views of fetuses after transplacental treatment. The corresponding fluorescence images show fetuses from HA1Fc-Alexa Fluor 680-injected (white) and PBS-injected pregnant mice (no fluorescence) after laser excitation. The lower panels show the dissected thymi from corresponding fetuses. **(C-D)** Levels of HA1Fc (**C**) and HA1 (**D**) in plasma and urine of pregnant mice (n=3) and in plasma of the fetuses (n=18), as measured by ELISA. The blood from 3 to 6 fetuses was pooled. The optical density obtained with the mother‟s plasma 5 min. after injection was set at 100%. The y-axes represent the levels of HA1Fc or HA as % relative to the starting levels in pregnant mice. Data are representative of three independent experiments. **(E)** Proliferation of CellTrace Violet (CTV)-labeled splenic $CD4^+$ T cells from HA-specific 6.5 TcR-Tg mice was analyzed in presence of mIgG1, $HA_{110-119}$ peptide or HA1Fc (0.06-1.6 µM, three-fold dilutions). The representative histogram on the left shows proliferation in the presence of 0.06 µM of the three Ags. Percent proliferation at different Ag concentrations is shown on the right, as determined by gating on dividing 6.5 TcR-Tg $CD4^+$ T cells based on CTV dilution. Results are representative of two independent experiments.


A
CD3+
CD4+
CD4+ 6.5+ Tg
CD4+ 6.5- Non-Tg
mIgG1
HA1Fc
88±0.2%
7.6±0.2%
Non-Tg
Tg
71±0.8%
10.7±0.4%
1.4±0.1%
7.4±0.2%
88±0.4%
7.3±0.2%
72±0.5%
10±0.2 %
4.2±0.2%
6.9±0.2%
CD4
CD8
6.5
Foxp3
CD25
B
C
CD4+ 6.5+ Tregs
CD4+ 6.5- Tregs
p < 0.0001
p = 0.0004
ns
CD4+ 6.5+ Teffs
CD4+ 6.5- Teffs
p = 0.04
CD8+ 6.5+ T cells
CD8+ 6.5- T cells
Cell Number (× 10^5) / 10^6 of CD4+ T cells
Cell Number (× 10^5) / 10^6 of CD3+ T cells
mIgG1
HA1Fc
Spleen
Thymus

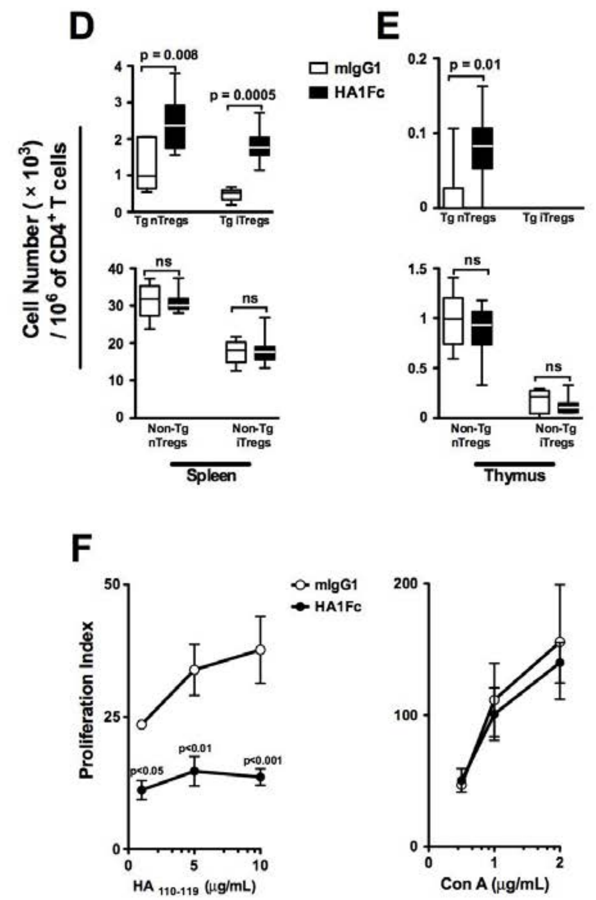

D
E
p = 0.008
p = 0.0005
p = 0.01
mIgG1
HA1Fc
Tg nTregs
Tg iTregs
Non-Tg nTregs
Non-Tg iTregs
ns
Cell Number (× 10^3) / 10^6 of CD4+ T cells
Spleen
Thymus
F
mIgG1
HA1Fc
Proliferation Index
p<0.05
p<0.01
p<0.001
HA 110-119 (μg/mL)
Con A (μg/mL)

Fig. 3. Transplacentally transferred HA1Fc shapes the T-cell repertoire in HA-specific 6.5 TcR-Tg mice. (A) Representative contour plots describing the gating and total percent population of T cells in the spleen of 2-week-old mice treated transplacentally with HA1Fc or mIgG at E16, E17 and E18 of gestation. The $CD4^+$ and $CD8^+$ T-cell subsets are gated on $CD3^+$ live T cells. TcR-Tg cells were identified using the 6.5 clonotypic antibody. Tregs were identified as $CD3^+CD4^+CD25^+Foxp3^+$ cells. **(B-C)** TcR-Tg (6.5^+) and non-Tg (6.5^-) T-cell subsets in spleens (**B**) and thymi (**C**) of 2-week-old mice treated transplacentally with HA1Fc (full circles) or mIgG (empty circle). The y-axes represent cell numbers out of 10^6 $CD4^+$ T cells or 10^6 $CD3^+$ T cells in case of $CD8^+$ cells. **(D-E)** Graphs depicting TcR-Tg or non-Tg natural Tregs (nTregs; Nrp-1^{hi}) and induced Tregs (iTregs; Nrp-1^{lo}) in spleens (**D**) and thymi (**E**) of 2-week-old transplacentally treated mice. The y-axes indicate cell numbers out of 10^6 $CD4^+$ T cells. **(F)** Proliferation of splenocytes from 2-week-old mice treated transplacentally with HA1Fc (full circles) or mIgG (empty circle). Splenocytes were stimulated with either $HA_{110-119}$ peptide (left panel) or concanavalin A (Con A, right panel). The y-axes denote the proliferation index, calculated as the ratio of incorporated [^{3}H]-thymidine in stimulated vs. non-stimulated cells. Results depicted in (A-F) are representative of four independent experiments. Data are means±SEM for 12 to 16 mice in each group. Statistical significance was assessed using two-sided Mann-Whitney U-test (A-E) or two-way ANOVA with Bonferroni post-test correction (F). ns, not significant.

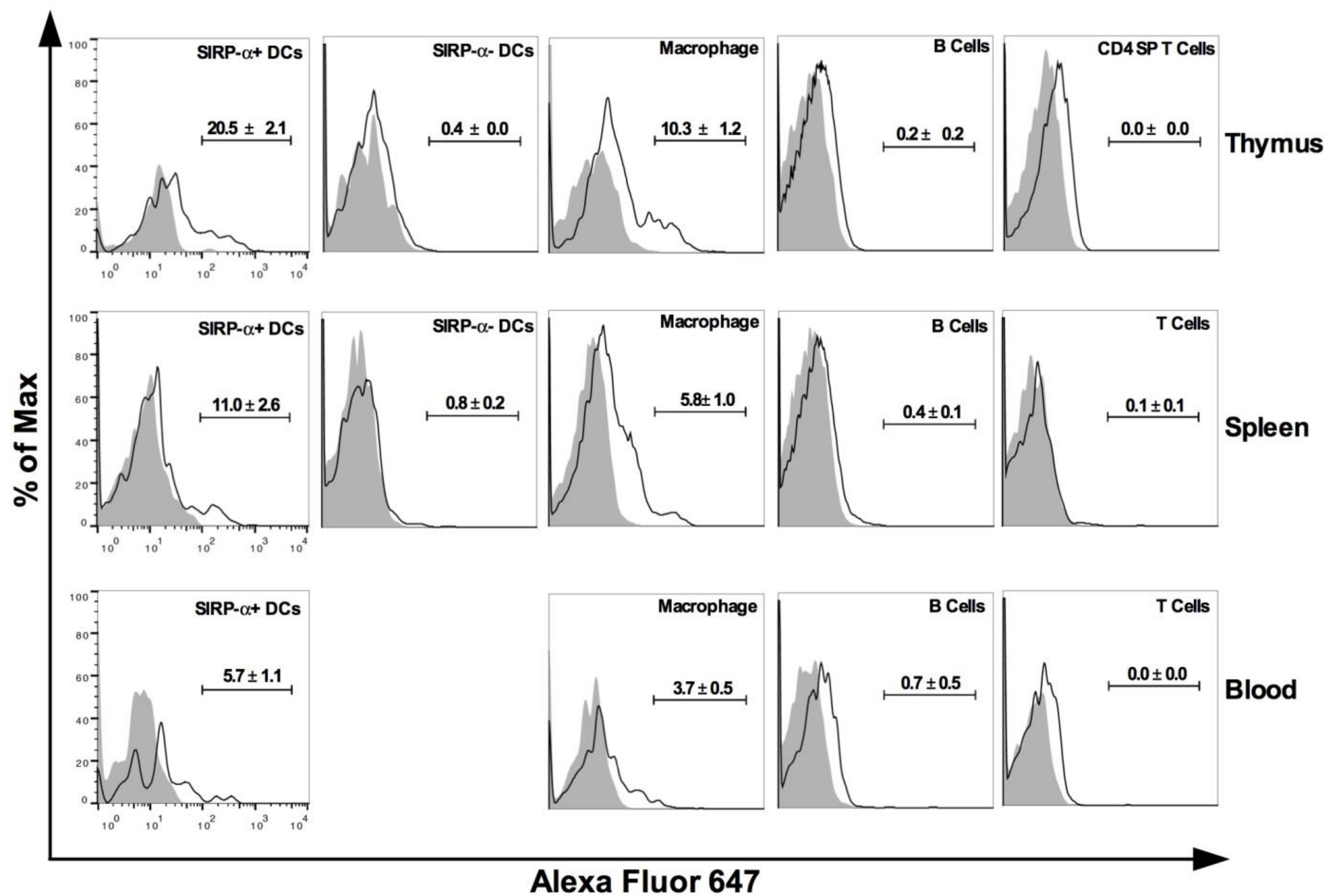
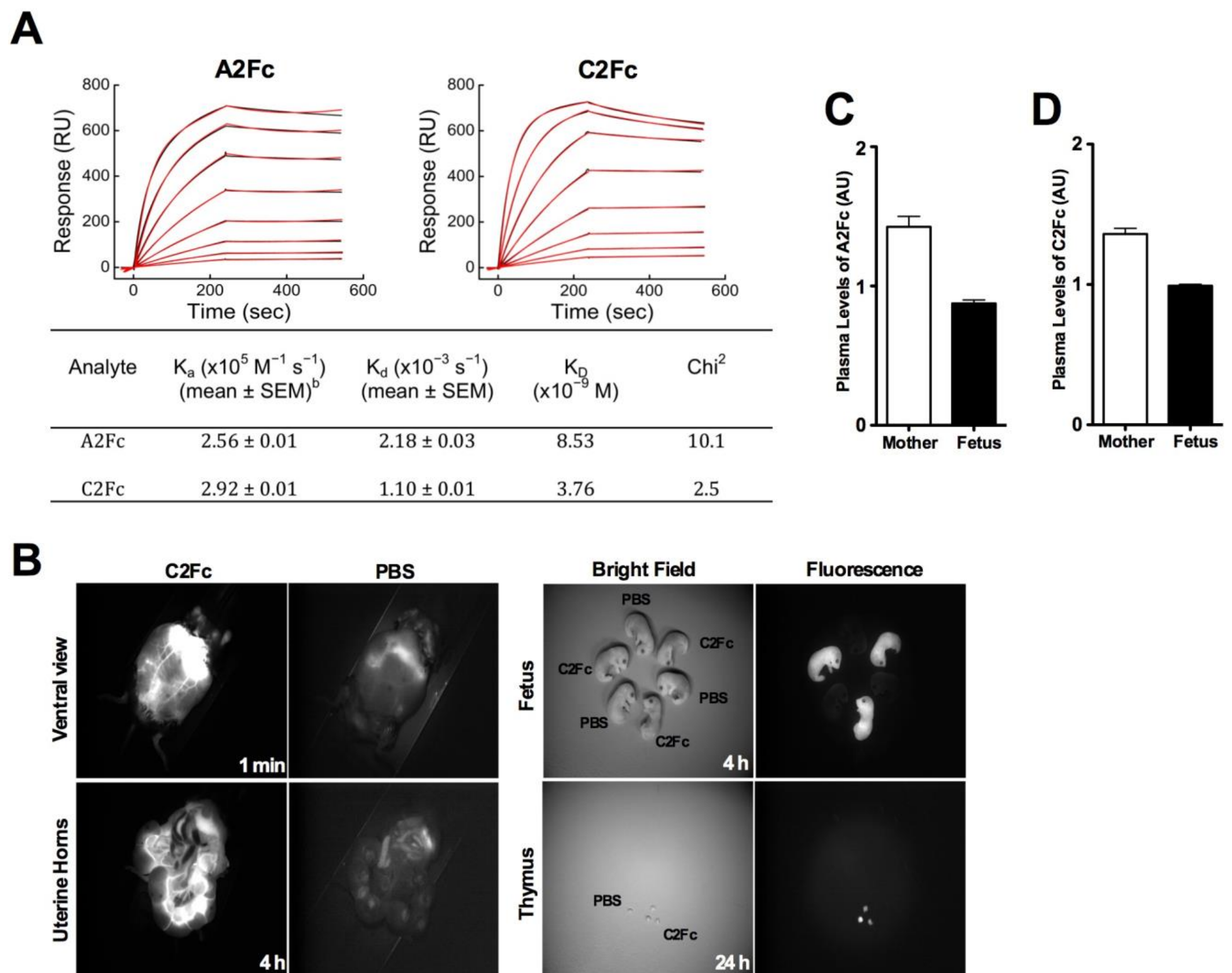


Fig. 4. Transplacentally transferred HA1Fc is endocytosed by fetal myeloid cells.

Pregnant mice were injected with either HA1Fc-Alexa Fluor 647 or PBS on E18. Fetuses were removed 24 hr after treatment to collect thymi (top row) spleens (middle

row), and blood (bottom row). Single cell suspensions were obtained by pooling spleens, thymi or blood from at least 3 fetuses. The histograms show the indicated cellular subsets in fetuses from pregnant mice injected with either HA1Fc-Alexa Fluor 647 (black lines) or PBS (grey profiles). The y-axes depict cell counts and x-axes show the HA1Fc-Alexa Fluor 647 fluorescence. Percentages of HA1Fc-Alexa Fluor 647^+ cells (means±SEM of three independent experiments, each including 16-20 fetuses) are shown among live, $CD11b^+CD11c^+SIRP\text{-}\alpha^+$ cells (circulating DCs); $CD11b^+CD11c^+SIRP\text{-}\alpha^-$ cells (thymic resident DCs); $CD11b^+CD11c^-F4/80^+$ cells (macrophages); $CD11b^-CD45R/B220^+$ cells (B cells); splenic T cells ($CD3^+TcRV\beta8.1/8.2^+$); thymic $CD4^+$ single positive (SP) T cells ($CD3^+CD4^+CD8^-$). The gating strategy is shown in Fig. S4.



Analyte	K_a ($x10^5$ M^{-1} s^{-1}) (mean ± SEM)[b]	K_d ($x10^{-3}$ s^{-1}) (mean ± SEM)	K_D ($x10^{-9}$ M)	Chi^2
A2Fc	2.56 ± 0.01	2.18 ± 0.03	8.53	10.1
C2Fc	2.92 ± 0.01	1.10 ± 0.01	3.76	2.5

Fig. 5. Transplacental transfer of Fc-fused FVIII domains to the fetal circulation. **(A)** SPR affinity measurements. Real-time interaction profiles of the binding of increasing concentrations (0.78 to 100 nM, two-fold dilutions) of A2Fc (left) and C2Fc (right) to immobilized recombinant mouse FcRn. The lower panel shows the kinetics of the interaction. **(B)** *In vivo* and *ex vivo* fluorescence imaging of pregnant

female BALB/c mice intravenously injected with C2Fc-Alexa Fluor 680 protein (100 μg) or PBS at E18. The left panels show the fluorescence at the indicated time points after injection in pregnant mice. Images on the top right panel are optical views of the fetuses from pregnant mice treated with either C2Fc-Alexa Fluor 680 protein or PBS. The corresponding fluorescence image shows the fetuses from C2Fc-Alexa Fluor 680-injected pregnant mice (white) and PBS-injected mothers (no fluorescence) after laser excitation. The bottom right panels show dissected thymi of fetuses from the same experiment in bright field light (left) or after laser excitation (right). Results are representative of 3 pregnant mice in each group, each carrying 6 to 8 fetuses. (**C-D**) Plasma levels of A2Fc (**C**) and C2Fc (**D**) in 18 fetuses and 3 pregnant mice (blood pooled from 3-6 fetuses) were determined by ELISA 4 hr after injection to pregnant mice. The y-axes represent the plasma levels of Fc-fusion proteins depicted in arbitrary units (AU). Results are representative of three independent experiments.

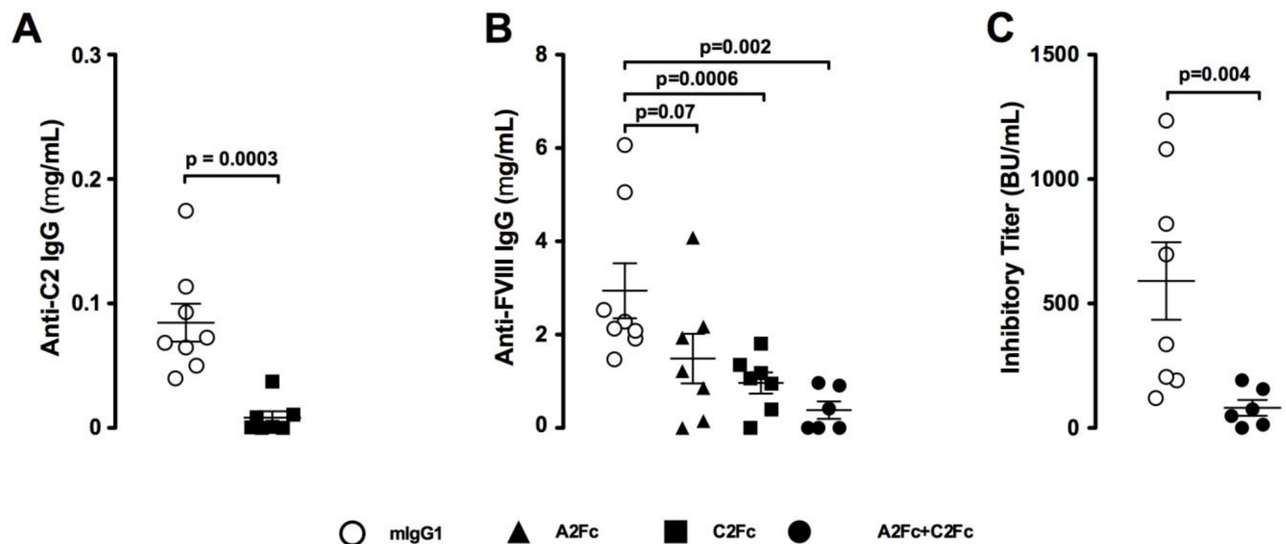


Fig. 6. Induction of tolerance to FVIII in hemA mice. (A) Anti-FVIII IgG plasma titers of mice treated transplacentally at E16, E17 and E18 with mIgG1 (empty circles) A2Fc (filled triangles), C2Fc (filled squares) and A2Fc+C2Fc (filled circles) after replacement therapy with therapeutic FVIII (1 IU/mouse). The y-axis represents the anti-FVIII IgG concentration (mg/mL), as calculated by normalization to a mouse anti-FVIII monoclonal antibody standard curve. **(B)** Anti-C2 IgG plasma titers of mice transplacentally treated as in (A). The y-axis shows the anti-C2 IgG concentration (mg/mL), as calculated by normalization to a mouse anti-C2 monoclonal antibody standard curve. **(C)** FVIII inhibitory plasma titers expressed as Bethesda units (BU)/mL of mice transplacentally treated with mIgG1 (empty circles)

or A2Fc+C2Fc (filled circles). Results are depicted as means±SEM and are representative of three independent experiments. Statistical significance was calculated by two-tailed Mann-Whitney U-test. ns, not significant.

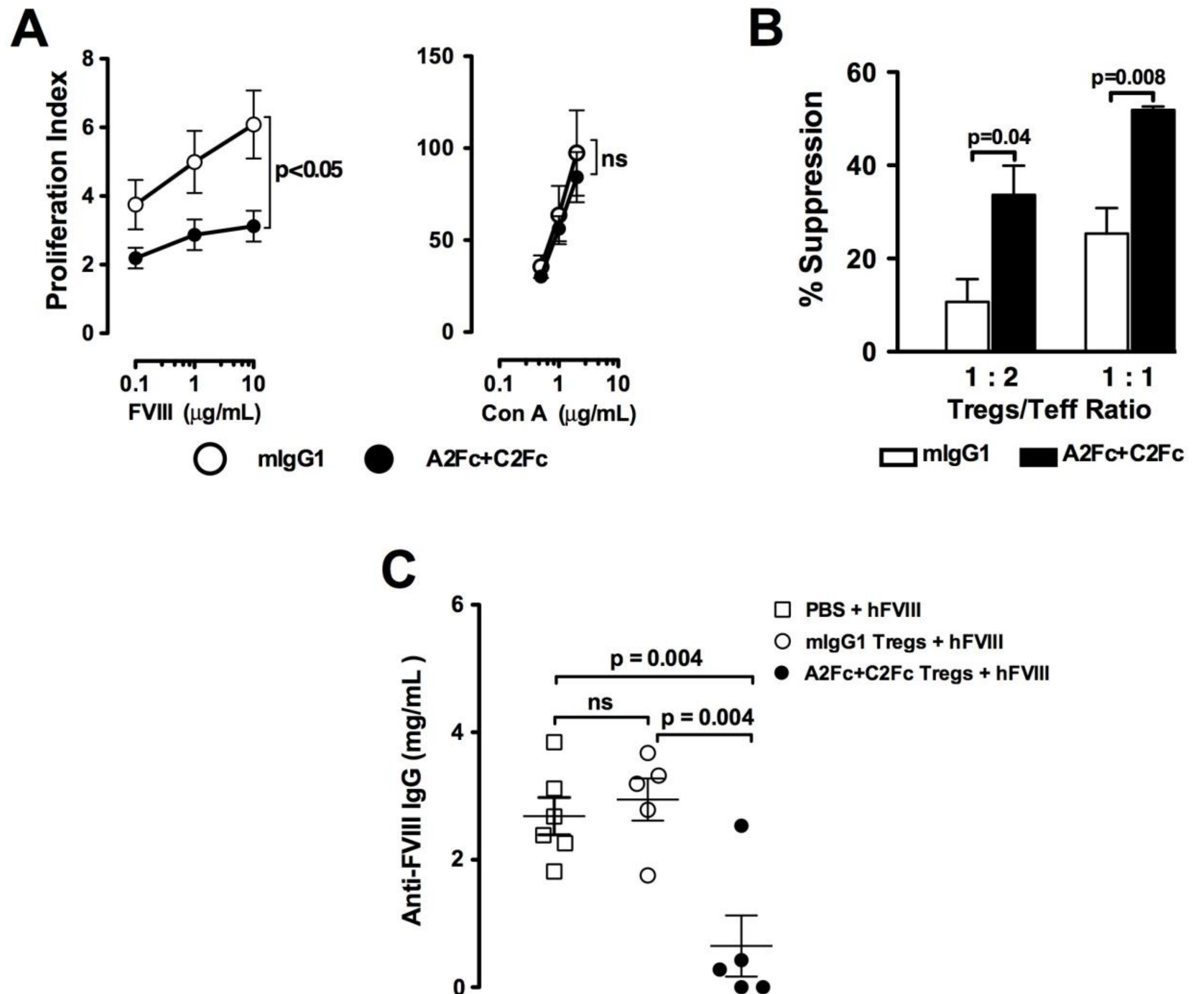


Fig. 7. Functional assessment of Tregs generated by transplacental treatment in hemA mice. (A) Proliferation of splenocytes from mice treated transplacentally with mIgG1 (empty circles) or A2Fc+C2Fc (filled circles). Splenocytes were stimulated with either FVIII (left) or concanavalin A (Con A, right). The y-axes shows the proliferation index, calculated as in Fig. 3F. **(B)** Suppression of proliferation of responder $CD4^{+}CD25^{-}$ Teff cells from FVIII-primed mice (n=8), in presence of FVIII, co-cultured with different ratios of Tregs from mice treated transplacentally

with mIgG1 (Tregs pooled from 22 mice) (empty bar) or A2Fc+C2Fc (Tregs pooled from 16 mice) (filled bar). Y-axis indicates the percent suppression of proliferation in responder $CD4^{+}CD25^{-}$ Teff cells. **(C)** Anti-FVIII IgG titers in mice challenged with FVIII (1 IU/mouse) after adoptively transferred with either PBS (empty squares) or 1×10^{6} Tregs from mice treated transplacentally with either mIgG1 (Tregs pooled from 32 mice) (empty circles) or A2Fc+C2Fc (Tregs pooled from 42 mice) (filled circles). The y-axis shows FVIII-specific IgG titers determined as in Figure 6A. Results are depicted as means±SEM and are representative of three (A) or two independent experiments (B, C), with 5 to 8 mice per group. Statistical significance was calculated by two-way ANOVA with Bonferroni post-test (A) or by two-tailed unpaired t-test (B, C). ns, not significant.

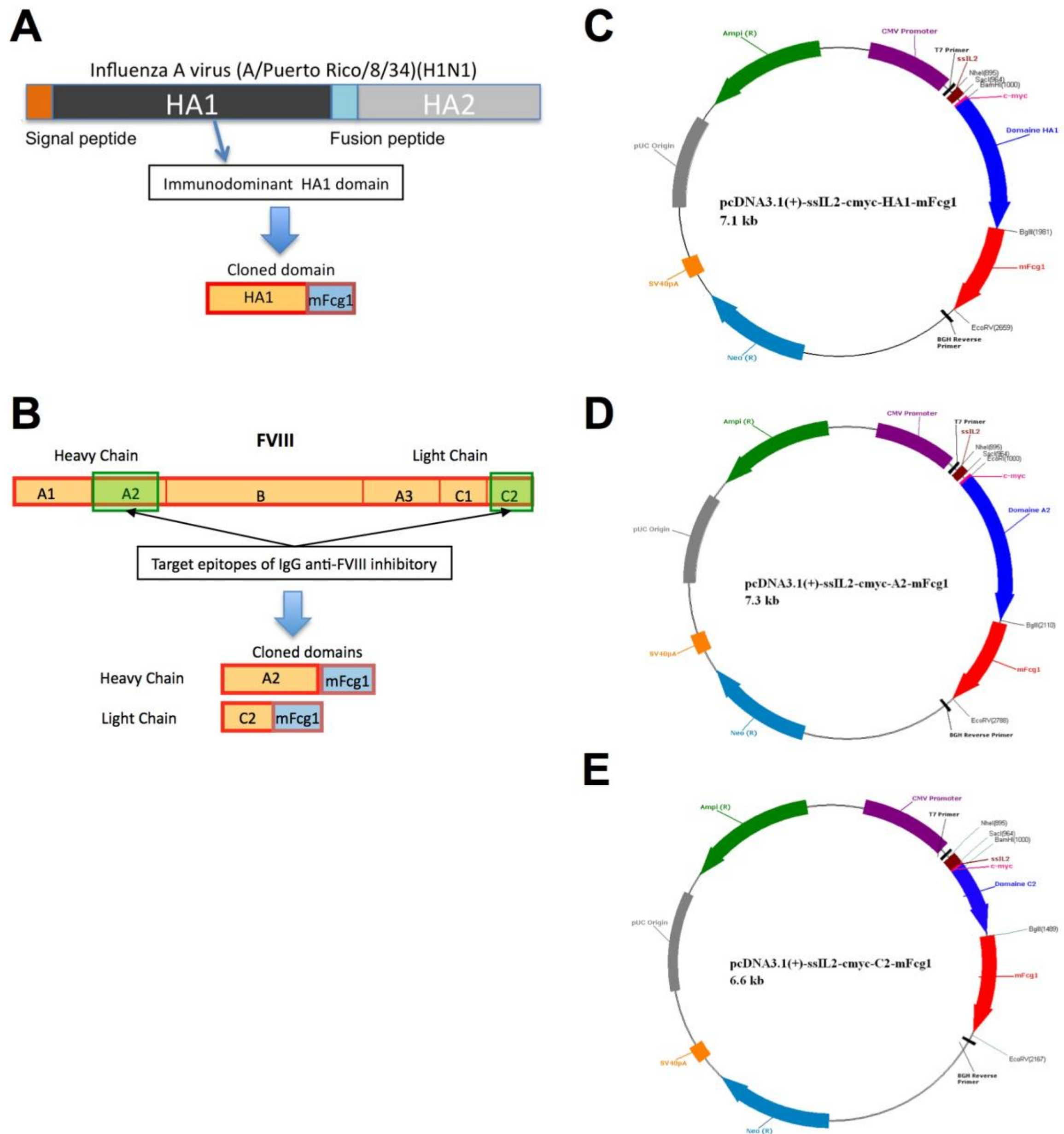


Fig. S1. Schematic representation of Fc fusion constructs. (**A, B**) The selected domains of Influenza A virus (A: HA1) and of FVIII (B: A2 and C2) are shown. **(C, D, E)** The complete construct maps for the HA1Fc (C), A2Fc (D) and C2Fc fusion proteins (E) are depicted.

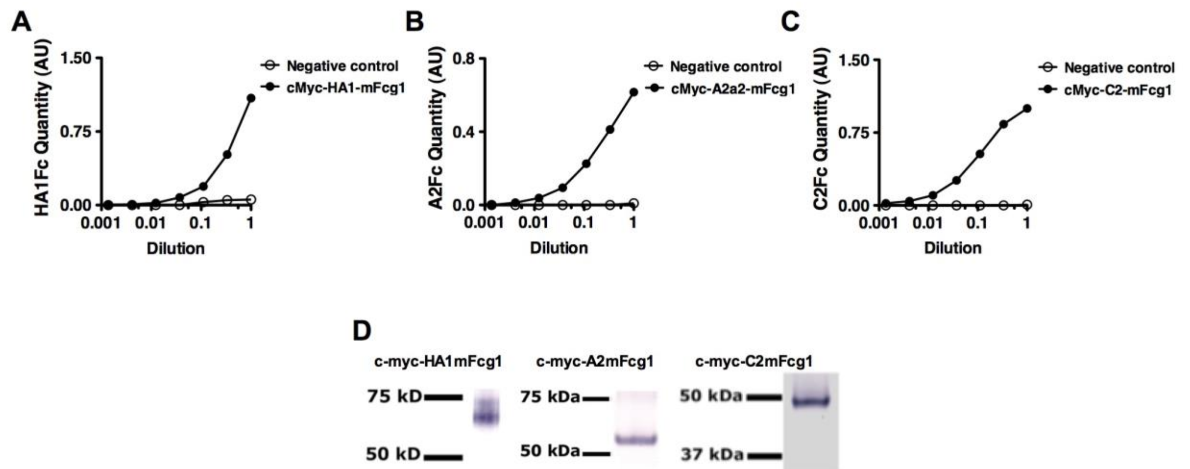


Fig. S2. Validation of Fc-fusion proteins. (**A, B, C**) Secreted HA1Fc (**A**), A2Fc (**B**) and C2Fc (**C**) chimeric Fcγ1-coupled proteins expressed in stably transfected eukaryotic cells, as measured by ELISA at the indicated dilutions of culture supernatants using domain specific monoclonal antibodies and expressed as arbitrary units (AU). The supernatant from non-transfected cells was used as negative control (**D**) The Fcγ1-coupled constructs validated by Western blotting with an anti-c-myc antibody (clone 9E10) after SDS-PAGE under reducing conditions.

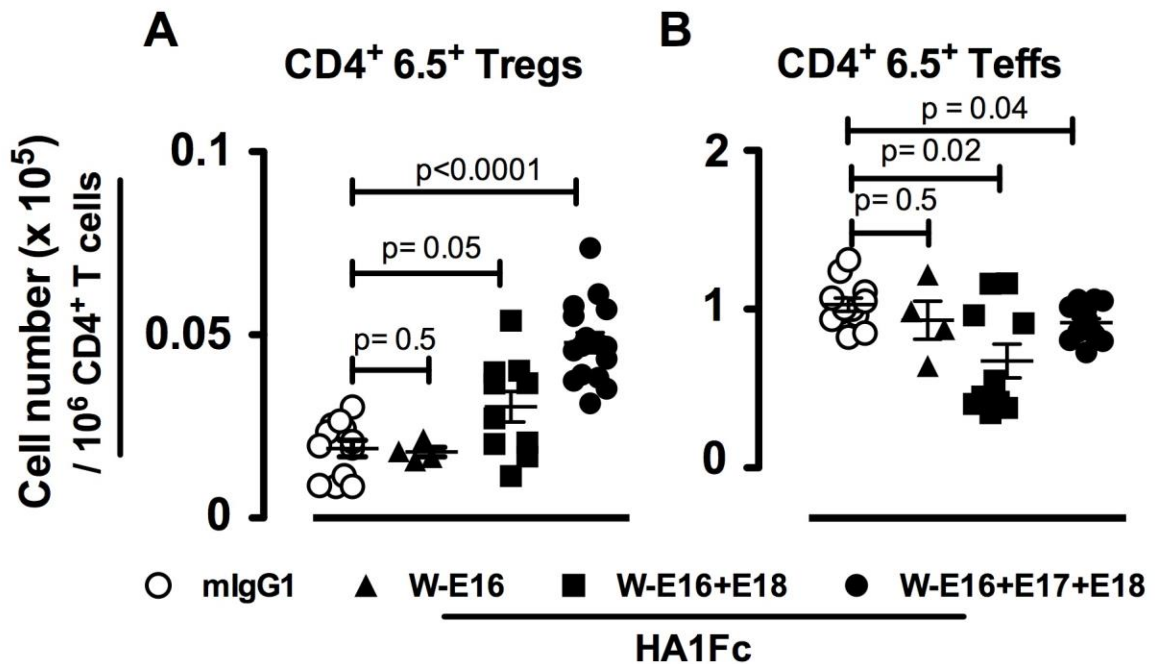


Fig. S3. Selection of the optimal time window of transplacental treatment for induction of Ag-specific Tregs in HA 6.5 TcR-Tg mice. (**A-B)** Modulation of spleen TcR-Tg $CD4^+$ Treg (A) and Teff cells (B) after transplacental treatment at different gestational time points. Cell numbers out of 10^6 $CD4^+$ T cells are shown. Representative results out of two independent experiments are shown, with bars indicating means±SEM. Statistical significance was calculated by two-tailed Mann-Whitney U-test.

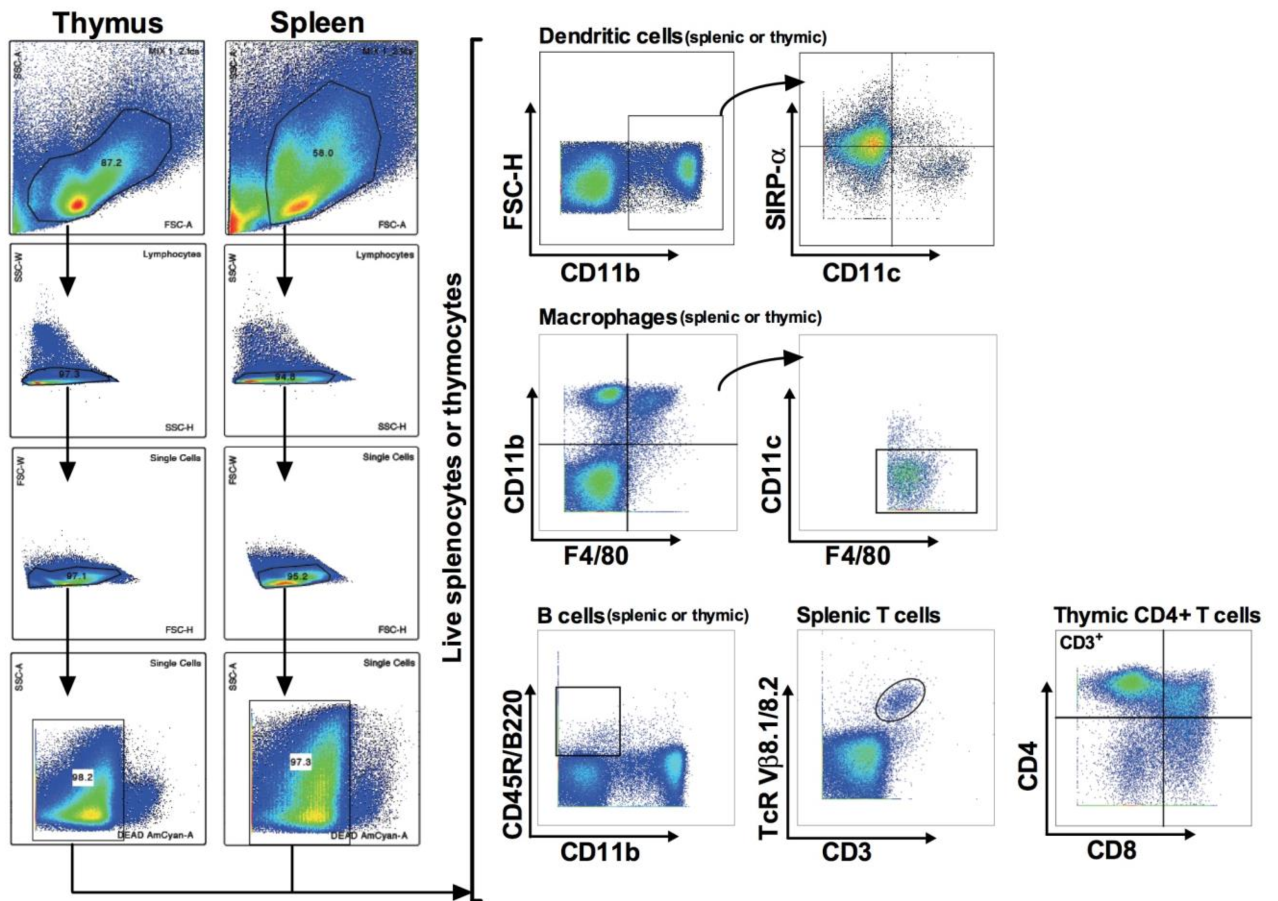


Fig. S4. Gating strategy used to identify cell subsets uptaking HA1Fc in fetal spleen and thymus. The plot ancestry for each cell subset is shown, as analyzed by FlowJo software.

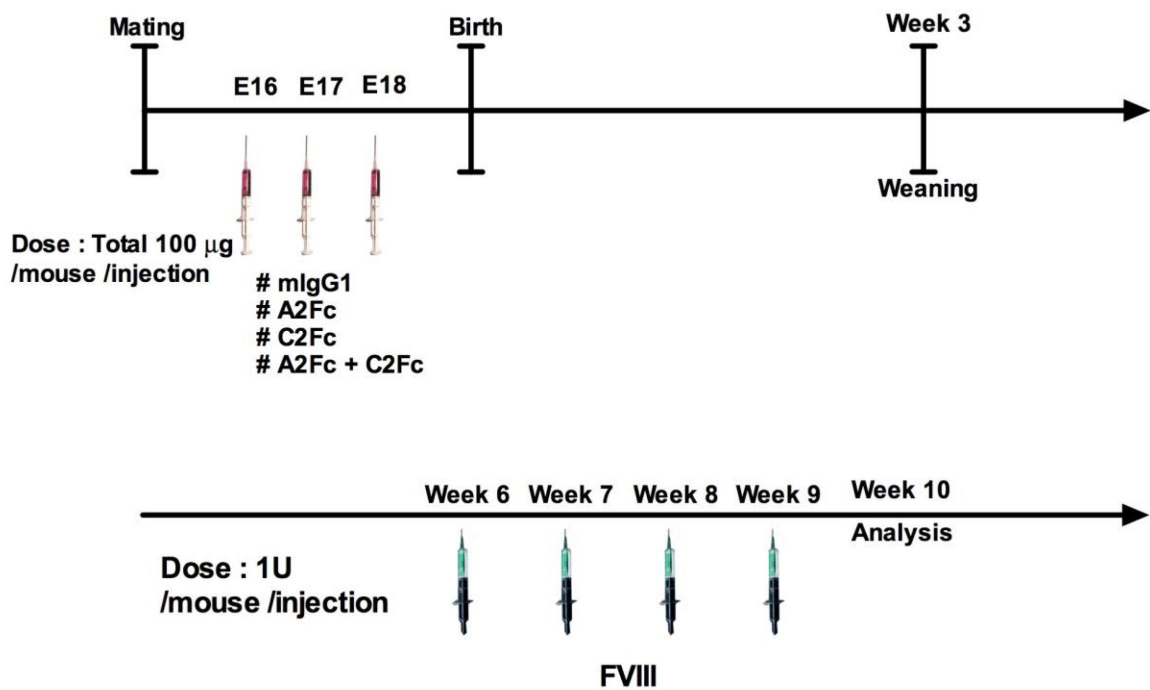


Fig. S5. Treatment Regimens in the mouse model of hemophilia A. The indicated combinations of Fcγ1-fusion proteins were injected into pregnant mice at E16, E17 and E18. The mice and their progeny were bled at the time of weaning. The progeny was further bled before (week 6) and after (week 10) replacement therapy with full-length FVIII administered weekly between week 6 and 9.

Table S1: Oligonucleotide sequence of the primers used in cloning of Fcg1-fusion chimeric proteins.

ID	Primer Sequence (5'-3')
Signal peptide ssIL-2	NheIIL-2 s1: CTA GCT AGC ACC ATG TAC AGG ATG CAA CTC IL2sacI as1: ATT GCA CTA AGT CTT GCA CTT GCT ACG AAC TCG GAG CTC GAG CAG AAA CTC ATC
Tag c-myc	MyclinkbamHI s1: GC GAG CTC GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG GGA TCC AGA TCT TC MyclinkEcoRI s2: GC GAG CTC GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG GAA TTC AGA TCT TC
Domain HA1	HA1BamH1 s1: CGC GGA TCC GAC ACA ATA TGT ATA GGC HA1BglII as1: GGA AGA TCT GGA TTG AAT GGA CGG AGT
Domain A2	A2EcoRI s1: GGA TTC TCA GTT GCC AAG AAG CAT A2BglII as1: GCG AGA TCT TGG TTC AAT GGC ATT
Domain C2	C2BamHI s1: CG GGA TCC AAT AGT TGC AGC ATG CCA C2BglII as1: GCC AGA TCT GTA GAG GTC CTG TGC CT
Mouse IgG1Fc	IgG1mBglII s1: GCG AGA TCT GGT TGT AAG CCT TGC ATA TG IgG1mEcorRV as1: CG GAT ATC GGA TCA TTT ACC AGG AGA GT
T7 & BGH	T7: TAA TAC GAC TCA CTA TAG GG BGH reverse: TAG AAG GCA CAG TCG AGG