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Stimulation Through Tlr4 Increases Fviii Inhibitor Formation In A Mouse Model Of Hemophilia A

Claire K. Holley

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A

THESIS

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by

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Houston, Texas

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To my parents,

for always believing in me and never letting me give up.

First and foremost, I would like to thank my advisor, Keri C. Smith, Ph.D., for starting me on my journey to earn my degree, keeping me on the right path throughout my research, and always urging me to continually think, ask, learn, and discover.

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STIMULATION THROUGH TLR4 INCREASES FVIII INHIBITOR FORMATION IN A MOUSE MODEL OF HEMOPHILIA A

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Hemophilia A is a clotting disorder caused by functional factor VIII (FVIII) deficiency. About 25% of patients treated with therapeutic recombinant FVIII develop antibodies (inhibitors) that render subsequent FVIII treatments ineffective. The immune mechanisms of inhibitor formation are not entirely understood, but circumstantial evidence indicates a role for increased inflammatory response, possibly via stimulation of Toll-like receptors (TLRs), at the time of FVIII immunization. I hypothesized that stimulation through TLR4 in conjunction with FVIII treatments would increase the formation of FVIII inhibitors. To test this hypothesis, FVIII K.O. mice were injected with recombinant human FVIII with or without concomitant doses of TLR4 agonist (lipopoysaccharide; LPS). The addition of LPS combined with FVIII significantly increased the rate and the production of anti-FVIII IgG antibodies and neutralizing FVIII inhibitors. In the spleen, repeated *in vivo* TLR4 stimulation with LPS increased the relative percentage of macrophages and dendritic cells (DCs) over the course of 4 injections. However, repeated *in vivo* FVIII stimulation significantly increased the density of TLR4 expressed on the surface of all spleen antigen presenting cells (APCs). Culture of splenocytes isolated from mice revealed that the combined stimulation of LPS and FVIII also synergistically increased early secretion of the inflammatory cytokines IL-6, TNF-α, and IL-10, which was not maintained throughout the course of the repeated injections. While cytokine secretion was relatively unchanged in response to FVIII re-stimulation in culture, LPS re-stimulation in culture induced increased and prolonged inflammatory cytokine secretion. Re-stimulation with both LPS and FVIII induced cytokine secretion similar to LPS stimulation alone. Interestingly, long term treatment of mice with LPS alone resulted in splenocytes that showed reduced response to FVIII in culture. Together these results indicated that creating a pro-inflammatory environment through the combined stimulation of chronic, low-dose LPS and FVIII changed not only the populations but also the repertoire of APCs in the spleen, triggering the increased production of FVIII inhibitors. These results suggested an anti-inflammatory regimen should be instituted for all hemophilia A patients to reduce or delay the formation of FVIII inhibitors during replacement therapy.

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ABBREVIATIONS

aa: amino acid

AP-1: activator protein 1

APCs: antigen-presenting cells (macrophages, dendritic cells, and B-cells)

ATP: adenosine tri-phosphate

B.U: Bethesda unit

BCR: B-cell receptor

BIP: binding immunoglobulin protein

bp: base pairs (kbp: kilobase pairs)

BSA: Bovine serum albumin

Ca^{2+}: calcium ions

CD: cluster of differentiation

CTLA-4: Cytotoxic T-Lymphocyte Antigen-4

Da: Dalton (kDa: kilodalton)

DCs: dendritic cells

DNA: deoxyribonucleic acid

dPBS: Dulbecco's phosphate buffered saline

FI: Factor I, fibrinogen (FIa: activated Factor I, fibrin)

FII: Factor II, prothrombin (FIIa: activated Factor II, thrombin)

FIX: Factor IX (FIXa: activated Factor IX)

FV: Factor V (FVa: activated Factor V)

FVII: Factor VII (FVIIa: activated Factor VII)

FVIII: Factor VIII (FVIIIa: activated Factor VIII)

FX: Factor X (FXa: activated Factor X)

FXI: Factor XI (FXIa: activated Factor XI)

FXII: Factor XII (FXIIa: activated Factor XII)

FXIII: Factor XIII (FXIIIa: activated Factor XIII)

HRP: horseradish peroxidase

HSPG: heparin sulfate proteoglycans

IFN-γ: interferon γ

IgG: immunoglobulin type G

IL: interleukin

IRAK: IL-1 receptor-associated kinase

IRF: interferon regulatory factor

ITI: immune tolerance induction

LBP: LPS binding protein

LPS: lipopolysaccharides, TLR4 agonist

LRP: lipoprotein receptor-related protein

LRR: leucine-rich-repeat

MD-2: Lymphocyte antigen 96

MHC: major histocompatability complex

MyD88: myeloid differentiation primary response gene 88

NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells

PAM: Pam3CysSK4, TLR1:2 agonist

PAMP: pathogen-associated molecular patterns

PBS: Phosphate-buffered saline

PD-1: programmed cell death protein

PL: phospholipids

PRR: pattern recognition receptor

PT: prothrombin time

PTT (aPTT): partial thromboplastin time

RNA: ribonucleic acid

TCR: T-cell receptor

Tenase complex ("Xase"): FXa/FVa

TF (FIII): Tissue Factor (Factor III)

TGF-β: transforming growth factor β

T_H1: helper T-cell type 1

T_H2: helper T-cell type 2

TIR: Toll/Interleukin-1 receptor

TIRAP: TIR-domain-containing adaptor protein

TLR: Toll-like receptor

TNF-α: tissue necrosis factor α

TRAM: TRIF-related adaptor protein

Treg: regulatory T-cell

TRIF: TIR-domain-containing adapter-inducing interferon-β

VWF: von Willebrand's Factor

INTRODUCTION

Hemophilia is a blood disorder characterized by the inability to properly clot due to the lack of an essential clotting protein or reduced activity of that protein. To understand this disorder, its complications, and how to treat patients, it is important to first understand the process of coagulation.

1. Coagulation

Coagulation, from Latin *coagulare* 'to cause to curdle[1],' is the process in which enzymatic activation of a series of proteins initiates the conversion of fibrinogen into fibrin to facilitate healing of a damaged blood vessel. Coagulation is a highly regulated[2] part of hemostasis, from the Greek *haima* 'blood' and *stasis* 'standing still[1],' the complex process of wound healing in which blood remains in its fluid state within the vascular system but takes on a semi-solid state at the site of a breach in a blood vessel.

In the "classic" view of the coagulation cascade, there are two major pathways that initiate formation of fibrin. As reviewed in Roberts et.al[3] (Fig.1), the intrinsic (cell-contact) pathway is controlled by vitamin K-dependent protein cofactors, namely factor XII (FXII), factor XI (FXI), factor IX (FIX), and factor VIII (FVIII), produced in the liver with circulation in the blood. Exposure to prekallikrein, high-molecular-weight kininogen, and collagen on the surface of platelets activates FXII (FXIIa) and initiates the clotting cascade. Each successive factor in the cascade is similarly activated by the previously activated factor. It is important to note that FVIII and factor V (FV) are activated by residual thrombin present in the blood and act as cofactors in the activation of FIX and factor X (FX) respectively. The FVIIIa/FIXa complex catalyzes the activation of FX in the presence of FVa and initiates the formation of the tenase (FVa/FXa) complex. The extrinsic (trauma) pathway, on the other hand, is initiated by factors that are not normally circulating in the blood, namely tissue factor (TF, FIII) which is located on the surface of endothelial cells. Once TF is exposed to the blood by tissue injury, factor VII (FVII) is activated, binds to TF to form a complex that activates FX, and leads to formation of the tenase complex. The tenase complex is common to both the intrinsic and extrinsic clotting pathways. This complex is stabilized by von Willebrand factor (VWF) on activated platelets and is responsible for catalyzing the conversion of prothrombin (Factor II, FII) into thrombin, which catalyzes the transition of fibrinogen (Factor I, FI) into fibrin. Factor XIII (FXIII), which is also activated by thrombin, is responsible for fibrin cross-linking in the finished clot.

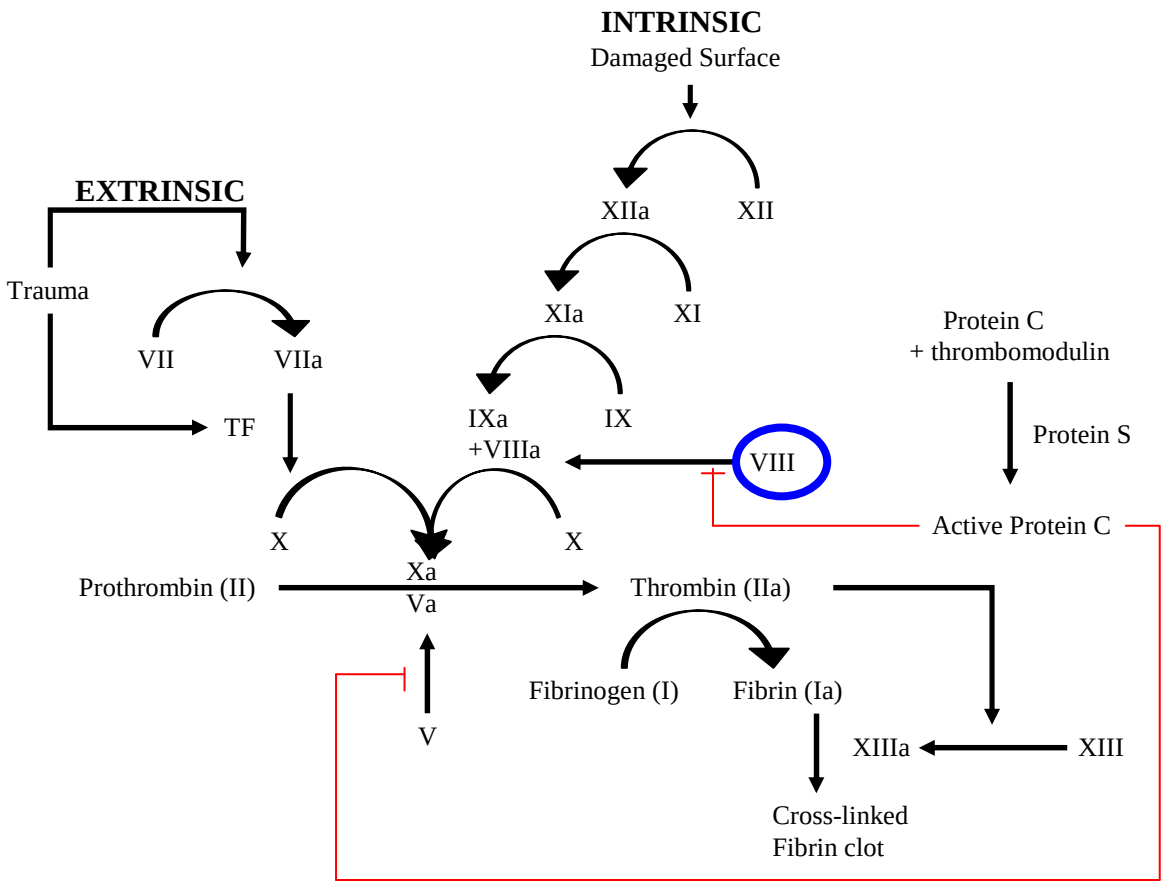


Fig.1: Intrinsic and extrinsic pathways as defined in the classic clotting cascade
The extrinsic cascade is triggered by trauma and utilizes TF while the intrinsic clotting cascade is triggered by damaged cell surfaces and utilizes FVIII. Both cascades activate the tenase complex that catalyzes the formation of thrombin. The cascade is controlled by negative regulators such as Protein C. Adapted with permission from *Anesthesiology*[3] and *Molecular Pathology*[4].

However, this classic view of coagulation is really a simplified view of the complex interplay and feedback that occurs during the coagulation process. As reviewed by Roberts et.al.[3] and Lee et.al.[5], the intrinsic and extrinsic pathways are inter-dependent because a deficiency in one of the essential clotting factors such as FVIII, FIX, or FVII is not completely compensated for by the other intact pathway of the clotting cascade. Therefore, the current model of clotting more closely resembles a cross-over positive-feedback loop (Fig.2). The process begins with FVIIa binding to TF, anchored to the activated phospholipid (PL) membrane of a TF-bearing endothelial cell, which can then activate FX and FIX. FXa, remaining near the TF-bearing endothelial cells, activates FV and becomes part of the tenase complex, catalyzing the formation of small amounts of thrombin. This initial production of thrombin, in combination with residual levels of thrombin in the blood, will catalyze the conversion of fibrinogen to fibrin to form an initial clot, activate platelets and essential intrinsic clotting factors (FVIII, FV, FXI), and separate FVIII from VWF

to form FVIIIa. FVIIIa will then act as an enzyme cofactor to increase formation of the tenase complex five-fold, producing substantially more thrombin for final clot formation. Once a clot is formed, FVIIIa and FVa are inactivated by FIXa and activated protein C, which leads to an overall down-regulation of the clotting cascade and restoration of hemostasis (Fig.1).

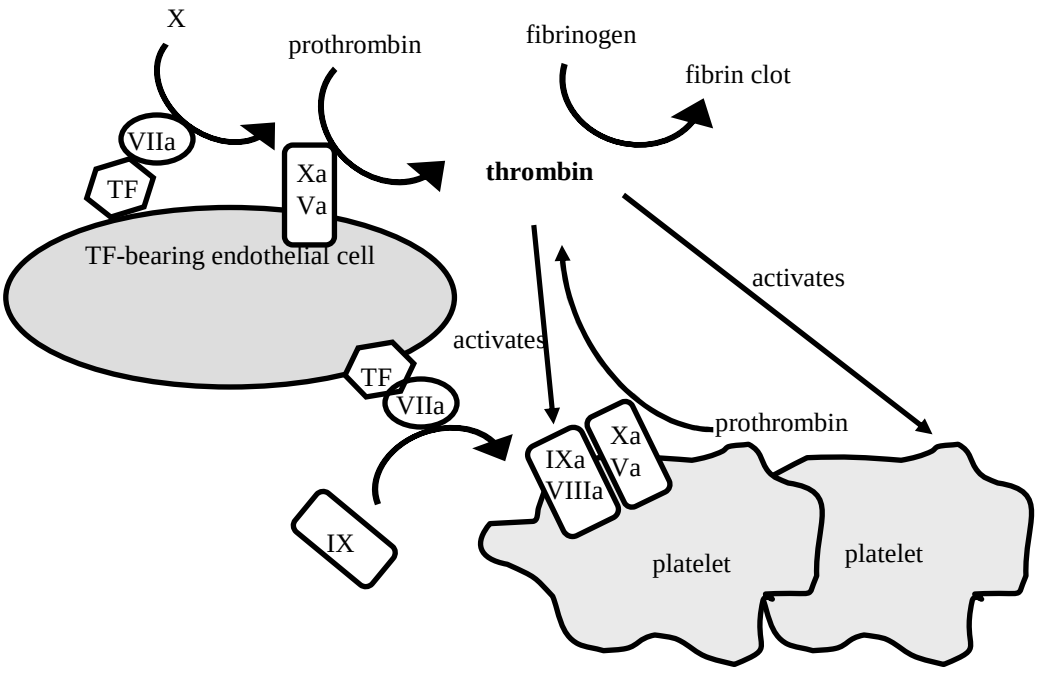


Fig.2: Currently accepted model of interdependent clotting pathways
Trauma triggers the extrinsic cascade, activating the tenase complex and creating a usable pool of thrombin. Thrombin activates intrinsic clotting factors and platelets, increasing tenase activating and thrombin formation. The cascades activate each other in order to form clot. Adapted with permission from *Anesthesiology*[3].

2. Hemophilia

Hemophilia, from the Greek *haima* 'blood' and *philia* 'love'[1], is a general term that encompasses three coagulation disorders (hemophilia A, B, and C) caused by the lack of a functional form of an essential clotting factor protein. Patients with hemophilia A produce little or no functional FVIII. The prevalence of "classic" hemophilia is one in 5,000-10,000 male births[6, 7]. Patients with hemophilia B produce little or no functional FIX. Hemophilia B is rare in that its prevalence is one in 25,000-40,000 male births[8, 9]. Patients with hemophilia C produce little or no functional FXI. It is the rarest and least severe form of hemophilia with a prevalence of only one in 100,000 individuals, usually individuals of Ashkenazi Jewish descent[9].

Hemophilia A is further classified by how the disease first manifests: congenital, due to a mutation in the FVIII gene on the X chromosome; or acquired, due to spontaneous production of

anti-FVIII antibodies. The majority of hemophilia A patients are congenital and have complications that manifest at birth or in early childhood. Acquired hemophilia A, on the other hand, affects only one in 1,000,000 persons, most of whom are adults over the age of 50 with no previous personal or family history of bleeding disorders or other underlying medical conditions. In some patients, acquired hemophilia has been associated with postpartum bleeding as well as autoimmune, dermatologic, infectious, or oncologic diseases; however the cause in these cases remains unclear and therefore patient prognosis and treatment are variable[10].

3. Clinical Diagnosis of Hemophilia A

There are two routine clinical tests used to identify a clotting disorder, the prothrombin time (PT) and the partial thromboplastin time (PTT, aPTT). The PT test evaluates the extrinsic (trauma) coagulation pathway by measuring the functionality of FVII, FV, FX, prothrombin, and fibrinogen (Fig.3A). Intrinsic coagulation cascade proteins, thromboplastin, and calcium (Ca^{2+}) are added to citrated patient plasma. Clotting time is measured; delayed clotting indicates a problem with one of the extrinsic clotting factors. Similarly, the PTT (or aPTT) test evaluates the intrinsic (cell contact) coagulation pathway by measuring the functionality of FVIII, FXII, FXI, FIX, FX, prothrombin, and fibrinogen (Fig.3B). Extrinsic coagulation cascade proteins, PL, kaolin, and Ca^{2+} are added to citrated patient plasma. Clotting time is measured; delayed clotting indicates a deficiency in one of the intrinsic clotting factors[5, 11]. Patients with a normal PT but a prolonged aPTT are diagnosed with hemophilia[12].

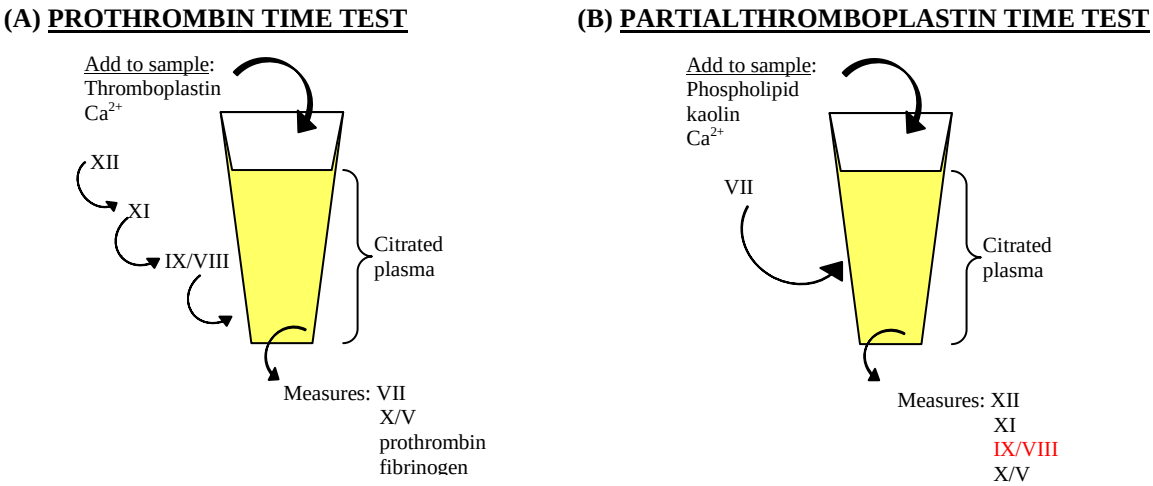


Fig.3: Clinical assays for identifying clotting disorders
(A) Prothrombin Time Test identifies problems with the extrinsic clotting cascade and (B) Partial Thromboplastin Time Test identifies problems with the intrinsic clotting cascade. Adapted with permission from the *McGill Virtual Physiology lab*[11]

Hemophilia A severity is classified by the amount of functional FVIII present in the blood, which is directly related to the time required for clot formation during the aPTT assay. Severity is divided into three classes: severe, moderate, and mild. Patients are “severe” if they have less than 1% of the normal functional levels of FVIII, “moderate” if they have 1-5% of the normal functional levels of FVIII, and “mild” if they have less than 5-25% of the normal functional levels of FVIII[12-14].

4. Factor VIII Gene Structure and Protein Formation

The *F8* gene, first sequenced in 1984[15], is approximately 186 kbp[16] long and located on the X chromosome (Xq28). The gene, as reviewed in Lee et.al.[5] and White[17], encodes a 300 kDa glycoprotein that is synthesized in the liver and, when released into the bloodstream, acts as a pro-cofactor for FIX enhancing its activity by 200,000 fold[18]. In hepatocytes, the gene is transcribed into pre-mRNA that undergoes cleavage and splicing until the mature mRNA contains the essential 26 exons. The mRNA is translated into a 2332 amino acid (aa)-long polypeptide chain which can be divided into 6 domains (A1, A2, B, A3, C1, and C2) and 3 linker regions (a1, a2, and a3 acidic residues) based on function (Fig.4)[18].

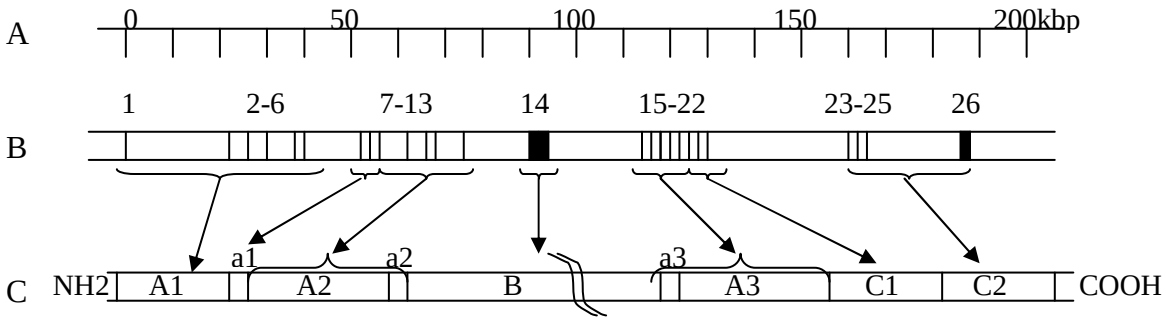


Fig.4: FVIII gene and protein organization
(A) The 200 kbp FVIII gene is transcribed and translated into (B) mRNA containing 26 essential exons which are cleaved together to form (C) a 300 kDa FVIII protein. Adapted with permission from *Molecular Pathology*[4] and *Br J Haematol*[14].

FVIII is a cofactor for FIX; the activation of FIX is performed by the A2+a2 protease domain. The other protease domain (A1+a1), in the presence of FIXa, is responsible for activating FX. The B domain undergoes multiple and complex post-translational glycosylations but does not contain binding sites for any other essential clotting proteins and is later cleaved out of the final active FVIII protein complex. Similar to the B domain, the a3 acidic domain is later

cleaved out of the final active FVIII protein complex. The C1 and C2 domains are responsible for binding and stabilizing the FVIII protein. Both of these domains bind VWF, stabilizing the FVIII protein structure in the blood until it is needed for clotting. The C2 domain also binds PL and lipoprotein-receptor related proteins (LRP) which stabilize the protein structure on the surface of platelets to enable FVIII to properly activate FIX and FX (summarized in Table 1).

Domain	Amino Acids	Exons	Function	Binding Sites
A1	1-336	1-6	FX protease domain	FXa, FIXa, activated protein C
a1 acidic residues	337-372	7-8		FX, FXa, thrombin
A2	373-710	9-13	FIX protease domain	FIXa, LRP, HSPGs, activated Protein C
A2 acidic residues	711-740			FXa, thrombin
B	741-1648	14		
A3 acidic residues	1649-1689	15-19	EGF-like domain	thrombin, VWF, FXa
A3	1690-2019			FIXa, LRP
C1	2020-2172	20-22	Binding domains	VWF
C2	2173-2332	23-26		VWF,PL, LRP, FXa

Table 1: Components and functions of the FVIII protein domains

5. Factor VIII Post-Translational Processing

The nascent polypeptide chain is translocated from the cytosol into the ER lumen of hepatocytes, chaperoned by binding immunoglobulin protein (BIP), and undergoes signal peptide cleavage and N-glycosylation. BIP is released after adenosine tri-phosphate (ATP) hydrolysis to allow the polypeptide chain to begin disulfide bond formation during the initial stages of protein folding in the presence of Ca^{2+}. This initial FVIII structure is exported to the Golgi where it undergoes complex N-glycosylation, S/T-glycosylation, and tyrosine sulfation. The protein is cleaved into two chains: the heavy chain (200 kDa) consisting of the A1, a1, A2, a2, and part of the B domains; and the light chain (80 kDa) consisting of the a3, A3, C1, and C2 domains. These two chains, coordinated and stabilized by Ca^{2+}, form the final FVIII structure that is secreted into the blood.

Once in the blood, the inactive form of FVIII is bound and stabilized by VWF so that the half-life of the protein is approximately 8-12 hours[3, 19]. Thrombin activates FVIII by initiating cleavage of the remaining portion of the B domain and the a3 acidic domain. The a1 acidic domain and the A2 domain are also cleaved, separating the protease domains. The final, activated structure is a 170 kDa heterotrimer, coordinated by Ca^{2+}. If not bound by VWF, FVIIIa is quickly degraded when activated protein C cleaves the A1 domain and A2 domains, destroying the functional protease domains and essential structure of FVIIIa (reviewed in Table 2).

Protein	Size	Modifications	Result	Location
Whole protein	300 kDa	Transcription/translation of gene Translocation into ER N-glycosylation, disulfide bonds	Formation	Hepatocyte
Hetero-dimer	H chain (200 kDa) L chain (80 kDa)	Complex N, S/T glycosylation, Tyr sulfation in Golgi Cleavage of B domain Stabilization by VWF	Secretion (FVIII)	Hepatocyte Blood
Hetero-trimer	A1 chain (50 kDa) A2 chain (43 kDa) A3,C1,C2 chain (73 kDa)	Cleavage by thrombin Removal of B, a3 acidic domains Separation of A1, A2 domains	Activation (FVIIIa)	Blood
Degraded particles of FVIII		Cleavage of A2, a1 acidic domains by activated protein C, FIXa	Inactivation	Blood

Table 2: Cleavages and post-translational modifications of FVIII protein

The topology of FVIIIa is crucial for its proper function. The protease domains are oriented facing out to allow access to the clotting factors and thrombin. The binding domains are oriented to face the PL surface of the platelets to which they will bind to stabilize the protein[12, 20, 21] (Figs.5-6).

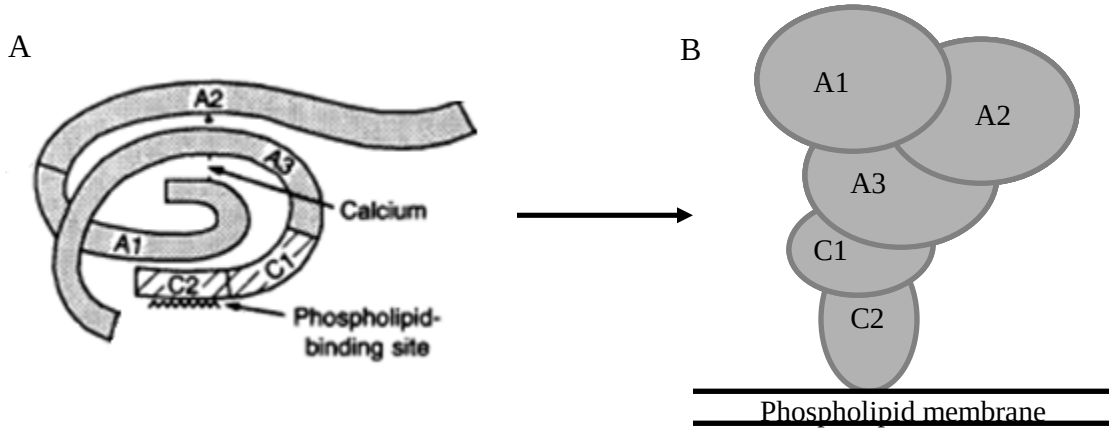


Fig.5: FVIII protein structure

(A) Spatial organization of FVIII protein domains and (B) 3-D model representing the topology of FVIII protein. Adapted with permission from *Haemophilia*[21] and *N Eng J Med,* Copyright Massachusetts Medical Society [12].

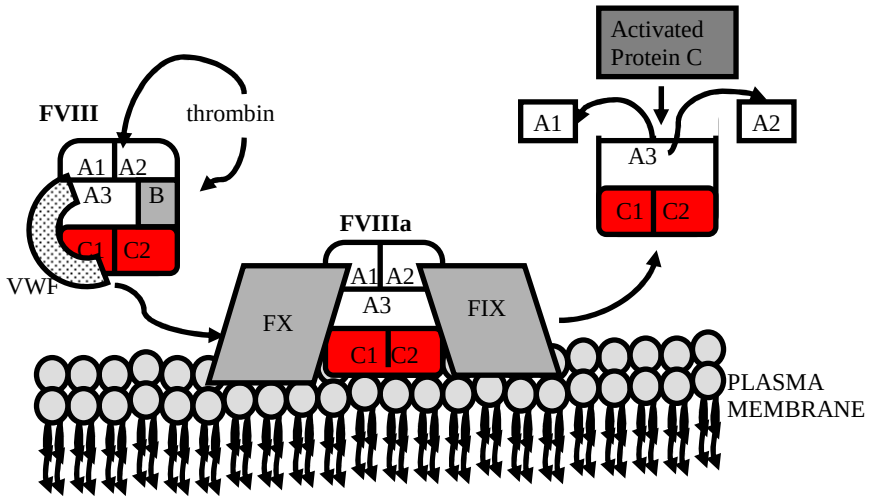


Fig.6: FVIII protein interactions during coagulation
FVIII is activated by thrombin before binding to PL on the surface of platelets where it activates FIX and FX. Once coagulation is complete, FVIII is deactivated and degraded by activated protein C.

6. Factor VIII Mutations

FVIII gene mutations lead to incorrect protein transcription, translation, and post-translational processing. The type and location of the mutation affects whether the protein will be produced and if it will be functional. As described by Bowen[4], FVIII gene mutations include missense and nonsense point mutations, deletions, insertions, and chromosomal rearrangements/inversions.

Point mutations, which may or may not change the encoded amino acid, are the most common mutations, affecting 90% of patients and resulting in variable hemophilia severity. Some missense point mutations, where the transcribed amino acid is unchanged or changed to an amino acid with similar properties, have little to no effect on the final protein structure leading to production of relatively normal FVIII proteins and only moderate symptoms. Other missense mutations can create alternative mRNA splice sites or, where the encoded amino acid has completely different properties from the original, alter the final protein structure. These missense mutations lead to the production of non-functional or misfolded FVIII proteins and mild-severe complications for the patient. Nonsense point mutations, which lead to the formation of a premature stop codon, can cause exon skipping, resulting in a truncated and non-functional protein and severe patient complications.

Deletions, the removal of a piece of the FVIII gene, the second most common gene defect, are found in 5-10% of patients. Deletions can range in size from whole gene deletions to micro-deletions and do not appear to cluster to any specific area of the gene. Deletions often also cause a frameshift which leads to non-functional protein and severe patient symptoms. Similar to

deletions, insertions into the FVIII gene are also very detrimental to functional protein formation by altering mRNA splice sites and/or introducing frameshifts.

Rearrangements (inversions) due to homologous recombination also lead to severe hemophilia and are usually the rarest of the FVIII gene mutations. The only exception is the intron 22 inversion which is found in 40-50% of patients suffering from severe hemophilia A.

Bowen[4] also discussed FVIII gene mutations that do not directly effect the transcription, translation, and post-translational processing of the FVIII protein but instead affect the secretion, stability, and interaction of the FVIII protein with other clotting factors. Missense mutations in certain arginine residues lead to increased intracellular accumulation, decreased levels of circulating FVIII protein, and mild patient symptoms. Mutations in the FVIII binding and protease domains can prevent proper FVIII interaction with VWF, Ca^{2+}, FIX, FX, or PL. Inability to interact with VWF or Ca^{2+} leads to reduced FVIII stability and shorter half-life in the blood[22]. At the same time, mutations that prevent proper Ca^{2+} binding and coordination will prevent proper FVIII interaction with FIX, FX, and PL, such that FVIII is no longer able to participate in the activation of downstream clotting factors.

7. Pathology of Hemophilia A

Clinical complications depend on a combination of disease severity and environmental factors. As reviewed by Hoyer[12] and Coppola[23], common complications include intramuscular bleeding, bleeding into the joints, hemarthrosis (deterioration of the joints due to bleeding), swelling, numbness, pain, difficulty with wound healing and inflammation/infection, and the formation of FVIII inhibitors (discussed later). Intramuscular bleeding and hemarthrosis, often caused by external trauma or surgery, are often the first indication of a bleeding episode due to the accumulation of blood in one of the 'key' joints, usually a knee or elbow. Swelling, pain, and numbness from pinched nerves is usually severe enough that there is a reluctance to use the affected joint leading to further joint damage, bone fusion, muscle atrophy below the joint, and eventually the need for joint replacement. Inflammation and infections can also lead to and exacerbate complications[24, 25], slowing down wound healing compared to people unaffected by this disease. Inflammation can be triggered by open or improperly-treated injuries, surgery, or already-present infections/immune conditions aggravated by a compromised immune system. Infections can be introduced by contaminated blood transfusions or therapeutic treatments. Viral infections such as human immunodeficiency virus or hepatitis virus were a major problem in the mid-1980's. Due to the lack of knowledge about these viruses and proper screening technology, the use of clotting factor concentrates isolated from contaminated human serum made

hemophiliacs more likely to contract these viruses; by 1987, 78% of patients receiving FVIII replacement therapy were infected. Treatment has since become much safer with the introduction of recombinant and porcine clotting factors, heat-treated clotting concentrates, and better donor screening techniques for those patients who are still being treated with human FVIII[26].

Non-clinical factors include the age of the patient, frequency of replacement therapies, type and availability of clotting factor concentrates, and the cost of treatments[23, 27]. Hemophilia A is a chronic, incurable disease with a cost of around $80,000-$150,000 a year[28], depending on the patient's FVIII dose regimen and the presence of inhibitors. Costs can often be too much for a patient to handle even with medical insurance. Limited availability of the clotting factor concentrates is also a barrier to the diffusion of prophylaxis, especially in developing countries[23].

8. Current Treatments

The current treatment for hemophilia A is the intravenous application of FVIII, most often a recombinant form of the protein that was first synthesized and used in 1987[17]. Recombinant human FVIII is derived through transfection of non-human mammalian cell lines capable of performing all of the complex posttranslational modifications required for proper protein function, either Chinese hamster ovary or baby hamster kidney[19]. Patients can also be treated with porcine or human-derived FVIII. The dosage and frequency of FVIII applications is individualized for each patient based upon weight, age, frequency of bleeds, type of replacement FVIII, and the physician's choice of treatment regimen[23]. Patients with congenital hemophilia are usually diagnosed as infants and placed on a prophylactic FVIII treatment regimen between the ages of 1-2 years or after the first joint bleed. This type of treatment is used to regularly replenish serum levels of FVIII to minimize bleeding and attempt to convert the severe hemophilia into a milder form with reduced clinical complications and increased quality of life[23, 29]. Prophylactic treatments are much preferred to on-demand FVIII treatments and high dosage FVIII applications in cases of emergency bleeds, which have been associated with increased instances of anti-FVIII antibody formation[30].

9. Factor VIII Inhibitors

During FVIII replacement therapy, about 25-33% of patients with severe hemophilia develop neutralizing antibodies or "inhibitors" against FVIII[14]. Anti-FVIII antibodies are polyclonal IgG antibodies, usually IgG_1, IgG_2 and IgG_4 subclasses, which target the functional and/or non-functional domains of the FVIII protein, blocking its pro-coagulant activity and preventing activation of downstream clotting factors. These antibodies act by: (1) sterically hindering

epitopes required for FVIII interaction with other coagulation molecules, including VWF, FIXa, FX or PL; (2) destabilizing the FVIII protein so that it less effective at activating other clotting factors; or (3) degrading FVIII either by direct hydrolysis or through formation of immune complexes[31-34]. Therefore, the presence of FVIII inhibitors renders FVIII replacement therapy less effective. The level of inhibitors in a patient's circulation is quantitated by the Nijmegen modification of the Bethesda assay (Fig.7)[35]. Inhibitor levels are reported in Bethesda Units (B.U.), in which one Bethesda unit is the amount of inhibitor required to reduce clotting by 50%. For this assay, the cut-off for inhibitor detection is ~0.6 B.U[13, 25, 35, 36].

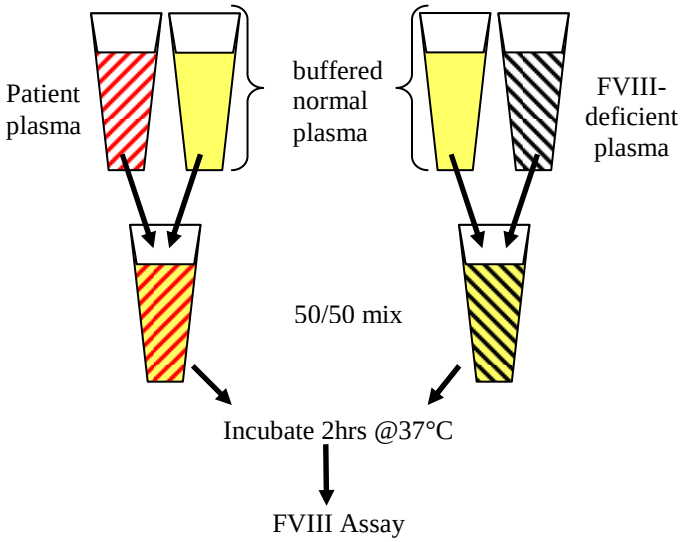


Fig.7: Nijmegen modified Bethesda assay for inhibitor detection
Patient plasma (being tested) is mixed 50/50 with buffered normal plasma, incubated, and undergoes FVIII assay. The results are compared to those obtained from a 50/50 mixture of known FVIII-deficient plasma and buffered normal plasma. Adapted with permission from *Thromb Haemost*[35, 36].

10. FVIII Inhibitor Risk Factors

The formation of anti-FVIII antibodies is determined by a delicate balance between genetic and environmental risk factors[25, 37](reviewed in Fig.8). Genetic risk factors include the type and location of the FVIII gene mutation, family history of inhibitor development, ethnicity, and the immuno-genotype of certain inflammatory cytokines. The type of FVIII gene mutation has the greatest influence on a patient's risk for developing inhibitors. Patients with large deletions, nonsense mutations, and chromosomal inversions have the highest incidence of inhibitor formation. This may be because the complete deficit of endogenous FVIII production prevents establishment of central tolerance to FVIII[24, 38, 39]. Replacement FVIII is seen as "foreign" protein by the immune system. Patients with smaller FVIII gene mutations, such as missense

mutations or small deletions, may produce the FVIII protein even if it is non-functional, thereby enabling central tolerance to the protein and lowering the risk of inhibitor formation during replacement therapy. Similarly, family history of inhibitor development also predisposes a patient to inhibitor formation[14, 24, 39]. Ethnicity/race is also a risk factor for inhibitor development. African-American patients are twice as likely to develop inhibitors as Caucasian patients[40]. The immuno-genotype of certain inflammatory cytokines is linked to the increased development of FVIII inhibitors. Patients with certain polymorphisms in the promoter regions of the IL-10, TNF-α, and MHC II genes are more likely to develop inhibitors[41, 42] whereas patients with polymorphisms in the CTLA-4 gene are less likely to develop inhibitors[42, 43].

Non-genetic risk factors for the formation of FVIII inhibitors include the type, concentration, and frequency of therapeutic FVIII infusions, along with immunological influences/disorders that occur during treatment. Although no significant risk of increased inhibitor formation has yet been linked to the type of therapeutic FVIII product, whether it is human plasma-derived or recombinant, whole length or B-domain deleted, switching products during treatment carries a small risk of inhibitor formation[29]. There is come indication that viral infections can promote inhibitor development; increased inhibitor development has been found in patients with Hepatitis A, Hepatitis B, and HIV[26, 44]. The largest risk factors for developing inhibitors are the type and concentration of therapeutic treatments. Initiating FVIII treatments at an early age and in a prophylactic manner carries lower risks of inhibitor development, whereas large, on-demand infusions during severe bleeds and major surgery are associated with increased risk of inhibitor formation[25]. Although there are many factors that can influence the risk of inhibitor formation, there is no definite way to determine if and when a particular patient will begin producing FVIII inhibitors.

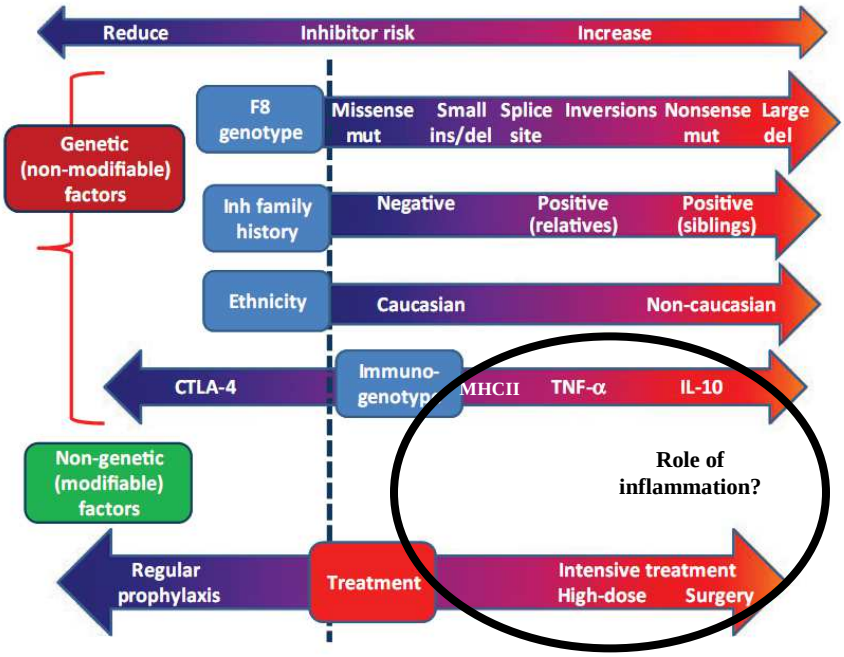


Fig.8: Risk Factors for the development of FVIII inhibitors

Both genetic and environmental factors have been found to increase the risk of FVIII inhibitor development in hemophilia patients. Viewed together, these risk factors indicate that inflammation might also be driving the formation of inhibitors. Adapted with permission from *Haemophilia*[37]

11. Current Treatments after FVIII Inhibitor Formation

FVIII replacement therapy must be adjusted due to anti-FVIII antibodies. Patients with inhibitor titers of <5 B.U. can still receive replacement FVIII therapy, just at higher and more frequent doses to replenish FVIII levels sufficient to maintain hemostasis[14]. Patients with inhibitor titers of >5 B.U. must receive one of several bypass therapies which include the application of prothrombin complex concentrates or recombinant human FVIIa, both of which work through the extrinsic clotting pathway to activate the tenase complex without requiring FVIII. Patients with high inhibitor titers can also undergo immune tolerance induction (ITI), a course of treatment designed to eradicate existing FVIII inhibitors and induce FVIII-specific immune tolerance[14]. The various ITI protocols utilize regular applications of large doses of FVIII and immunosuppressive drugs over the course of several years. High doses of FVIII down-regulate the FVIII adaptive immune response, specifically by inducing anergy and depleting the anti-FVIII antibody secreting plasma cells[45]. If ITI treatment is effective, which occurs in approximately 80% of the cases, the patient can restart normal FVIII treatments[45].

12. Role of Inflammation in the Formation of FVIII Inhibitors

Research has indicated that there might be a relationship between inhibitor production and inflammation[14]. The development of inhibitors is caused by highly regulated interactions between different cells of the innate and adaptive immune systems[25]. Since recombinant human FVIII is immunogenic, repeated therapeutic administration of FVIII can be identified as a foreign protein by the immune system triggering a FVIII-specific immune response. During this immune response, the FVIII binds to receptors on the surface of APCs, mainly macrophages and DCs. The elimination of macrophages and $CD11c^{+}/CD8a^{-}$ dendritic cells abrogated the onset of anti-FVIII immune response[33, 44, 46, 47]. APCs then migrate to the spleen where they co-localize with T-cells. Removal of the spleen has been shown to prevent inhibitor formation and eradicate any inhibitors already present[45]. APCs will internalize and degrade FVIII for presentation on MHC II while also secreting inflammatory cytokines (such as IL-6, IL-12, and TNF-α) and up-regulating co-stimulatory molecules (such as CD40, CD80, and CD86). Previous research has indicated that polymorphisms in the TNF-α and IL-10 genes increase a patient's likelihood to develop inhibitors[42, 43]. APCs bind the T-cell receptor (TCR) *via* MHC II and induce T-cell activation through co-stimulatory molecules on T-cells. Lack of $CD4^{+}$ T-cell stimulation due to loss of CD40L, CD80, CD86 or CTLA-4 signaling impairs cellular cross-talk between APCs and CD4+ T-cells, preventing initial inhibitor formation and removing any inhibitors already present in the blood, as seen from studies in patients infected with AIDS with low CD4+ T-cell counts[33, 48]. The activated helper T-cells will then bind the B-cell receptor (BCR) to activate B-cells, stimulating production of FVIII-specific plasma cells that produce large amounts of anti-FVIII antibodies[33]. B-cell depletion, utilizing anti-CD20 therapy, significantly decreases FVIII inhibitor titers in a mouse model[46]. Similarly, re-stimulation using high doses of FVIII inhibits FVIII-specific memory B-cells, preventing further differentiation of FVIII plasma cells and decreasing the production of anti-FVIII antibodies[45].

The stimuli that initially trigger and drive the FVIII immune response are not well defined. Toll-like receptors (TLRs) might play a role in triggering the FVIII immune response because immune cells that express TLR and cytokines secreted after TLR stimulation are key components in the FVIII immune response[14]. Previous data from the Smith lab utilizing cytokine multiplex analysis and statistical algorithms to model *in silico* the anti-FVIII immune response (not published) suggested that TLR4 might be up-regulated early during the FVIII response in mice. To test this prediction, I studied the role of TLR4 stimulation on the formation of FVIII inhibitors in a mouse model of hemophilia A.

TOLL-LIKE RECEPTORS (TLRs)

It has been suggested that inflammation triggers the FVIII immune response, leading to the production of FVIII inhibitors. Previous experiments in the Smith lab identified Toll-like receptor 2 (TLR2) and TLR4 as two potential receptors that, when stimulated in conjunction to FVIII, will drive this inflammatory response. In order to understand how inflammation is affecting the formation of inhibitors, it is important to understand how TLRs trigger the inflammatory response and the effect this signaling has on the interaction between the innate and adaptive immune responses.

1. History of Toll-Like Receptors

TLRs are evolutionarily conserved homologs of the Toll protein, a developmental protein first identified in *Drosophila* that also conveyed anti-fungal protection in adult flies[49]. Due to domain homology, TLRs are defined as members of a larger superfamily of proteins that includes the interleukin-1 receptor (IL-1R) and IL-18[50]. TLRs contain an extracellular binding domain consisting of a 31 amino acid (aa) N-flanking region, 19-25 leucine-rich-repeat (LRR) tandem motifs that are directly involved in ligand binding, and a cysteine-rich terminal domain. Each LRR is 24-29 aa long and contains an xLxxLxLxx motif[50]. TLRs also contain in intracellular signaling domain that is homologous to the IL-1R signaling domain, called the Toll/Interleukin-1 receptor (TIR) domain. These two domains are separated by a transmembrane domain.

2. The Location of Toll-Like Receptors

Ten human TLRs have been identified. TLR1, 2, 4, 5, and 6 are located in the plasma membrane and bind any bacterial, fungal, or other pathogenic proteins that come into contact with the TLR-expressing cells. TLR3, 7, 8, and 9 are located in the endosome and bind any nucleic acids, mainly viral, that is phagocytosed by the cell[51]. The location and function of TLR10 are not yet known. TLRs are expressed in varying degrees and combinations on both immune cells, including monocytes, macrophages, DCs, T-cells, and B-cells[52], and non-immune cells, including fibroblasts, endothelial cells, adipocytes, epithelial cells, and glial cells[50]. Mice and other animals have been shown to possess a greater number of TLRs (TLR11-13).

3. The Function and Agonists of Toll-Like Receptors

Toll-like receptors are type I integral membrane glycoprotein receptors that act as part of an early warning system for infection. They have been dubbed “adjuvant receptors” because they bind ligands that are potent adjuvants and trigger a vigorous innate immune response in the

attempt to clear bacterial or viral infections[50, 53]. This immune response will eventually lead to the induction of the adaptive immune response and the production of antibodies targeting those pathogens[52].

As pattern recognition receptors (PRR), each of the ten TLRs are responsible for binding a subset of pathogen-associated molecular patterns (PAMPs) associated with immunological danger and stress. The majority of known TLR agonists is derived mainly from bacteria and viruses but can also include endogenous ligands (reviewed in Table 3); there are many more potential TLR agonists that have yet to be identified including those specific for TLR10.

Toll-Like Receptor	Agonist	Agonist Origin
TLR1	N-terminus triacylated lipopeptides	Bacteria
	Soluble factors	*Neisseria* bacteria
TLR2 (forms heterodimer with TLR1 or TLR6)	Glycolipids	Bacteria
	Lipopeptides/lipoproteins	Bacteria
	Lipoteichoic acid	Gram- bacteria
	Peptidoglycan	Gram- bacteria
	Heat-shock protein (HSP)70	Host cells
	Zymosan (β-glucan)	Fungi
	Porins	*Neisseria* bacteria
TLR3	double-stranded RNA	Viruses
	poly I:C (double stranded RNA analog)	(synthetic)
TLR4 (forms homodimer)	lipopolysaccharide (LPS)	Gram- bacteria
	HSPs	Bacteria, host cells
	Fibrinogen	Host cells
	Heparin sulfate fragments	Host cells
	Hyaluronic acid fragments	Host cells
	Nickel	
	Opioid drugs	
TLR5	Flagellin	Bacteria
TLR6	N-terminus diacylated lipopeptides	Mycoplasma
	Soluble tuberculosis factor	
TLR7	Imidazoquinoline	(synthetic)
	Loxoribine (a guanosine analogue)	(synthetic)
	Bropirimine	(synthetic)
	G/U-rich single-stranded RNA	RNA viruses
TLR8	Small synthetic compounds	
	G/U-rich single-stranded RNA	RNA viruses
TLR9	CpG DNA	Bacteria, DNA viruses
	CpG ODN (unmethylated CpG dinucleotides)	(synthetic)
TLR10	(unknown)	?

Table 3: Known human toll-like receptors and their agonists

4. Toll-Like Receptor Pathways and Signaling

As reviewed by Akira[50, 54], TLR signaling is triggered when the TLR LRR-horseshoe binding domains recognize and bind specific microbial PAMPs. After ligand binding, TLRs undergo a conformational change, sometimes after receptor dimerization, that is required for recruitment of downstream intracellular adaptor proteins including TIR-domain-containing adaptor protein (TIRAP), myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor protein (TRAM), and IL-1 receptor-associated kinase (IRAK). These adaptor proteins, all of which contain TIR domains, propagate the signal via cascading phosphorylations. The signal eventually activates transcription factors including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor (IRF), and activator protein (AP-1). These factors are responsible for activating the transcription of inflammatory cytokines and co-stimulatory molecules (summarized in Fig.9).

The majority of TLRs, excluding TLR3, utilize a MyD88-dependent pathway[50, 54]. Plasma membrane-bound TLRs activate NF-κB and trigger the transcription of inflammatory cytokines while TLRs located in the endosome-bound TLRs activate IRF7 and trigger the transcription of type 1 interferons. The resulting immune response is dependent upon the type of agonist, the TLR that is stimulated, and the cell types activated by TLR stimulation[55]. For example, TLR2 signaling preferentially induces a helper T-cell type 2 (T_H2) response[55], a humoral response characterized by the secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 along with the proliferation and maturation of B-cells and the production of antibodies. This response is normally triggered by extracellular parasite infections and allergic responses, such as asthma[51]. TLR3 utilizes a MyD88-independent pathway to trigger the transcription of type 1 interferons. Instead of MyD88, this TLR utilizes TRIF and IRF3 to initiate an immune response.

TLR4 is unique in that it can utilize either the MyD88-dependent and MyD88-independent pathways. It acts in cooperation with LPS-binding protein (LBP), which sequesters LPS from the plasma and presents it to the CD14 receptor[52], and MD-2, which associates with TLR4 and confers responsiveness to LPS TLR4 is capable of activating multiple transcription factors, leading to the transcription of a variety of inflammatory cytokines and the up-regulation of co-stimulatory molecules[50, 52, 54], to elicit an immune response.

For example, TLR4 preferentially, and especially in the presence of high doses of LPS, triggers a helper T-cell type 1 (T_H1) response, a cell-mediated response characterized by the secretion of IFN-γ, TGF-β, IL-2, and IL-10 along with the increased killing capability of macrophages and cytotoxic ($CD8^+$) T-cells. Along with the activation of lymphocytes and

macrophages, the up-regulation of co-stimulatory molecules enhances T-cell activation, expansion, and survival. T_H1 responses are normally triggered by intracellular infections like leishmaniasis and inflammatory diseases.

However the pattern of TLR expression and the diversity of cytokines induced by TLR stimulation can trigger alternative immune responses depending on how and where the receptor has been activated[55]. TLR2 activation normally triggers a T_H2 response; however, it also increases vascular permeability and neutrophil trafficking to facilitate pathogen clearance during inflammation; increases TF expression and fibrinolysis to promote coagulation and wound healing. Importantly, signaling through TLR2 can trigger the activation and proliferation of regulatory T-cells (Tregs)[51], which play a critical role in tolerance to self antigens and protection against autoimmunity[55]. Along those same lines, TLR4 activation normally triggers a T_H1 response; however, low doses of LPS usually found in low level, chronic infections can instead trigger a T_H2 response[51].

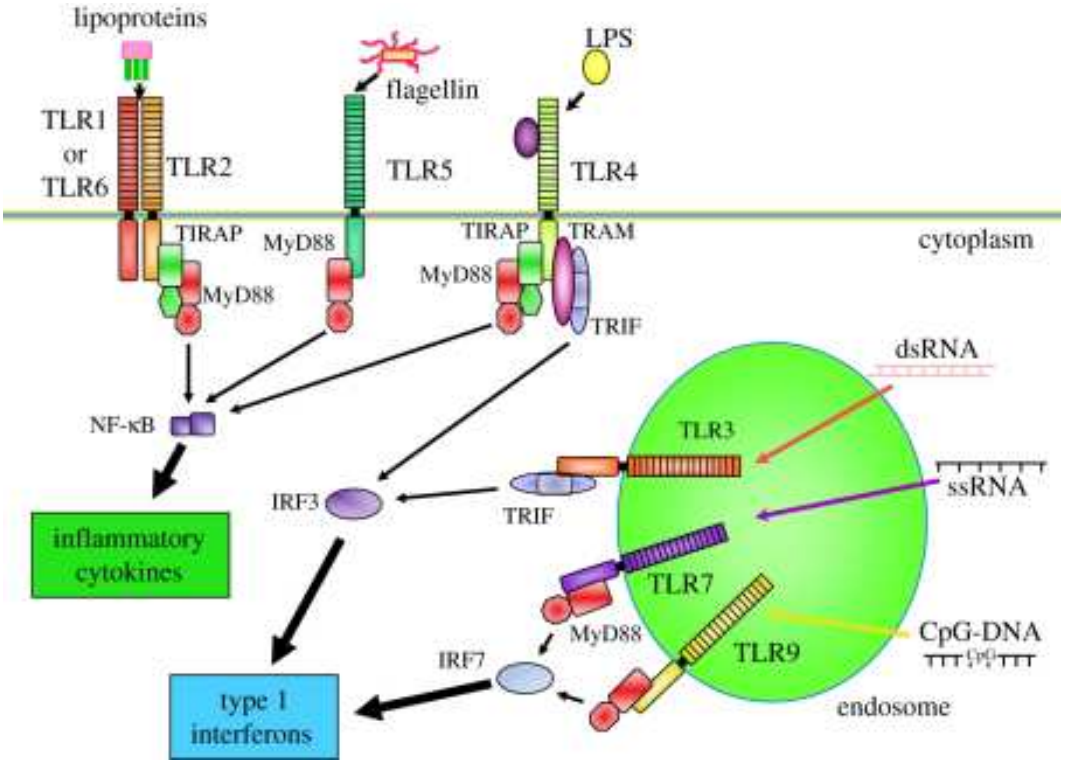


Fig.9: Interlinking pathways of the toll-like receptor family
Toll-like receptors bind PAMPs and trigger intracellular signaling, via MyD88-dependent and MyD88-independent pathways, to initiate the production of inflammatory cytokines and the up-regulation of co-stimulatory molecules as part of the innate immune response during infection. Figure used with permission from *Phil Trans R Soc B*[50].

5. Toll-Like Receptors and the Immune Response

TLR stimulation drives the transition from the innate immune response to the adaptive immune response by (1) regulating the activation, proliferation, and survival of APCs and T-cells; (2) triggering the secretion of inflammatory cytokines; and (3) triggering the maturation of B-cells into plasma cells. During the innate immune response, macrophages and DCs are the principle producers of inflammatory cytokines after TLR stimulation[53]. For example, LPS binding to TLR4 triggers an intracellular cascade that activates NF-κB transcription and secretion of inflammatory cytokines such as IL-6, TNF-α, and IL-12 and the up-regulation of CD40, CD80, CD86, and MHC II[56]. These cytokines, especially TNF-α and IL-12, enhance the activation, expansion, and survival of T-cells[57]. The production of these cytokines is important for up-regulating the inflammatory response, but overproduction can lead to organ damage and septic shock[53]. Therefore, macrophages also secrete IL-10 which inhibits continued TNF-α and IL-12 secretion. IL-10 is secreted by APCs and acts as a negative regulator to inhibit further inflammatory cytokine secretion, including TNF-α, IL-1β, and IL-12. While IL-10 also reduces APC differentiation, it does not affect T-cell development or activity[56]. Activated T-cells move to nearest draining lymph nodes and initiate the adaptive response by activating B-cells, turning them into antibody-secreting plasma cells[58]. Therefore, TLR stimulation during the FVIII immune response may contribute to the increased production of anti-FVIII antibodies (Fig.10).

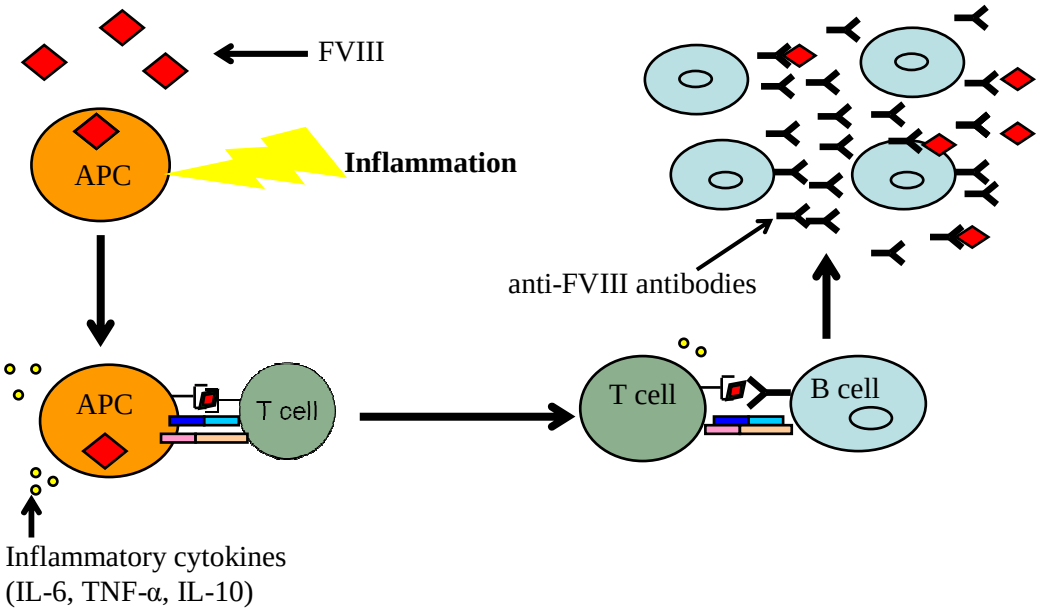


Fig.10: Model of enhanced development of FVIII inhibitors due to inflammation
Inflammation activates APCs to secrete inflammatory cytokines and better present FVIII antigens to T-cells, activating them. Activated T-cells move to the spleen and activate B-cells, triggering them to secrete anti-FVIII antibodies. Figure used with permission from Keri C. Smith, Ph.D.

HYPOTHESIS

Hemophilia patients receiving replacement FVIII therapy mount an immune response against exogenous FVIII, due to lack of central tolerance, and produce neutralizing anti-FVIII antibodies (inhibitors). Although the FVIII immune response is well documented, the stimulus that initially triggers and drives this response is not well defined. Previous research has indicated that this FVIII immune response might be enhanced by inflammation. It was suspected that toll-like receptors (TLRs) might play a role in triggering this response because immune cells that express TLR and cytokines that are secreted after TLR stimulation have been identified as key components in the FVIII immune response[14]. Therefore, I hypothesized that stimulation through TLR4 in conjunction with FVIII treatment triggers inflammation and drives the increased production of anti-FVIII antibodies.

Specific Aims:

(1) The production of anti-FVIII antibodies and FVIII inhibitors

(2) Effects on antigen presenting cell (APC) populations in the spleen

(3) Effects on inflammatory cytokine (IL-6, TNF-α, and IL-10) secretion

SCIENTIFIC METHODS AND REAGENTS

1. Recombinant Human Factor VIII Preparation and Dialysis

Lyophilized rhFVIII (Kogenate FS, Bayer Heathcare Pharmaceuticals, Tarrytown, NY) was serially reconstituted, 6-8 vials at a time, by addition of sterile water combined in a final volume of 1.5 mL. The rhFVIII was then dialyzed into solution (10mM Hepes and 150mM NaCl, pH 7.5) using a membrane with 10,000 MWCO (ThermoFisher Scientific, Waltham, MA). The concentration of the dialyzed rhFVIII was determined by Pierce MicroBCA Protein Assay Kit (ThermoFisher Scientific).

2. Mice

FVIII deficient, exon 16 deleted, mice backcrossed onto the C57BL/6 mouse strain were kindly provided by David Lillicrap (Queens University, Ontario, Canada). This mouse model is known for having a robust T_H1 and T_H2 inflammatory cytokine response and produces high antibody titers in response to FVIII replacement therapy[59]. A breeding colony was established and maintained in the Center for Laboratory Animal Medicine and Care facility at the University of Texas Health Science Center-Houston under an Animal Welfare Committee approved protocol. Mice were housed in IVC (Individually Ventilated Cages, Tecniplast, Buguggiate, VA, Italy) under pathogen-free conditions and fed sterile food and water ad libitum.

3. Experimental Treatments

Reagents used in the mouse treatments were rhFVIII, TLR2 agonist (synthetic Pam3CSK4 "PAM", InvivoGen, San Diego, CA), and/or TLR4 agonist (Lipopolysaccharides "LPS" from *E.coli* O111:B4, Sigma, St. Louis, MO). At 6-8 weeks, mice were given treatment intravenously based on Table 4. Mice received treatment once every seven days for up to four weeks. If mice received a dual treatment of TLR agonist and rhFVIII, the two compounds were dissolved together in 100 μL of PBS and given as a single intravenous dose *via* tail vein.

Treatment Group	Administered Treatment (100 μL total volume)
1 (control)	100 uL of phosphate-buffered saline (PBS)
2	2 μg/mouse (10 U) rhFVIII in PBS
3	10 μg/mouse PAM in PBS
4	10 μg/mouse LPS in PBS
5	10 μg/mouse PAM + 2 μg/mouse rhFVIII in PBS
6	10 μg/mouse LPS + 2 μg/mouse rhFVIII in PBS

Table 4: Mouse experimental treatments

Mice were euthanized at indicated times and the blood and spleen were harvested. Blood samples were also collected at weekly intervals during the treatment process *via* tail snips. Blood samples were collected in 10% sodium citrate/blood volume (Becton-Dickinson, Franklin Lakes, NJ), centrifuged at 1400 rpm for 20 minutes, and stored at -80^{o}C. Fresh splenocytes were isolated by mechanical dissociation, water lysis to remove red blood cells, and filtration through a μM screen. Splenocytes were counted on a hemocytometer using 0.4% Trypan Blue (Amresco, Solon, OH).

4. Culture Media

Splenocytes were cultured in RPMI-1640 media containing L-glutamine, HEPES, sodium pyruvate, and glucose (American Type Culture Collection, Manassas, VA) to which 50 μM 2-Mercaptoethanol (Sigma), 1.6 mM L-glutamine, 100 U and 100 μg/mL penicillin/steptomycin (all from Gibco, Grand Island, NY) was also added. Peritoneal macrophages were cultured in DMEM media containing glucose, L-glutamine, and sodium pyruvate (Dulbecco's Modification on Eagle's Medium, Mediatech, Herndon, VA) to which 100 U and 100 μg/mL penicillin/streptomycin and 10 mM L-glutamine was also added. Fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) was heat-inactivated at 56^{o}C for 30 minutes.

5. Cell Cultures

Splenocytes were cultured in RPMI-1640 media with 10% FBS at a concentration of 1x10^{6} cells/mL. Cultures were re-stimulated based on Table 5. Cultures were incubated for 24 hours at 37^{o}C with 5% CO_2 saturation. After incubation, culture supernatants were harvested and stored at -80^{o}C.

Treatment Group	Administered Treatment (in 1mL cultures)
1 (control)	media only
2	0.5 μg rhFVIII
3	increasing concentrations of PAM (0-4.0 μg/mL)
4	increasing concentrations of LPS (0-4.0 μg/mL)
5	increasing concentrations of PAM+0.5 μg rhFVIII
6	increasing concentrations of LPS+0.5 μg rhFVIII

Table 5: Splenocyte culture re-stimulation treatments

6. Cytokine Enzyme-Linked Immunosorbent Assay (ELISA)

Cell culture supernatants were tested for IL-6, TNF-α, IL-10, and IL-12p70 using commercially available DuoSet ELISA detection kits (R&D, Minneapolis, MN). The plates were developed with either 3,3',5,5'-tetramethylbenzidine (TMB) solution, included in the DuoSet kit,

or OPD solution containing 0.2 M Na_2HPO_4, 0.1 M citric acid, OPD (*o*-phenylenediamine, Sigma) and 0.05% H_2O_2 (Sigma). The substrate was allowed to react at room temperature in the dark for 20 minutes. The reaction was stopped by the addition of 2 N H_2SO_4 and absorbance was determined at 450 nm on a Bio-Rad 3550 Plate Reader (Bio-Rad, Hercules, CA). Unknowns were determined from a standard curve using GraphPad Prism5 (GraphPad Software, La Jolla, CA).

7. Anti-FVIII IgG Enzyme-Linked Immunosorbent Assay (ELISA)

Flat-bottom, medium-binding, Microlon 96-well ELISA plates (Greiner Bio-One, Monroe, NC) were coated with 1 μg/mL of rhFVIII dissolved in 100 mM $NaHCO_3$, pH 9.5 and incubated at 37°C for 1 hour. The plate was then blocked with 5% skim milk dissolved in 0.05% PBS/T (1X PBS mixed with Tween-20 (Sigma) at 37°C for 1 hour. The mouse plasma samples were then serially diluted 1:2, starting at 2^4 dilution, in 1% skim milk dissolved in 0.05% PBS/T and incubated at 37°C for 2 hours. The plate was then incubated with a 1:1000 dilution of goat anti-mouse-horseradish peroxidase (HRP) (Sigma) and incubated at 37°C for 1 hour. The HRP was detected by addition of OPD substrate solution and 0.05% H_2O_2 and allowed to react at room temperature in the dark for 20 minutes. The reaction was stopped by the addition of 2N H_2SO_4 and absorbance was determined at 490 nm on a Bio-Rad 3550 Plate Reader. Results were expressed as the lowest dilution above the endpoint titer of 0.200 OD over the background.

8. Flow Cytometry

Antibodies used to mark cells for flow cytometry were anti-mouse CD11b-PerCP Cy5.5 (clone M1/70), anti-mouse CD11c-PerCP Cy5.5 (clone N418), anti-mouse CD19-PerCP Cy5.5 (clone eBio1D3), anti-mouse TLR2/CD282-FITC (clone 6C2, all from eBioscience), rat IgG_{2b} κ-FITC isotype control (eB149/10HS, all from eBioscience), monoclonal anti-rat TLR4-PE (clone 267518, R&D Systems), and rat IgG_{2a}-PE isotype control (clone eBR2a, eBioscience).

Splenocytes were re-suspended in 1mL of RPMI-1640 media. The cells were blocked with anti-mouse CD16/32 Fc block (clone 93, eBioscience, San Diego, CA) for 30 minutes at 4°C. The cells were then washed 1 mL of flow buffer (1% FBS in PBS) and centrifuged at 2000 rpm for 5 minutes. The splenocytes were then incubated for 30 minutes at 4°C with 0.5 ug/mL of the appropriate antibodies based on Table 6. The cells were washed, centrifuged, and re-suspended in 1 mL of 50% fixation buffer (4% paraformaldehyde in PBS, Sigma) and flow buffer and stored at 4°C. At the time of analysis, the cells were centrifuged and re-suspended in 500 μL of fresh flow buffer. The cells were analyzed on a BD FACSCalibur (Becton-Dickinson) using CellQuest Pro Software (Becton-Dickinson). 50,000 events per tube were collected.

Staining Group	Flow Cytometry Staining Protocol
1	CD11b-PerCP Cy5.5, TLR2-FITC, TLR4-PE
2	CD11b-PerCP Cy5.5, TLR2-FITC isotype, TLR4-PE isotype
3	CD11c-PerCP Cy5.5, TLR2-FITC, TLR4-PE
4	CD11c-PerCP Cy5.5, TLR2-FITC isotype, TLR4-PE isotype
5	CD19-PerCP Cy5.5, TLR2-FITC, TLR4-PE
6	CD19-PerCP Cy5.5, TLR2-FITC isotype, TLR4-PE isotype

Table 6: Flow cytometry antibody staining protocol

9. Bethesda Assays

FVIII:Coagulant inhibitor levels were measured using a modified Bethesda method[60]. Mouse plasma serially diluted in Owren's veronal buffer (Seimens, Marburg, Germany) was mixed with an equal volume of normal human pooled plasma (George-King Bio-Medical, Overland Park, KS). For the control mixture, normal human pooled plasma was mixed with an equal volume of Owren's veronal buffer. Both mixtures were incubated at 37^{o}C for 2 hours.

The remaining FVIII:C activity in the test and control mixtures was determined using a one-stage clotting assay (ACL 300 Beckman Coulter, Lexington, MA) with reagents from the manufacturer with the exception of FVIII-deficient plasma (George-King Bio-Medical, Overland Park, KS). The residual FVIII activity in the test mixture was determined as a percentage of the activity present in the control mixture and the inhibitor activity of the test mixture. Activity was then calculated using a linear regression of inhibitor titer versus log of residual activity. One Bethesda unit is defined as the amount of inhibitor that reduces the FVIII:C activity to 50% after two hours of incubation at 37^{o}C.

10. TLR4 Competition Assay

Peritoneal macrophages were isolated from naïve FVIII deficient C57BL/6 mice[61]. The peritoneum was exposed and cold Dulbecco's phosphate-buffered saline (dPBS) without calcium and magnesium was injected into the peritoneal cavity, massaged, and then extracted. The fluid was centrifuged at 1000 rpm for 10 minutes at 4^{o}C at 400 x *g*. The macrophages were counted using 0.4% Trypan Blue and then cultured in DMEM media at 1-3x10^6 cells/mL. To upregulate TLR4 expression, the culture was stimulated with 1μg/mL of LPS for 24 hours at 37^{o}C with 5% CO_2 saturation. After incubation, the cells were harvested, centrifuged at 2000 rpm for 5 minutes, and re-suspended in fresh medium. The cells were then blocked with anti-mouse CD16/32 Fc block for 30 minutes at 4^{o}C. Next, the cells were incubated with media only, 5 μg/mL rhFVIII, or 5 μg/mL BSA (Bovine Serum Albumin, Sigma) for 30 minutes at 4^{o}C. The cells were then washed with 1mL of flow buffer and centrifuged. The cells were then incubated with 0.5 ug/mL

anti-mouse TLR2/CD282-FITC, monoclonal anti-rat TLR4 or polyclonal rabbit anti-mouse TLR4 followed by goat anti-rabbit IgG-FITC (both from Abcam, Cambridge, MA) for 30 minutes at 4^{o}C. The cells were then washed, centrifuged, and re-suspended in 1 mL of 50% fixation buffer and flow buffer and stored at 4^{o}C. At the time of analysis, the cells were centrifuged and re-suspended in 500 μL of fresh flow buffer. The cells were analyzed on a BD FACSCalibur using CellQuest Pro Software. 50,000 events per tube were collected.

11. Endotoxin Assay

Common laboratory solutions (sterile water, media, flow buffer, reagent diluent, PBS/T, PBS, and FVIII dialysis buffer) were tested for trace levels of endotoxin using a commercially available ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GeneScript, Piscataway, NJ). Absorbance was determined at 545 nm on a Molecular Devices SPECTRAmax250 Microplate Reader (GMI, Ramsey, CA). Unknowns were determined from a standard curve using GraphPad Prism5.

STIMULATION OF TLR4 INCREASES FVIII INHIBITOR PRODUCTION

I hypothesized that stimulation of TLR4 in conjunction with FVIII treatment would drive the increased production of anti-FVIII antibodies. To test this hypothesis, I i.v. injected FVIII-deficient mice once a week for four weeks with PBS, FVIII, TLR4 agonist (LPS), or LPS+FVIII. It was important to utilize several different methods to accurately assess the presence and magnitude of anti-FVIII antibodies present in the serum samples because anti-FVIII antibodies have been identified as being directed towards both functional and non-functional domains of the FVIII protein[34]. Therefore, blood samples were collected every seven days post-primary injection and analyzed using (1) enzyme-linked immunosorbant assay (ELISA) for the presence of total (functional and non-functional) anti-FVIII IgG antibodies, and (2) Bethesda assay for the presence of only functionally inhibitory anti-FVIII IgG antibodies (FVIII inhibitors). ELISA results were reported as FVIII specific IgG antibody end titer, the lowest dilution of the plasma in which antibodies are detectable above the standardized clinical threshold of 0.200 OD over the background. Bethesda results were reported as Bethesda Units (B.U.) where one B.U. is the amount of inhibitor that reduces the FVIII:C activity by 50% after two hours of 37^{o}C incubation.

To first confirm the kinetics and magnitude of the antibody response to therapeutic treatment, mice injected with only FVIII were tested for anti-FVIII antibodies over the course of the injections. End titer levels of approximately 2^{7} were detected at day 21 post-primary injection (Fig.11). Antibody levels continued to increase as FVIII injections continued, with a maximum end titer level of 2^{11} at day 28 post-primary injection. Similarly, FVIII inhibitor levels of approximately 20 B.U. were also detectable by day 21 post-primary injection (Fig.12). Inhibitor levels also increased significantly as FVIII treatments continued, with a maximum level of 330 B.U. at day 28 post-primary injection ($p<0.05$). This is the normal course of anti-FVIII antibody and FVIII inhibitor development in hemophilic mice that have no central tolerance to FVIII[59]. It should be noted that control mice injected with PBS or LPS never developed anti-FVIII IgG antibodies as there was no FVIII present in the blood. Since the production of total anti-FVIII IgG antibodies and functional anti-FVIII antibodies (inhibitors) have a direct correlation[62], the control mice were not tested for inhibitors.

To determine if TLR4 stimulation during FVIII treatment would increase the production of anti-FVIII antibodies, I next measured the kinetics and magnitude of the antibody response in mice injected with LPS+FVIII over the course of time. End titer levels were approximately 2^{14} at day 21 and 2^{15} at day 28 post-primary injection, which was significantly increased ($p<0.001$) compared to mice that received FVIII alone (Fig.11). At the same time, anti-FVIII IgG antibody levels were also detectable earlier during the course of treatment with end titer levels of

approximately 2^7 at day 14 post-primary injection. Similarly, inhibitor levels were approximately 270 B.U. at day 21 (*p<0.01*) and 1075 B.U. at day 28 post-primary injection (*p=0.0135*), which was also significantly increased compared to mice that received FVIII alone (Fig.12). These data indicated that, as hypothesized, TLR4 stimulation significantly increased FVIII inhibitor production.

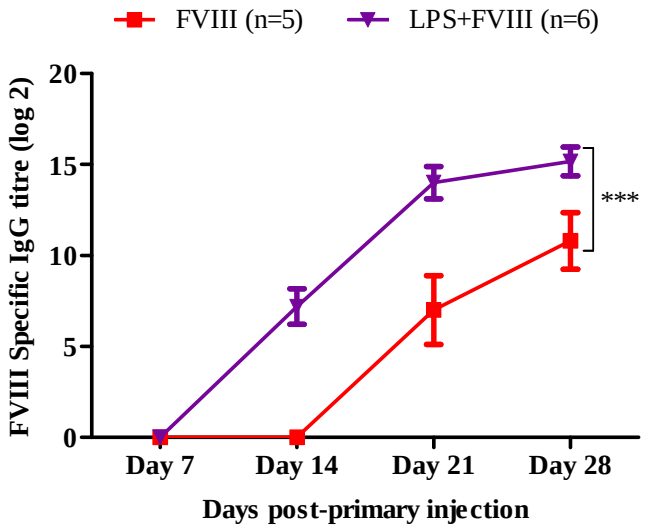


Fig.11: Repeated *in vivo* LPS and FVIII stimulation increases the production of anti-FVIII IgG antibodies. Antibody titers were measured from citrated plasma using a modified ELISA. Significance determine by 2-way ANOVA and unpaired t-test with Welch's correction where *p<0.05, ***p<0.001

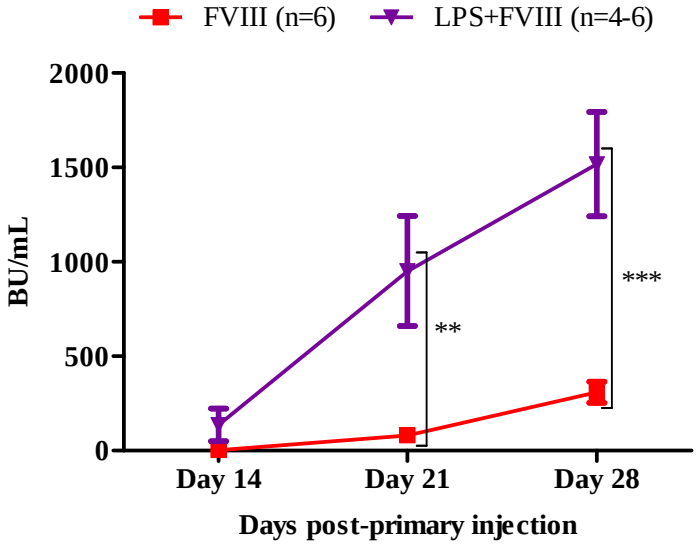


Fig.12: Repeated *in vivo* LPS and FVIII stimulation increases the production of FVIII inhibitors. Inhibitors were measured from citrated plasma using a modified Bethesda assay. Significance determine by 2-way ANOVA and unpaired t-test with Welch's correction where *p<0.05, **p<0.01, ***p<0.001

STIMULATION OF TLR4 INCREASES PERCENTAGE OF APCS EXPRESSING TLR4 BUT DECREASES TLR4 DENSITY ON APCs

I hypothesized that stimulation of TLR4 in conjunction with FVIII injections would increase the formation of FVIII inhibitors due to increased expression of TLR4 on the surface of antigen presenting cells (APCs). In order to test this hypothesis, I i.v injected FVIII-deficient mice once a week for four weeks with FVIII, TLR4 agonist (LPS), or LPS+FVIII. Splenocytes were harvested every seven days post-primary injection and stained for flow cytometry analysis as previously described in Chapter IV. The cell plots were gated in order to observe live splenocyte populations (Fig.13). The resultant flow plots (Fig.14) then underwent quadrant analysis (Fig.15) to determine if the different in vivo injections over time changed the relative percentage of APCs in the spleen and the expression of TLR4 on those splenic APCs

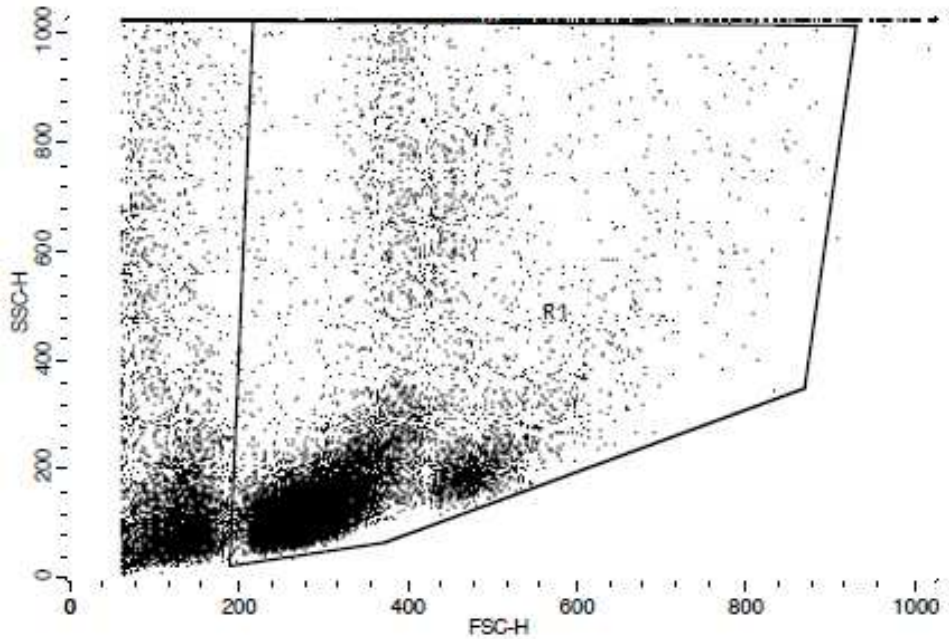


Fig.13: Splenocyte population analyzed in flow cytometry experiments.
Splenocytes were analyzed using Forward Scatter (FSC) to identify relative cell size and Side Scatter (SSC) to identify relative cell complexity. The depicted gate (R1) identifies the spleen cell population, consisting of lymphocytes and granulocytes, later utilized in TLR4/APC analysis.

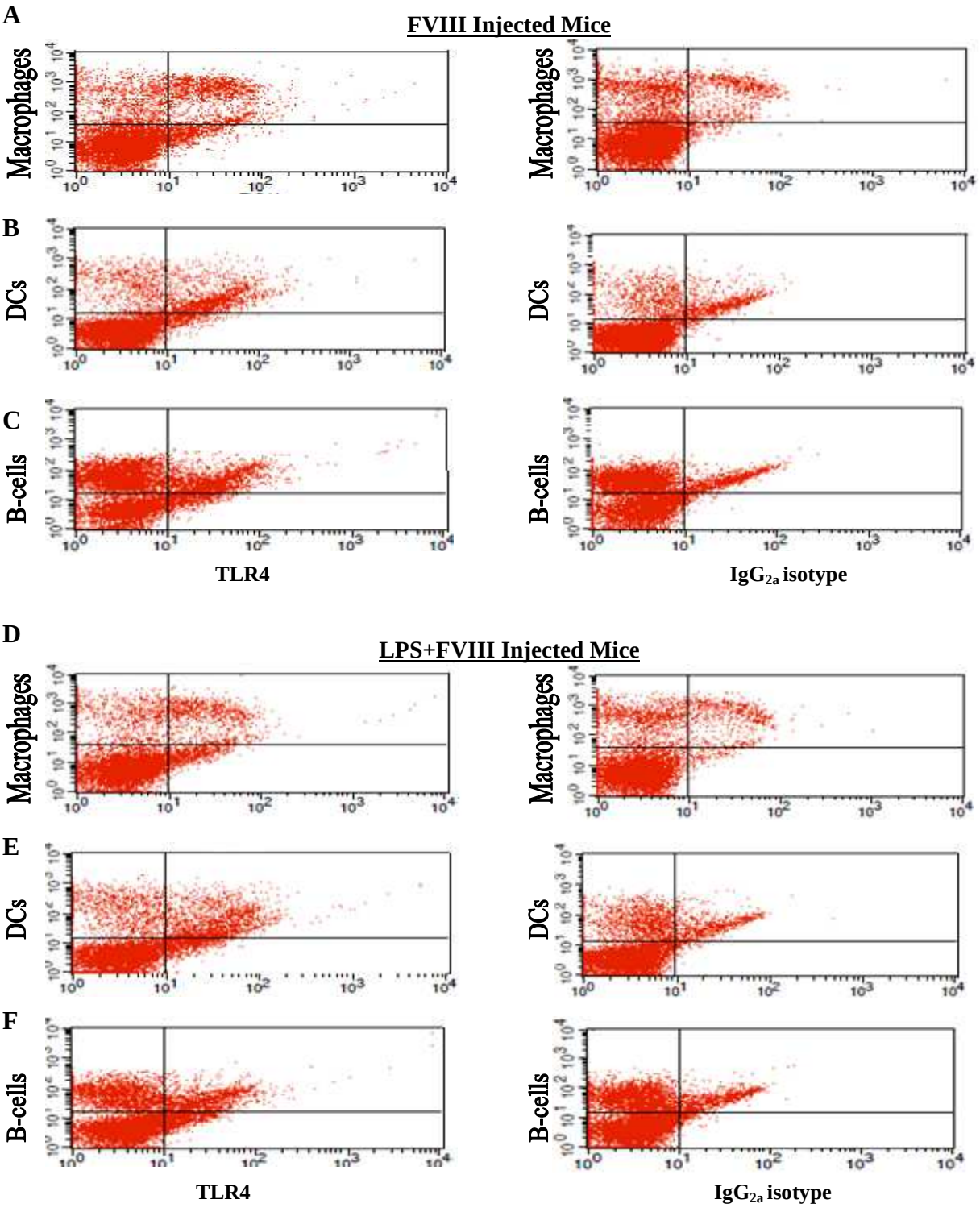
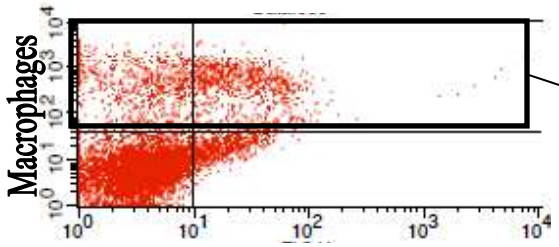


Fig.14: Analysis of splenocyte populations for APC and TLR4 expression.
Splenocytes, from (A-C) FVIII and (D-F) LPS+FVIII injected mice 28 days post-primary injection, incubated with fluorescently-labeled antibodies specific for **(A,D) CD11b+ macrophages, (B,E) CD11c+ DCs, or (C,F) CD19+ B-cells** together with antibodies specific for TLR4 or IgG_{2a} isotype control. Antibodies detected *via* flow cytometry and analyzed based on fluorescence.



% cells in R1

fluorescence intensity

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	742	7.09	4.50	1.45	1144.90
UR	2250	21.51	13.65	42.31	393.25
LL	2043	19.53	12.39	2.02	7.08
LR	5425	51.86	32.91	9.55	11.39

Fig.15: Quadrant analysis of gated splenocyte populations.
Quadrants drawn onto the flow cytometry plots represent cell populations that are positive for CD11b macrophages and TLR4 antibodies. Statistical analysis of the quadrants indicated the relative percentage and density of cells binding only APC antibodies or both APC and TLR4 antibodies.

First, I wanted to determine if stimulation through TLR4 would significantly increase the relative percentage of macrophages, DCs, and B-cells in the spleen in comparison to *in vivo* FVIII stimulation over the course of four injections. Results indicated that repeated LPS stimulation with or without concurrent FVIII injections significantly increased the relative percentage of macrophages ($p<0.001$) and DCs in the spleen ($p<0.001$) (Fig.16A-B). Repeated stimulation through TLR4 did not, however, increase the percentage of B-cells but instead significantly decreased ($p<0.05$) the percentage of B-cells detected in the spleen after only one injection. This lower percentage of B-cells remained constant over the course of the LPS injections. Interestingly, while initially spleens from FVIII injected mice consisted of 55% B-cells, the percentage of B-cells steadily decreased over the course of the FVIII injections (Fig.16C).

While stimulation through TLR4 increased in the relative percentage of macrophages and DCs in the spleen, it did not explain the relationship between TLR4 stimulation and the formation of FVIII antibodies. Therefore, I wanted to determine if stimulation through TLR4 would significantly increase the percentage of APCs expressing TLR4 and, since the level of TLR4 expression can change, I also wanted to determine if stimulation through TLR4 would significantly change the density, as measured by fluorescence intensity, of TLR4 expressed on APC surfaces in the spleen in comparison to *in vivo* FVIII stimulation over the course of four injections. Results indicated that, compared to *in vivo* FVIII stimulation, repeated stimulation through TLR4 increased the percentage of macrophages ($p<0.05$), DCs, and B-cells expressing TLR4 in the spleen (Fig.17A-C). However, TLR4 stimulation decreased the density of TLR4 expressed on the surface of all APCs ($p<0.05$) (Fig.17D-F). Concurrent stimulation with LPS+FVIII had the same effect as LPS stimulation on the percentage of APCs expressing TLR4 in the spleen and the density of TLR4 expressed on the APCs.

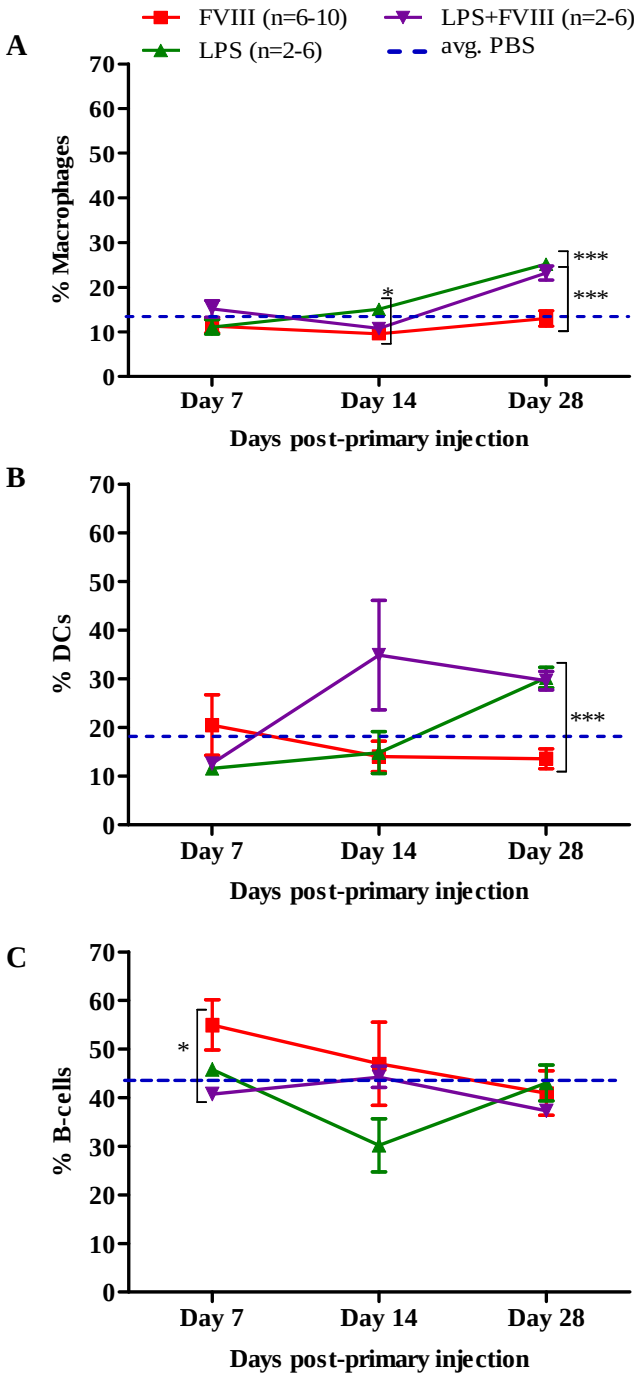


Fig.16: Repeated stimulation through TLR4 increases percentage of macrophages and DCs in the spleen compared to mice responding to FVIII alone. The percentage of total APCs was calculated by addition of the percentage of gated cells from quadrants with positive APC staining. (**A) CD11b+ macrophages, (B) CD11c+ DCs, or (C) CD19+ B-cells**. Avg. PBS depicts the average percentage of APCs isolated from untreated control mice. Significance determined by unpaired t-test with Welch's correction where *p<0.05, ***p<0.001

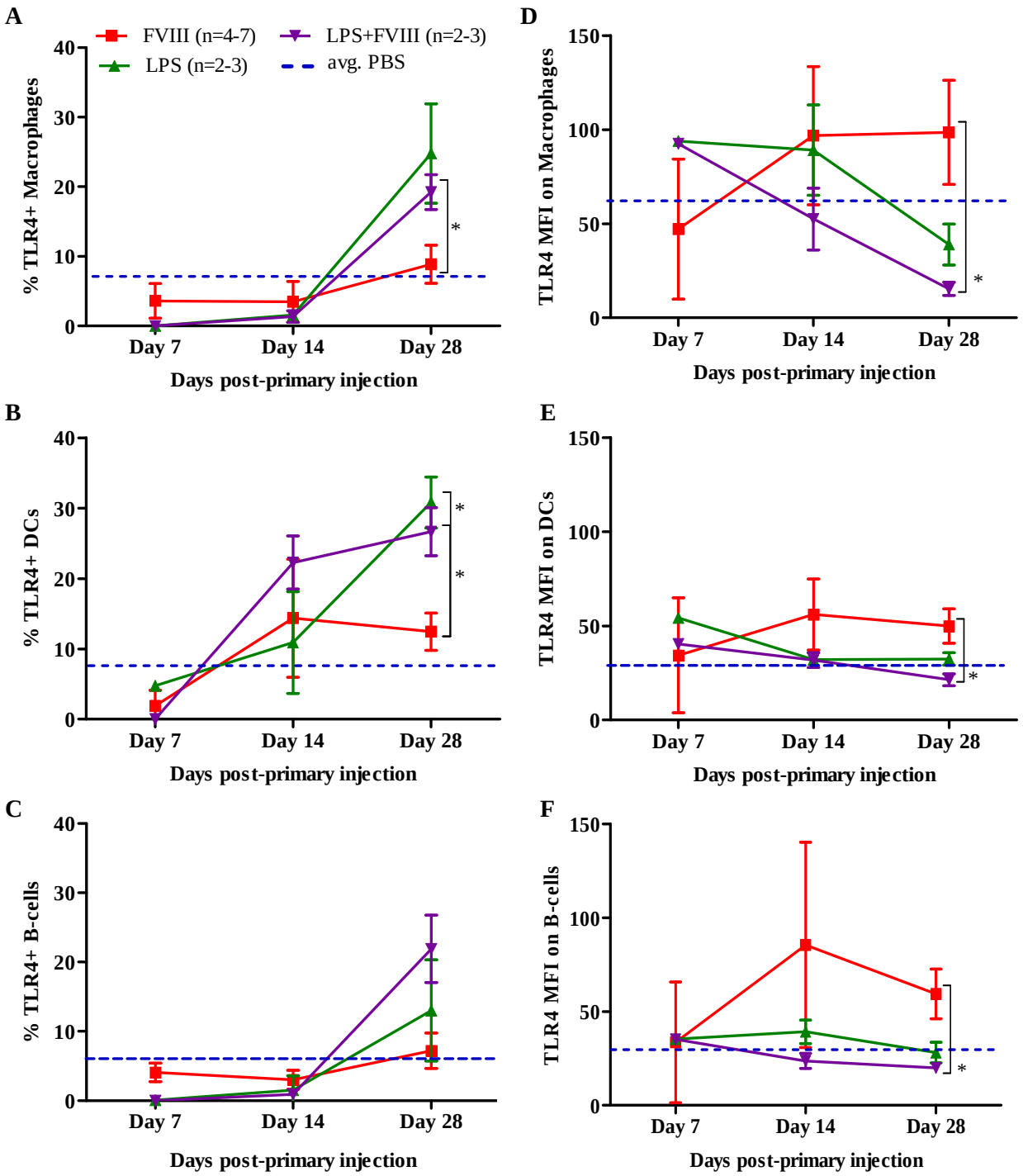


Fig.17: Repeated stimulation through TLR4 increases percentage of APCs expressing TLR4 but decreases density of TLR4. (A-C)The percentage of APCs expressing TLR4 was calculated from the change in APC+/TLR+ cells over total APC+ cells. (D-F)The mean fluorescence intensity (MFI) of APCs expressing TLR4 was calculated from the difference of TLR4+ MFI compared to isotype MFI. **(A,D) CD11b+ macrophages, (B,E) CD11c+ DCs, and (C,F) CD19+ B-cells.** Avg. PBS depicts the average percentage or MFI of APCs isolated from untreated control mice. Significance determined by unpaired t-test with Welch's correction where *p<0.05

When viewed together (Fig.18), these results indicated that, in comparison to in vivo FVIII stimulation, early stimulation through TLR4 only affected the relative percentage of B-cells in the spleen. The relative percentages of macrophages and DCs, as well as the expression of TLR4 on the APCs in the spleen, were unchanged. Over the course of the injections, the repeated stimulation of TLR4 increased not only the relative percentage of macrophages and DCs in the spleen but also the percentage of all APCs expressing TLR4 in the spleen. This was an indication that stimulation through TLR4 over time triggered the activation and proliferation of macrophages and DCs in the spleen and the up-regulation of TLR4. While repeated FVIII stimulation did not change the relative percentage of APCs in the spleen, it instead resulted in increased density of TLR4 expressed on specific populations of each APC. This was an indication that FVIII stimulation over time did not trigger APC proliferation but instead triggered the activation of a specific subset of APCs capable of increased TLR4 presentation.

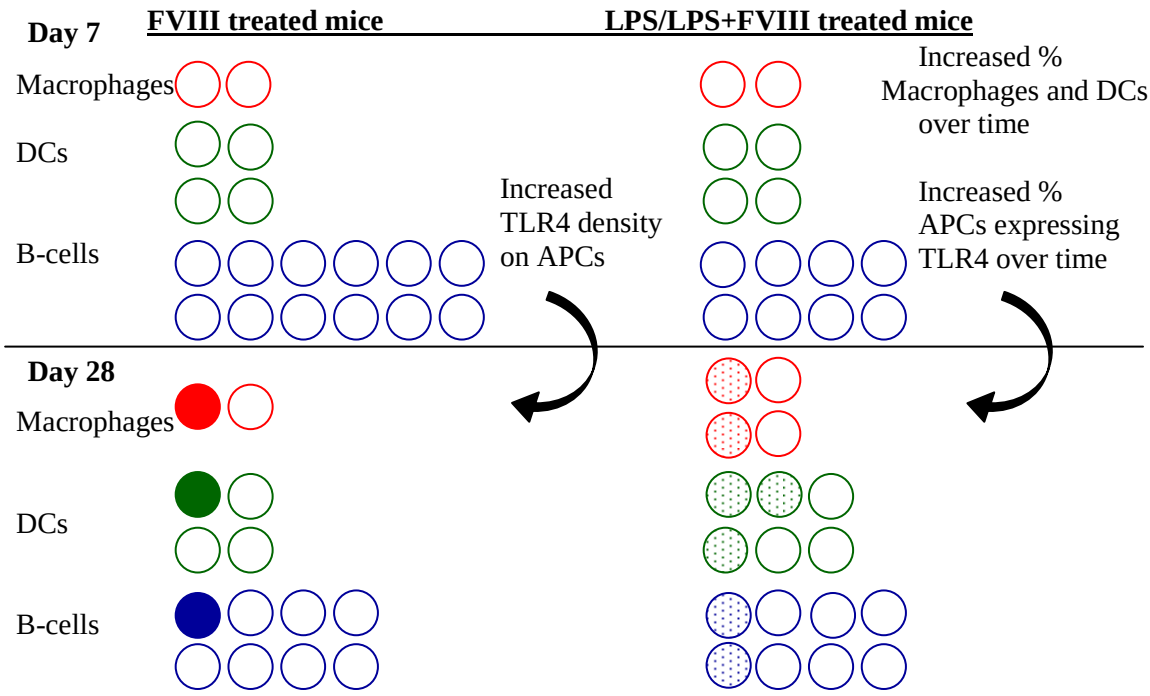


Fig.18: *In vivo* LPS stimulation increases percentage of macrophages and DCs in the spleen while *in vivo* FVIII stimulation increases density of TLR4 expression on APCs. Empty circles represent cell populations (red=macrophages, green=DCs, blue=B-cells)**,** filled circles represent APCs expressing TLR4 where "patterned" circles are APCs that have normal TLR4 expression and "solid" circles are APCs that have highly dense TLR4 expression.

STIMULATION OF TLR4 INCREASES EARLY INFLAMMATORY CYTOKINE SECRETION

It has been shown that LPS stimulates TLR4 triggering enhanced TNF-α and IL-6 secretion during an inflammatory response; IL-10 is secreted to regulate the production of TNF-α and prevent sepsis[51, 56]. I hypothesized that stimulation of TLR4 in conjunction with FVIII injections would increase the formation of FVIII inhibitors by triggering the secretion of TNF-α, IL-6 and IL-10. In order to test my hypothesis, I i.v. injected FVIII deficient mice once a week for four weeks with FVIII, TLR4 agonist (LPS), or LPS+FVIII. Splenocytes were harvested every seven days post-primary injection, cultured, and the secretion of IL-6, IL-10, and TNF-α was measured by ELISA as previously described in Chapter IV. I analyzed the change in inflammatory cytokine secretion from mice injected with LPS with or without concurrent FVIII compared to the level of cytokines secreted by mice injected with FVIII.

First, I wanted to determine the effects of *in vivo* TLR4 stimulation on the secretion of IL-6, IL-10, and TNF-α. In comparison to *in vivo* FVIII injections, injections of LPS with or without concurrent FVIII injections significantly increased early IL-6 ($p<0.001$), IL-10 ($p<0.05$-0.001), and TNF-α ($p<0.05$) secretion (Fig.19). All cytokine secretion decreased significantly ($p<0.01$-0.001) over the course of the remaining injections. IL-6 secretion remained low despite repeated TLR4 stimulation while secretion of IL-10 and TNF-α increased again by day 28. Interestingly, repeated injections of FVIII increased the secretion of IL-6, though not significantly, by day 28 indicating the beginning of a FVIII-specific response (Fig.19A). However, there was no indication of a FVIII-specific response in the secretion of IL-10 or TNF-α (Fig.19B-C).

Next, I analyzed the effect of FVIII re-stimulation in culture on IL-6, IL-10, and TNF-α secretion in order to determine if FVIII could generate a continued inflammatory response after repeated *in vivo* agonist stimulation. When re-stimulated with FVIII in culture, trends similar to those previously seen in un-stimulated cultures (Fig.19) were observed. *In vivo* injections of LPS again significantly increased early IL-6 ($p<0.001$), IL-10 ($p<0.01$), and TNF-α secretion (Fig.20). The secretion of all three cytokines ($p<0.01$-0.001) synergistically increased after *in vivo* injections of LPS+FVIII. Again all cytokine secretion decreased significantly ($p<0.01$-0.001) over the course of the remaining injections. Unlike un-stimulated cultures, FVIII re-stimulation induced increased secretion of IL-6 ($p<0.001$) and TNF-α by day 28 (Fig.20A,C). The secretion of IL-6 and TNF-α after LPS stimulation did not increase at this time indicating a FVIII-specific response in the secretion of IL-6 and TNF-α.

A comparison of cytokine secretion after in vitro FVIII re-stimulation (Fig.20) relative to cytokine secretion without any re-stimulation (Fig.19) indicated that there was relatively no

difference in IL-6 or IL-10 secretion between the different treatment groups during the early inflammatory response after FVIII re-stimulation (Fig.20D-E). By 28 days post-primary injection, IL-6 secretion increased 2-fold (*p<0.01*) from splenocytes of mice injected with FVIII, indicating the presence of a long-term FVIII recall response (Fig.20D). TNF-α secretion presented the opposite trend in that secretion increased 2-fold (*p<0.05*) during the early (day 7) inflammatory response splenocytes of mice injected with FVIII and re-stimulated with FVIII in culture (Fig.20F).

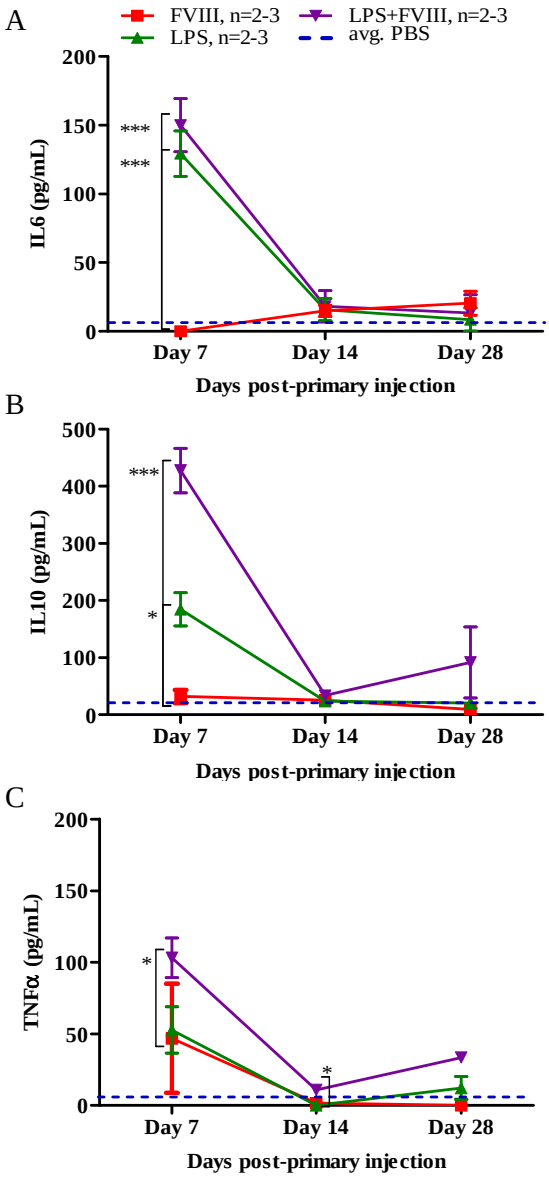


Fig.19: *In vivo* TLR4 stimulation increases early inflammatory cytokine secretion. Splenocytes were isolated every 7 days post-primary injection and cultured for 24hrs. without additional re-stimulation. The culture supernatants were collected and measured *via* ELISA for **(A) IL-6, (B) IL-10, and (C) TNF-α**. Avg. PBS depicts the average cytokine detected from untreated control mice. Significance determined by unpaired t-test with Welch's correction where *p<0.05, ***p<0.001

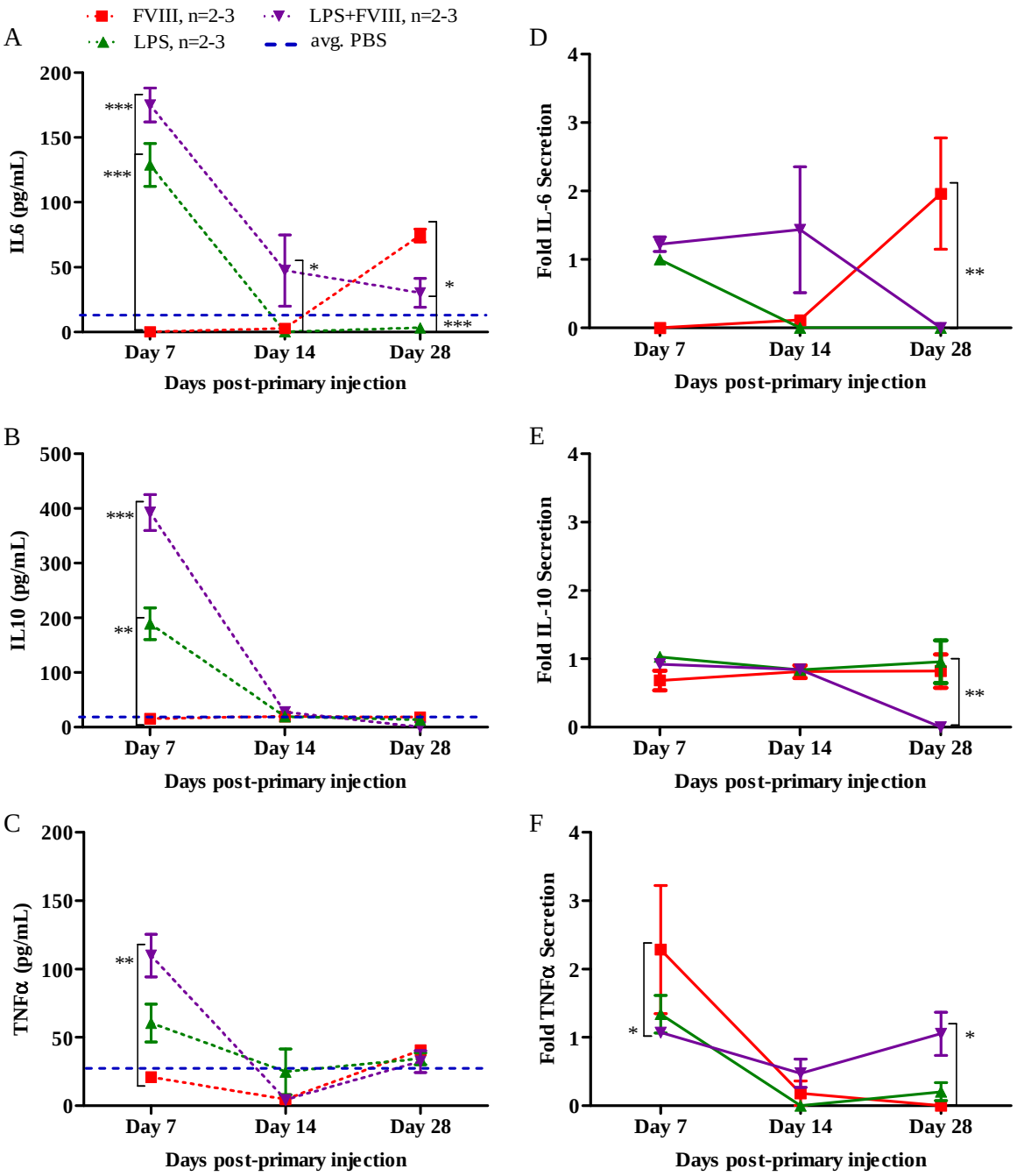


Fig.20: *In vivo* TLR4 stimulation synergistically increases early inflammatory cytokine secretion in response to FVIII re-stimulation in culture. Splenocytes isolated every 7 days post-primary injection and re-stimulated in culture for 24hrs. with 0.5ug FVIII. (A-C) Culture supernatants collected and measured *via* ELISA for cytokine secretion. Avg. PBS depicts the average cytokine detected from untreated control mice. (D-F) Ratio comparison of cytokine secretion from splenocyte cultures re-stimulated with 0.5ug FVIII over cultures that were not re-stimulated. Values >1 indicate increased cytokine secretion from re-stimulated cultures. **(A,D) IL-6, (B,E) IL-10, and (C,F) TNF-α.** Significance determined by unpaired t-test with Welch's correction where *p<0.05, **p<0.01, ***p<0.001

Since FVIII stimulation alone was not inducing discernable differences in cytokine secretion, I then analyzed the effect of LPS re-stimulation on IL-6, IL-10, and TNF-α secretion in order to determine if cells were capable of responding to additional inflammatory stimulus in culture.

Previous studies have shown that stimulation through all TLRs can to some degree modulate both the re-stimulation and inhibition of FVIII-specific memory B-cells. While those studies were focused specifically on the adaptive immune response, it has been postulated that this same effect will also be observed on APCs during the inflammatory response[63]. When re-stimulated with LPS in culture, the kinetics of the inflammatory cytokine secretion changed compared to cultures that were un-stimulated or re-stimulated with FVIII alone. Compared to *in vivo* FVIII injections, *in vivo* injections of LPS+FVIII significantly increased ($p<0.01$) IL-6 and TNF-α secretion in response to LPS by day 14 post-primary injection (Fig.21A,C). However, this increased cytokine secretion was not maintained throughout the remainder of the injections, resulting in a significant decrease ($p<0.01$) in IL-6 and TNF-α secretion by day 28. Levels of IL-6 increased significantly ($p<0.001$) in mice injected with LPS alone, compared to mice injected with FVIII, and continued to increase significantly in a linear manner over the course of the injections so that, by day 28, the IL-6 secretion was significantly increased ($p<0.05$) compared to all other treatment groups (Fig.21A). Therefore, the decrease in IL-6 secretion from mice injected with LPS+FVIII was caused by the presence of FVIII as the secretion of IL-6 after *in vivo* LPS stimulation was not yet exhausted. The kinetics of IL-10 secretion also changed after LPS re-stimulation in culture but in a different manner than previously seen with IL-6 or TNF-α. IL-10 secretion was significantly increased ($p<0.05$) in mice injected with LPS, compared to mice injected with FVIII alone, and this level of secretion was maintained over the course of the injections. IL-10 was significantly increased ($p<0.001$) in mice injected with LPS +FVIII, compared to mice injected with FVIII, and continued to decrease significantly ($p<0.05$) in a linear manner over the course of the injections (Fig.21B). These results indicated that, unlike IL-6 and TNF-α, IL-10 secretion could not be extended as the result of re-stimulation.

A comparison of cytokine secretion after in vitro LPS re-stimulation (Fig.21) relative to cytokine secretion without any re-stimulation (Fig. 19) indicated that there was significantly increased secretion of IL-6, IL-10, and TNF-α after re-stimulation in culture (Fig21D-F). After LPS re-stimulation, IL-10 secretion was increased 8-fold for all mouse treatment groups and remained relatively consistent over time (Fig.21E). IL-6 secretion peaked for all mouse treatment groups by 14 days post-primary injection. Splenocytes from LPS+FVIII treated mice that were re-stimulated in culture secreted 7-fold increased IL-6 while splenocytes from FVIII treated mice that were re-stimulated in culture secreted significantly increased levels of IL-6 (34-fold, $p<0.05$)

compared to splenocytes that were not re-stimulated in culture. IL-6 secretion decreased in all mouse treatment groups by 28 days post-primary injection (Fig.21D). Similarly, TNF-α secretion also peaked for all mouse treatment groups by 14 days post-primary injection. For this cytokine, however, splenocytes from LPS+FVIII treated mice that were re-stimulated in culture secreted 17-fold increased TNF-α which was significantly increased ($p<0.001$) compared to splenocytes from FVIII treated mice that were re-stimulated in culture that secreted 4-fold increased TNF-α, which remained relatively consistent over time (Fig.21F). Again TNF-α secretion decreased in LPS+FVIII treated mice by 28 days post-primary injection.

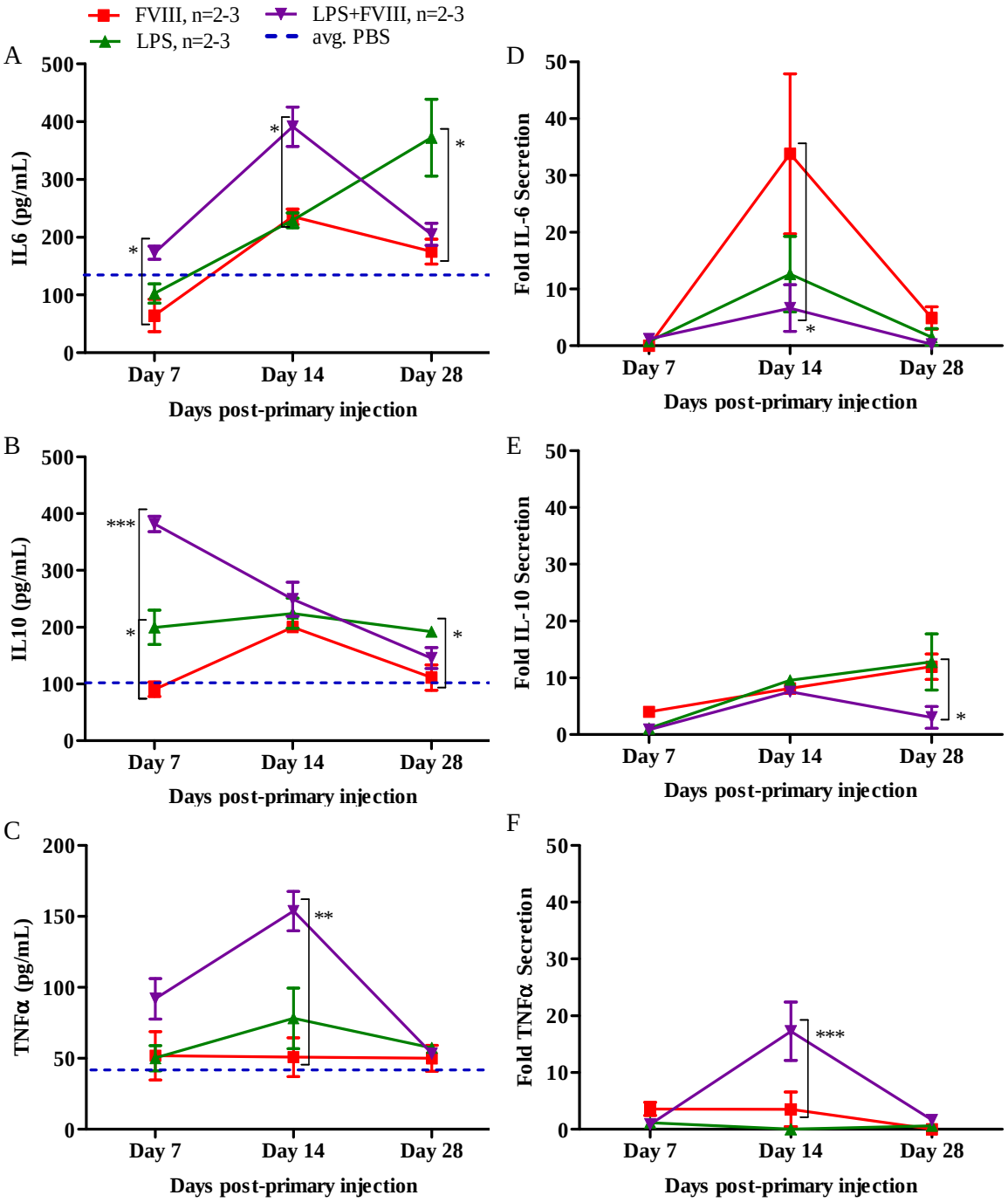


Fig.21: *In vivo* TLR4 stimulation changes the kinetics of inflammatory cytokine secretion in response to *in vitro* TLR4 re-stimulation. Splenocytes isolated every 7 days post-primary injection and re-stimulated in culture for 24hrs. with 1.0 ug LPS. (A-C) Culture supernatants collected and measured *via* ELISA for cytokine secretion. Avg. PBS depicts the average cytokine detected from untreated control mice. (D-F) Ratio comparison of cytokine secretion from splenocyte cultures re-stimulated with 1.0 ug FVIII over cultures that were not re-stimulated. Values >1 indicate increased cytokine secretion from re-stimulated cultures. **(A,D) IL-6, (B,E) IL-10, and (D,F) TNF-α**. Significance determined by unpaired t-test with Welch's correction where *p<0.05, **p<0.01, ***p<0.001

Lastly, since LPS stimulation changed the kinetics of the inflammatory cytokine secretion, I wanted to analyze the effect of LPS and FVIII re-stimulation together on IL-6, IL-10, and TNF-α secretion in order to determine if the co-administration of these agonists also affected the kinetics of cytokine secretion after *in vivo* re-stimulation. When re-stimulated with LPS and FVIII in culture, trends similar to those previously observed in LPS re-stimulated cultures (Fig.21) were observed (Fig.22). Again *in vivo* injections of LPS+FVIII significantly increased ($p<0.01$) IL-6 and TNF-α secretion by day 14 and significantly decreased ($p<0.01$) IL-6 and TNF-α secretion by day 28 (Fig.22A,C). IL-10 secretion was again significantly increased ($p<0.001$) in mice injected with LPS+FVIII, compared to mice injected with FVIII, and continued to decrease significantly ($p<0.05$) in a linear manner over the course of the injections (Fig.22B). Interestingly, it was observed that IL-6 secreted by mice injected with LPS, while still significantly increased ($p<0.05$) in comparison to mice injected with FVIII, was no longer increasing at day 28 as was observed in cultures responding to LPS alone (Fig.21A). Instead, IL-6 secretion was decreased compared to mice injected with LPS+FVIII (Fig.22A). These results suggested some level of stimulatory exhaustion which was only observed in mice injected with LPS but were naïve to FVIII.

A comparison of cytokine secretion after in vitro LPS+FVIII re-stimulation (Fig.22) relative to cytokine secretion after in vitro FVIII re-stimulation (Fig.20) indicated that the additional LPS in culture stimulated similar patterns of cytokine secretion seen after LPS re-stimulation alone; however, levels of all three cytokines were increased significantly (10-fold) after concurrent LPS+FVIII re-stimulation even over the levels of cytokines secreted after FVIII re-stimulation alone (Fig.23A-C). A comparison of cytokine secretion after in vitro LPS+FVIII re-stimulation relative to cytokine secretion after in vitro LPS re-stimulation indicated that the additional FVIII in culture had little/no effect on IL-6, IL-10, or TNF-α secretion between the different treatment groups throughout the inflammatory response (Fig.23D-F). These results indicated that the majority of cytokine was produced in response to LPS alone. Concurrent re-stimulation did have an effect on IL-6 secretion which increased significantly (4-fold, $p<0.01$) during the early inflammatory response from splenocytes of FVIII treated mice that were concurrently re-stimulated in culture. Together, these results indicated that concurrent LPS+FVIII re-stimulation in culture does synergistic affect early inflammatory cytokine secretion, specifically IL-6.

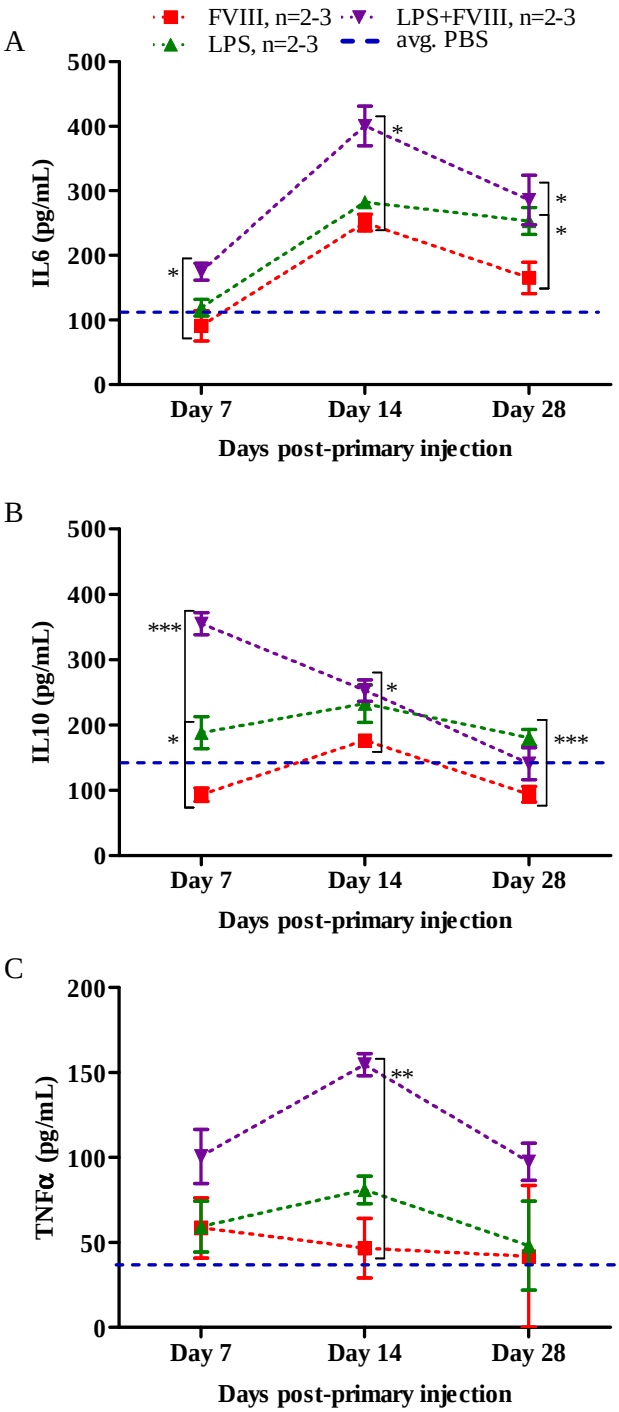


Fig.22: Concurrent LPS+FVIII re-stimulation induced same changes in inflammatory cytokine kinetics as LPS re-stimulation alone. Splenocytes were isolated from mice every 7 days post-primary injection and re-stimulated in culture for 24hrs. with 1.0ug LPS and 0.5ug FVIII. The culture supernatants were collected and measured *via* ELISA for **(A) IL-6, (B) IL-10, and (C) TNF-α**. Avg. PBS depicts the average cytokine detected from untreated control mice. Significance determined by unpaired t-test with Welch's correction where *p<0.05, **p<0.01, ***p<0.001

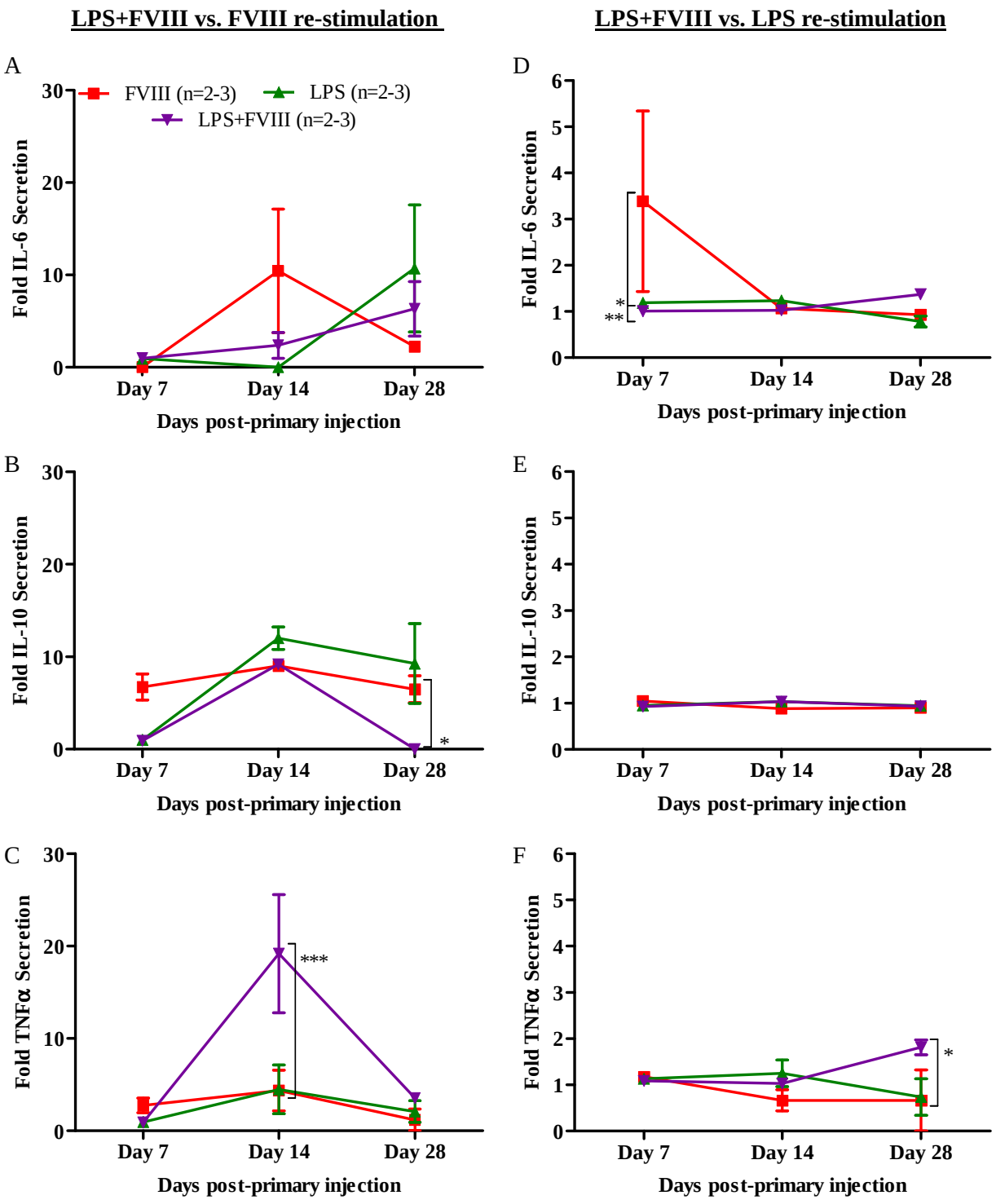


Fig.23: *In vitro* FVIII+LPS re-stimulation synergistically increased late inflammatory cytokine secretion. Ratio comparison of cytokine secretion from splenocyte cultures re-stimulated with 0.5 ug FVIII + 1.0 ug LPS over cultures that were re-stimulated with either (A-C) 0.5 ug FVIII or (D-F) 1.0 ug LPS. Values >1 indicate increased cytokine secretion from re-stimulated cultures. **(A,D) IL-6, (B,E) IL-10, and (C,F) TNF-α.** Significance determined by unpaired t-test with Welch's correction where *p<0.05, **p<0.01, ***p<0.001

In order to understand why FVIII stimulation in culture was decreasing cytokine secretion after long-term *in vivo* agonist stimulation, I further analyzed the IL-6 secretion from mice injected with LPS with or without concurrent FVIII injections which were then re-stimulated in culture with varying doses of LPS with or without constant FVIII (Fig.24). In both of the treatment groups, there was no difference in IL-6 secretion between cultures re-stimulated with LPS or LPS+FVIII during the early (day 7) inflammatory response (Fig.24A). In both mouse groups, IL-6 secretion significantly increased in response to LPS ($p<0.01$) in cultures containing FVIII compared to cultures that did not receive this extra re-stimulation at day 14 (Fig.24B). In mice injected with LPS+FVIII, this same trend continues at day 28 ($p<0.001$). However, by day 28, splenocytes from mice injected with only LPS and re-stimulated with FVIII in culture secreted significantly decreased ($p<0.001$) levels of IL-6 (Fig.24C). It has not yet been determined why mice stimulated only with LPS (naïve to FVIIII) have a reduced IL-6 response upon introduction to FVIII in culture during the late inflammatory response while mice that received concurrent *in vivo* agonist injections continued to have an increased IL-6 response to FVIII in culture. There is some implication that this might be due to splenocyte exhaustion or competition between LPS and FVIII to bind to TLR4.

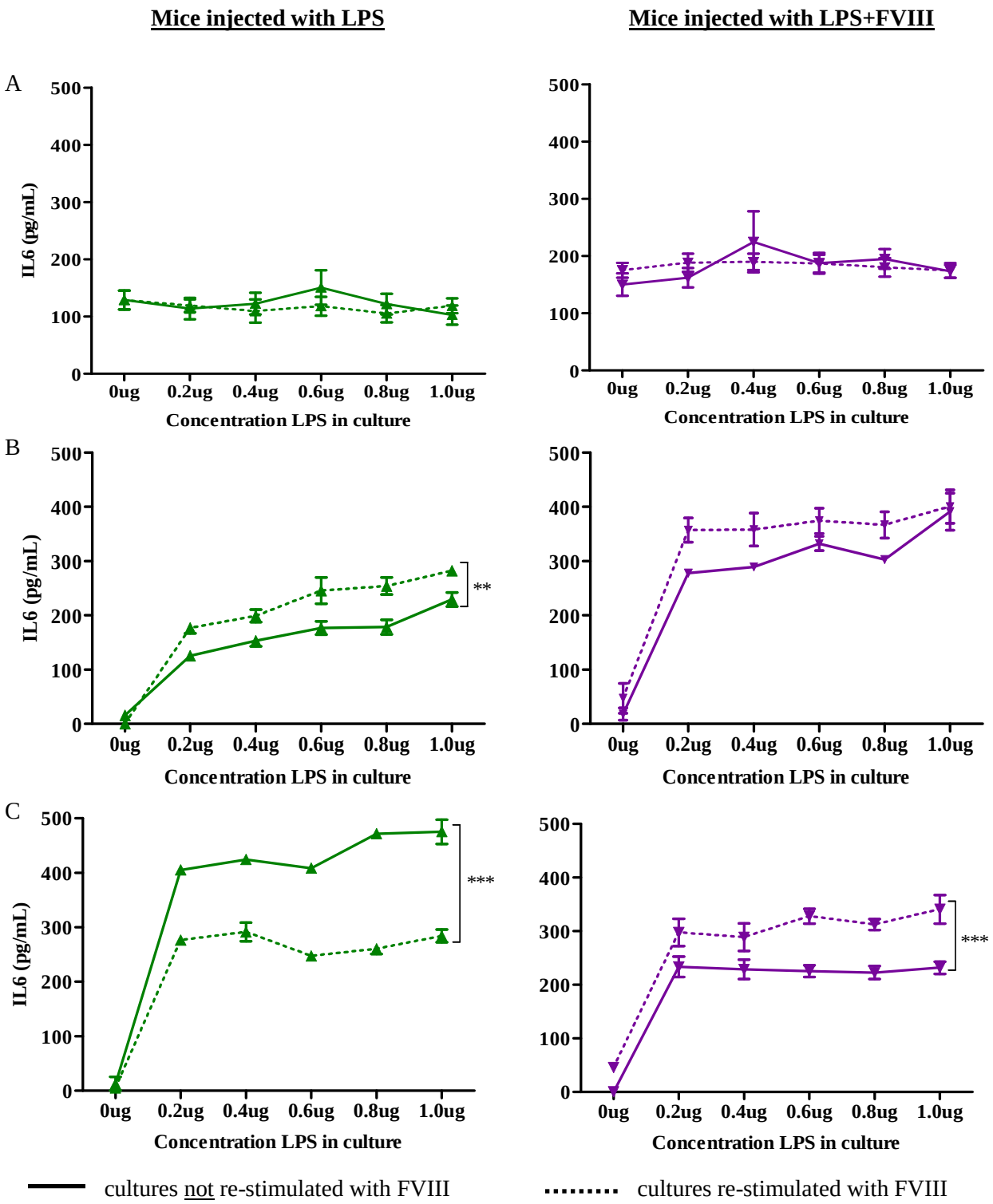


Fig. 24: Cells primed with multiple LPS doses secreted decreased IL-6 when treated with FVIII in culture. Splenocytes were isolated every 7 days post-primary injection of LPS (green) or LPS+FVIII (purple) and re-stimulated in culture for 24hrs. with increasing amounts of LPS without FVIII (—) or with 0.5ug FVIII (- - -). The culture supernatants were collected and measured *via* ELISA for IL-6 at **(A) 7 days, (B) 14 days, and (C) 28 days post-primary injection**. Significance determined by 2-way ANOVA where **p<0.01, ***p<0.001

To determine if there was competition between LPS and FVIII to bind TLR4, a competition assay was performed as previously described in Chapter IV. Peritoneal macrophages were stimulated with LPS for 24hrs. to up-regulate TLR4 and then pre-treated with either FVIII or BSA (as a control) to determine if FVIII was capable of preventing the binding of TLR2 (control), monoclonal TLR4, or polyclonal TLR4 antibodies. My results indicated that pre-treating peritoneal macrophages with FVIII had no significant effect on the percentage of macrophages that bound TLR4 antibody. This indicated that the presence of FVIII in culture is not directly competing with LPS to bind TLR4 (Fig.25). The different pre-treatments had no affect on TLR2 expression, used as a negative control, indicating that the FVIII pre-treatment was specific for TLR4.

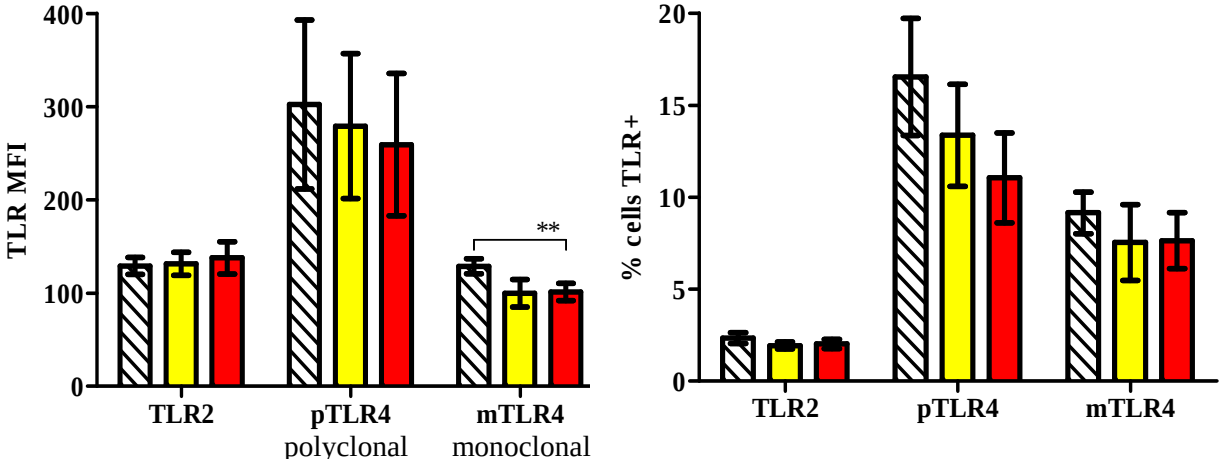


Fig.25: FVIII is not directly binding to TLR4.
Peritoneal macrophages were incubated with LPS for 24hrs, pretreated with BSA or FVIII, and incubated with TLR2, monoclonal TLR4 or polyclonal TLR4 antibodies. Antibodies were detected *via* flow cytometry, gated, and statistical analysis of the gates was performed. Significance determined by unpaired t-test with Welch's correction where **$p<0.01$

Overall, stimulation through TLR4 with or without concurrent FVIII injections increased the secretion of IL-6, IL-10, and TNF-α during the early inflammatory response, but was not maintained during repeated *in vivo* stimulation, indicating that TLR4-induced cytokine secretion is a tightly regulated, time specific process. Repeated LPS stimulation eventually over-stimulated the inflammatory response so as to have an antagonistic effect on IL-6 secretion. Even though I observed a FVIII-specific response during the late inflammatory response, FVIII was most likely not competing with LPS to bind TLR4.

ADDENDEUM: ENDOTOXIN ASSAY

To rule out the possibility that experimental solutions were contaminated with endotoxin (*E.coli* LPS) thereby introducing additional agonist and altering my results, I tested the PBS, culture medium, water, and FVIII dialysis buffer for trace levels of endotoxin as described in Chapter IV. The results (Fig.26) indicated that the PBS used to dissolve agonists for mouse injections, contained approximately 0.42 E.U./mL endotoxin. The FDA has determined that non-intrathecal drugs must have <5 E.U./kg endotoxin in order to be permissible for distribution. Since 1 E.U. (endotoxin unit) is equivalent to 100 pg LPS[64], the PBS I used contained approximately 42 pg/mL endotoxin, which was within the designated FDA guidelines. I could not discount the effect of trace levels of endotoxin in the PBS; however, since all of the mice received this extra endotoxin, the effect should be the same across all injection groups. I concluded that the relative changes, trends, and relationships detected in antibody production, APC populations, mean fluorescence, and cytokine production were indeed specific for LPS stimulation and not a by-product of contamination.

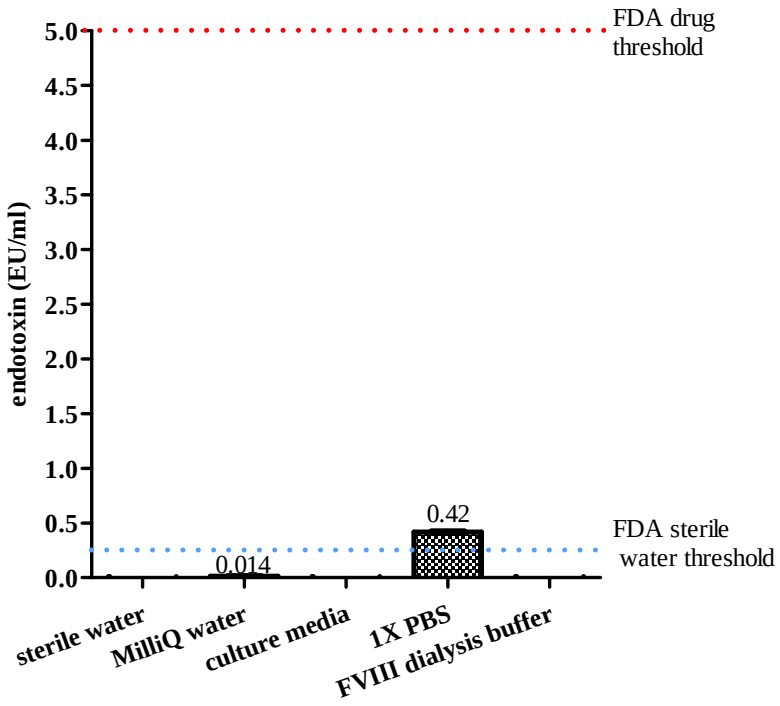


Fig.26: Presence of trace levels of endotoxin detected in PBS and MilliQ water.
Solutions were tested for trace levels of endotoxin *via* an LAL chromogenic assay. Results detected as absorbance. Unknowns were calculated from a standard curve.

DISCUSSION

Hemophilia patients receiving replacement FVIII therapy mount an immune response against the exogenous FVIII, due to lack of central tolerance, and may produce neutralizing anti-FVIII antibodies (inhibitors). During this immune response, FVIII binds to inflammatory receptors (like TLRs) on the surface of APCs[33, 44, 46, 47], which migrate to the spleen[65] where they co-localize with T-cells. APCs will internalize and degrade FVIII for presentation on MHC II while also secreting inflammatory cytokines (such as IL-6, IL-12, and TNF-α) and up-regulating co-stimulatory molecules (like CD40, CD80, and CD86). APCs bind TCR *via* MHC II and induce T-cell activation[33, 48] through co-stimulatory molecules, initiating either a T_H1 or T_H2 response. Activated helper T-cells will then stimulate B-cells to become FVIII-specific plasma cells that produce large amounts of anti-FVIII antibodies[33, 46]. Previous research has indicated that this FVIII immune response might be triggered and driven by inflammation. It was suspected that toll-like receptors (TLRs) might play a role in triggering this response because immune cells that express TLR and cytokines secreted after TLR stimulation have been suggested as key components in the FVIII immune response[14]. My model, which stimulated inflammation through TLR2 or TLR4 in a mouse model of hemophilia A, was designed to test this theory, identify the major components and cells involved, and discuss the possible implications this would have on future research and patient treatment.

1. The Effects of FVIII Stimulation Alone

Repeated *in vivo* injections of recombinant human FVIII over the course of four weeks increased anti-FVIII antibody and FVIII inhibitor production (Fig.11-12). Repeated *in vivo* FVIII stimulation, while having no significant effect on the relative percentage of macrophages or DCs in the spleen, decreased the relative percentage of B-cells present in the spleen (Fig.16). The overall decrease in splenic B-cells was most likely due to the fact that anti-CD19, the antibody used to identify B-cells using flow cytometry, is expressed on activated B-cells but not on plasma cells[66]. Since anti-FVIII antibody production increases after repeated injection of FVIII, it can be concluded that the decrease in B-cells in the spleen was most likely the direct result of increased FVIII-specific plasma cells. Interestingly, while repeated *in vivo* FVIII stimulation did not significantly change the percentage of APCs expressing TLR4, it instead significantly increased the density of TLR4 expressed on the surface of all APCs ($p<0.05$-0.01) (Fig.17). This up-regulation of the innate immune system receptors was an indication that FVIII stimulated an inflammatory response in APCs. This observation was further supported by the effect of FVIII stimulation on inflammatory cytokine secretion. TNF-α secretion was increased 7 days after the

first injection, after which it decreased and remained undetectable for the remainder of the injections. IL-10 did not appear to be significantly secreted in response to FVIII as it remained low throughout the course of the FVIII injections. IL-6 secretion, while initially undetectable, was slightly increased by the fourth injection of FVIII. FVIII re-stimulation in culture revealed increased TNF-α and IL-6 ($p<0.05$) secretion during the late (day 28) immune response indicating that APCs are capable of a FVIII recall response (Fig.19-20). These results indicated that FVIII did trigger an immune response but this response required multiple rounds of re-stimulation and time to develop. It is important to note that all my cultures were re-stimulated for 24hrs, which was determined as the optimal time frame for LPS stimulation. However, previous research has indicated that 72hr FVIII re-stimulations are usually required to produce more significant results. My cytokine results will need to be repeated utilizing this extended time frame.

2. The Effects of LPS Stimulation Alone

LPS stimulation alone cannot trigger the formation of FVIII antibodies so no inhibitors were detected. However, *in vivo* LPS stimulation significantly increased the relative percentage of macrophages ($p<0.001$) and DCs ($p<0.001$), but not B-cells, in the spleen. Interestingly, *in vivo* LPS injections increased the relative percentage of DCs more slowly than *in vivo* LPS+FVIII injections (Fig.16). *In vivo* LPS injections also increased the relative percentage of APCs expressing TLR4 in the spleen ($p<0.05$) but had no affect on the density of TLR4 expressed on APCs (Fig.17). Therefore, stimulation of TLR4 changed the repertoire of APCs in the spleen during the immune response. LPS stimulation significantly increased ($p<0.001$-0.05) the secretion of TNF-α, IL-6, and IL-10 7 days post-primary injection but secretion decreased after subsequent injections (Fig.19). Re-stimulation in culture increased the secretion of IL-6 in a linear manner over the course of the injections, which indicated that the APCs were not yet exhausted (Fig.21). The levels of IL-10 and TNF-α were also high ($p<0.05$) and maintained at that level throughout the repeated injections. Therefore, LPS stimulation is normally limited to an early inflammatory response but repeated stimulation additively increased cytokine secretion in the late inflammatory response (28 days post-primary injection). These results suggest that chronic inflammation not only changes the APC populations but also increases cytokine secretion which can lead to tissue damage.

3. The Effects of Concurrent LPS+FVIII Stimulation

Repeated *in vivo* injections of LPS and FVIII over the course of four weeks resulted not only in significantly increased anti-FVIII antibody and inhibitor titer ($p<0.001$), but inhibitors were also detectable earlier during the course of the injections (Fig.11-12). Repeated LPS+FVIII stimulation additively increased cytokine secretion during the early inflammatory response as seen from the significantly increased ($p<0.001$-0.05) secretion of IL-6, TNF-α, and IL-10 7 days post-primary injection (Fig.19). Concurrent re-stimulation in culture significantly increased and prolonged secretion of IL-6 ($p<0.05$) and TNF-α ($p<0.01$) in comparison to *in vivo* FVIII stimulation alone such that the highest levels of these cytokines were detected 14 days after the initial injection (Fig.19,22-23). Concurrent stimulation also significantly increased the overall percentage of macrophages ($p<0.001$) and DCs ($p<0.001$), but not B-cells, present in the spleen in comparison to *in vivo* FVIII stimulation (Fig.16). There was also an increased percentage of APCs that expressed surface TLR4 ($p<0.05$). Interestingly, the relative percentage of DCs in the spleen and the percentage of DCs expressing TLR4 quickly increased by the second injection (14 days post-primary injection), a trend not seen in mice injected with LPS. These results suggested that FVIII might be preferentially acting on DCs, changing the APC repertoire, while also expanding the APC populations overall.

Overall, these results indicated that repeated FVIII stimulation increased the density of TLR4 on the APCs, stimulated a late immune recall response capable of increasing IL-6 and TNF-α secretion (28 days post-primary injection), and decreased the relative percentage of B-cells in the spleen possibly due to an increase in plasma cells. Repeated LPS stimulation increased the relative percentage of macrophages and DCs in the spleen, increased the percentage of macrophages and B-cells expressing TLR4 in the spleen, and additively increased cytokine secretion in the late immune response (28 days post-primary injection). Taken together, repeated LPS+FVIII stimulation *should* have increased the percentage of APC expressing TLR4, especially DCs, and increased cytokine secretion throughout the inflammatory response which would eventually lead to inhibitor production, all of which were observed.

Only the increased density of TLR4 on APCs due to FVIII stimulation was not seen in mice injected with LPS+FVIII. The density of TLR4 expressed on APC surfaces did not change however (Fig.17) indicating that concurrent stimulation was not up-regulating TLR4, perhaps due to competition between LPS and FVIII. LPS is small, binds to a specific receptor on the surface of APCs, and is a potent adjuvant. Therefore it probably preferentially binds TLR4. FVIII, on the other hand, is large, complex, and there is evidence that it could be binding to one of several receptors, including low-density lipoprotein receptors, mannose receptors, or asialoglycoprotein

receptor which binds glycoproteins lacking terminal salicylic residues[33], however FVIII binding is not yet definitive. Therefore while my results indicated that FVIII is not directly competing with LPS to bind TLR4 (Fig.25), the slight decrease in antibody binding polyclonal TLR4 observed after both FVIII and BSA pre-treatment suggested that there might be some indirect competition for TLR4 binding due to steric hindrance from the massive size of these proteins, FVIII (170 kDa) and BSA (66.5 kDa) or interference with CD14. Competition does not explain the increased IL-6 secretion detected after concurrent re-stimulation in culture during the late immune response (Fig.22-24) suggesting that a change in APC repertoire due to concurrent stimulation is the most probable explanation for increased inhibitor production.

4. The Effects of Long-Term IL-6 Secretion

Interestingly, after the fourth *in vivo* LPS injection, IL-6 secretion significantly decreased after concurrent LPS+FVIII re-stimulation compared to IL-6 secretion after only LPS re-stimulation (Fig.24). This phenomenon was only observed during the late (day 28) immune response and only in cells naïve to FVIII before culture. This was unexpected because mice injected with both LPS+FVIII secreted significantly increased levels of IL-6 when concurrently re-stimulated compared to being re-stimulated with only LPS.

There are several possible explanations for this decreased IL-6 secretion. First, as previously discussed, FVIII could be competing with LPS to bind TLR4 (discussed above). If so, the presence of FVIII in culture would hinder LPS binding and reduce IL-6 secretion. However, this does not explain why concurrent LPS+FVIII re-stimulation triggers increased IL-6 secretion. Second, repeated LPS stimulation could be triggering the internalization or down-regulation of TLR4 during that late stage of the immune response. This decrease in available receptor would prevent a further increase in cytokine secretion in the presence of additional FVIII in culture. Concurrent *in vivo* LPS+FVIII stimulation should still have moderate levels of TLR4 expression due to the presence of FVIII. However, TLR4 density indicated that this was not the case, which could again be due to LPS potency, or could indicate that there is come level of compensation from the increase percentages of APCs expressing TLR4. Lastly, repeated LPS stimulation (without FVIII) may have changed the repertoire of the APCs in the spleen such that it is no longer responding normally to FVIII. The chronic LPS stimulation might have “educated” the APCs, forcing them into a solely LPS-driven pathway of development and proliferation, while killing off other “unnecessary” APCs. Therefore, FVIII re-stimulation would not have the same binding capabilities and stimulatory effects normally observed, resulting in reduced IL-6 secretion[51, 56].

5. LPS Stimulation Triggers Coagulation through Tissue Factor Production

During the course of these experiments, I observed that mice injected with LPS or LPS+FVIII were able to sufficiently clot after tail snips. This was unexpected as mice injected with LPS+FVIII were also producing very high levels of inhibitors which impede clotting. Previous studies have shown that LPS up-regulates TF release from endothelial cells, which then initiates clotting through the extrinsic coagulation pathway and completely bypasses the need for FVIII in the intrinsic coagulation pathway (Fig.1)[3, 67, 68].

While this dual LPS effect needs to be studied further, it might be a different method by which concurrent agonist stimulation induces inhibitor production. As discussed in the interdependent model of coagulation (Fig.2), TF activates FVII, initiating the extrinsic clotting cascade. This cascade activates FX, forming the tenase complex, and triggering the formation of thrombin. While involved in the formation of a primary clot, thrombin also activates components of the intrinsic pathway (like FVIII, FIX, FV) which are also important for increased catalysis of tenase complex formation and formation of a solid secondary clot. This alternative activation of FVIII enables efficient clotting, observed in mice receiving concurrent LPS and FVIII injections. The FVIII-enhanced clotting would also be responsible for quickly using up, and then triggering the degradation or, the replacement FVIII. The FVIII particles could then be more readily phagocytosed and presented by APCs, driving the immune system to more readily produce anti-FVIII antibodies. While mice receiving LPS injections alone were also able to clot via the extrinsic pathway, they had no FVIII to increase this process. The lack of FVIII also means that the protein would not be degraded and presented to the immune system by APCs, which is why no anti-FVIII antibodies are produced. This theory needs to be confirmed with future experimentation.

6. Relevance to Clinical Treatment

There is currently little to no reported incidence of gram-negative bacterial infections in patients with Hemophilia A. So while this model of inflammation does not directly apply to patient treatment, we have supplied evidence that a pro-inflammatory environment leads to the increased formation of FVIII inhibitors. Therefore, patients receiving treatment, especially those patients that have not yet developed inhibitors, should be monitored for infections and inflammation especially in areas where bleeding is common like the joints. Patients should be placed on a low-dose anti-inflammatory regimen (ie. NSAIDs, COX-2 inhibitors). If infection is identified, patients should also be place on an appropriate antibiotic regimen. Patients receiving on-demand therapy, who are already at risk for FVIII inhibitor formation compared to patients

receiving prophylactic treatment, receive treatment in instances of injury or surgery where infection and inflammation are more likely to occur. Therefore, this anti-inflammatory regimen should definitely be administered with all on-demand treatments.

Other treatment options could include: (1) blocking/antagonizing innate immune system receptors like TLR4, (2) preventing the production and/or secretion of inflammatory cytokines like IL-6 or TNF-α[66, 69], a method already used to treat inflammatory diseases like rheumatoid arthritis or (3) temporary drug suppressing of APC or CD4+ T-cell function (ie. cyclosporin).

Unfortunately, most of these options involve large-scale suppression of the immune system which would leave a patient immuno-compromised and open to other disease. A more likely method of treatment would be the up-regulation of anti-inflammatory components of the immune system which would dampen but not completely eliminate the innate immune system. Some treatment options further discussed in Chapter IX could include: (1) the up-regulation of anti-inflammatory receptors like TLR2, (2) up-regulation of anti-inflammatory cytokines like IL-10, or (3) activation of immuno-suppressive cells like Tregs.

STIMULATION THROUGH TLR2 DOES *NOT* INCREASE FVIII INHIBITOR PRODUCTION

As discussed in previous chapters, it has been suggested that inflammation specifically through the stimulation of toll-like receptors (TLRs) might be playing a role in triggering the FVIII immune response, leading to the production of FVIII inhibitors. Previous data from the Smith lab utilizing cytokine multiplex analysis and statistical algorithms to model the anti-FVIII immune response *in silico* suggested TLR2 would also be up-regulated early during the FVIII immune response in mice and drive increased inhibitor formation. Therefore, I also studied the role of TLR2 stimulation on the formation of FVIII inhibitors in a mouse model of hemophilia A.

For these experiments, I i.v. injected FVIII-deficient mice once a week for four weeks with PBS, FVIII, TLR2 agonist (PAM), or PAM+FVIII. Blood samples were collected every seven days post-primary injection and analyzed using ELISA and Bethesda assay (as described in Chapter IV). To determine if TLR2 stimulation during FVIII treatment would increase the production of anti-FVIII antibodies as was observed with TLR4 stimulation, I measured the kinetics and magnitude of the antibody in mice injected with PAM+FVIII over the course of time. Unexpectedly, end titer levels were approximately 2^4 at day 21 post-primary injection, which was significantly decreased ($p<0.05$) compared to mice that received FVIII alone (Fig.27). By day 28 post-primary injection, end titer levels increased to approximately 2^7 such that they were no longer significantly different compared to mice that received FVIII alone. Inhibitor levels were approximately 104 B.U. at day 28 post-primary injection ($p<0.05$), which is significantly decreased compared to mice injected with FVIII alone (Fig.28). This data indicated that TLR2 stimulation did not significantly increase, but instead significantly decreased, FVIII inhibitor production. Since there is a linear relationship between anti-FVIII IgG production and FVIII inhibitor production[62], it was surprising to observe that concurrent PAM+FVIII IgG antibody secretion, while delayed, was not significantly different from IgG titers produced by mice injected with FVIII. This suggests that TLR2 stimulation might be affecting B-cell populations and the IgG subclasses they produce such that now more non-inhibitory IgG antibodies were being secreted. The anti-inflammatory effect of TLR2 needs to be studied further.

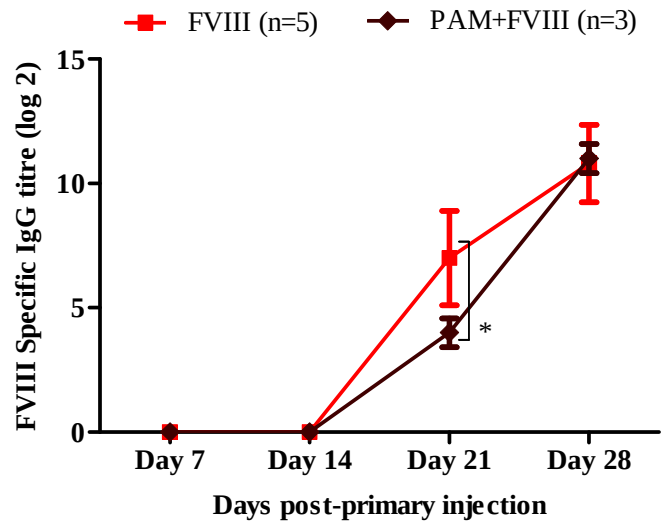


Fig.27: Repeated *in vivo* TLR2 stimulation delayed production of anti-FVIII IgG antibodies. Antibody titers were measured from citrated plasma using a modified ELISA. Significance determine by 2-way ANOVA and unpaired t-test with Welch's correction where *p<0.05

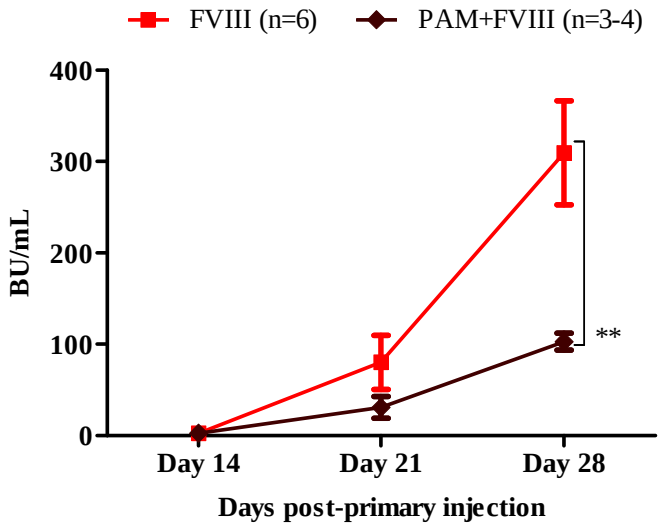


Fig.28: Repeated *in vivo* TLR2 stimulation decreases production of FVIII inhibitors. Inhibitors were measured from citrated plasma using a modified Bethesda assay. Significance determine by 2-way ANOVA and unpaired t-test with Welch's correction where **p<0.01

Unlike stimulation through TLR4, TLR2 stimulation appears to have an anti-inflammatory effect during the FVIII immune response, resulting in decreased inhibitor formation. This anti-inflammatory effect could be the result of (1) secretion of cytokines that switch immune system to an anti-inflammatory (tolerogenic) response, (2) activation of different APC populations, or (3) the up-regulation of anti-inflammatory receptors.

TLR2 and TLR4 share a MyD88-dependent NF-κB pathway that drives secretion of inflammatory cytokines including IL-6 and TNF-α. However, TLR4 has multiple routes by which to stimulate cytokine secretion and APC maturation; it can be concluded that the balance of secreted cytokines is what determines the type of inflammatory response. As previously discussed, TLR2 signaling preferentially induces a humoral, helper T-cell type 2 (T_H2) response[55], with increased IL-8, IL-23/p19, and MIP-1alpha secretion[70]. It was also observed that stimulation of TLR2 increased IL-12p40 secretion[70, 71], a subunit that it inhibitory on its own, which might explain some of the anti-inflammatory effects of TLR2.

Other studies have observed that there are distinct subclasses of DCs which are responsible for triggering the various immune responses during inflammation. Of the 3 different subclasses of DCs (monocytes, conventional myeloid, and plasmacytoid[72]), in humans only monocytes express TLR2 and TLR4. This subset of APCs will not only respond to bacterial infections but also, when stimulated by inflammation or TLR activation, are activated into mature APCs capable of migration, increased cytokine secretion, increased antigen presentation, and improved T-cells interactions[71, 73]. Due to its T_H2-driven effects, stimulation through TLR2 not only affects the proliferation and maturation of B-cells[55], but also triggers the activation and proliferation of regulatory T-cells (Tregs)[51]. The activation of Tregs might also explain the observed anti-inflammatory effects of TLR2 stimulation.

During monocyte activation, mature DCs will up-regulate migration/homing receptors such as CCR7, MHC, and T-cell co-stimulatory molecules CD80, CD86, and CTLA-4[71, 73]. Activated DCs have also been found to up-regulate PD-1 (programmed cell death protein 1) and its two ligands, PD-L1 and PD-L2. PD-L1 is found on resting DCs as well as other immune and non-immune cells while PD-L2 is found exclusively on activated APCs. Binding of PD-L1 to PD-1 protein creates a suppressive environment including inhibition of T-cell activation via blocking PI3K, suppression of cytokine secretion, induction of apoptosis of active APCs, and activation of Tregs[73]. It is possible that the up-regulation of this receptor is responsible for the anti-inflammatory effects observed after TLR2 stimulation. All of these possibilities are areas of future research in order to understand the FVIIII immune response and the formation of inhibitors in patients with hemophilia A.

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