FVIII Immunity: Early Events and Tolerance Mechanisms to FVIII

By

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Abstract

Among the complications of current treatments for hemophilia A, the development of anti-FVIII antibodies including “FVIII inhibitors” remains the major clinical problem in treating hemophiliacs. Factor VIII inhibitors work through neutralizing the coagulation cofactor activity of the infused FVIII and preventing the restoration of normal hemostasis. This thesis explains the influence of genetic background on the generation of FVIII inhibitors, introduces a new pre-clinical approach that reduces the immunological response towards FVIII and predicts the in vivo behavior of recombinant and plasma-derived FVIII products in hemophilic patients.

First, we studied the influence of the genetic background on the formation of FVIII antibodies by treating hemophilia A Balb/c and C57BL/6 mice with repetitive FVIII infusions. We observed that the C57BL/6 mice developed higher FVIII antibody titers than the Balb/c mice. Our results suggest that differences in the cytokine immune responses due to FVIII in Balb/c and C57BL/6 mice are responsible for the different FVIII antibody titers in each of these strains.

Second, we investigated the use of FVIII-pulsed immature dendritic cells in inducing immune tolerance against FVIII prior to the FVIII treatment. We showed that in vivo, FVIII does not induce the activation and proliferation of hemophilic T cells. Furthermore, infusing FVIII-pulsed immature dendritic cells into hemophilic mice resulted in a long-term reduction in immune reactivity towards FVIII. Also,

we have proposed methods on how to improve the tolerogenic abilities of dendritic cells. Our results indicate that the immature dendritic cells induced the formation of T regulatory cells and that these T regulatory cells were responsible for the observed reduction in immune reactivity.

Finally, we were able to identify the mechanisms behind the immune system activation in mice treated with either recombinant or plasma-derived FVIII products. We showed that plasma-derived FVIII results in reduced FVIII antibody titer formation in hemophilic mice. Our results demonstrate that the differences in antibody formation in hemophilic mice treated with either recombinant or plasma-derived FVIII products are due to the distinct cytokine micro-environment induced by each product.

This thesis contributes to the current knowledge on FVIII immunology and the in vivo behavior of FVIII in hemophilic mice. The results generated from this thesis can be used to modify the available FVIII treatments in order to minimize the immunological complications of FVIII and improve the quality of life of hemophilic patients.

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ADP	Adenosine-5'-diphosphate
Andro	Andrographolide
Anti-FVIII	Factor FVIII antibodies
Anti-VWF	von Willebrand factor antibodies
APCs	Antigen-presenting cells
APTT	Activated partial thromboplastin time
BP	Base-pairs
BU	Bethesda units
CD	Cluster differentiation
CD11c	Cluster Differentiation 11 c
CD25	Cluster Differentiation 25
CD28	Cluster Differentiation 28
CD40	Cluster Differentiation 40
CD80	Cluster Differentiation 80
CD86	Cluster Differentiation 86
cFVIII	Canine Factor FVIII
DCs	Dendritic cells
E16-Balb/c	Exon 16 knockout Balb/c mice
E16-C57BL/6	Exon 16 knockout C57BL/6 mice
ELISA	Enzyme-Linked ImmunoSorbent Assay
FEIBA	Factor VIII Inhibitor Bypassing Activity
FIIa	Thrombin
FIX	Factor IX
Foxp3	Forkhead transcription factor 3
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
GPIb	Glycoprotein 1b
GPIIb/IIIa	Glycoprotein II b/IIIa
H2B	Major Histocompatibility Haplotype 2D
H2D	Major Histocompatibility Haplotype 2B
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigens
iDCs	Immature Dendritic cells
IFN-γ	interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL1	Interleukin 1
IL2	Interleukin 2

IL4	Interleukin 4
IL5	Interleukin 5
IL6	Interleukin 6
IL10	Interleukin 10
IL12	Interleukin 12
ITI	Immune tolerance induction
LPS	Lipopolysaccharide
MHC-I	Major Histocompatibility Class I
MHC-II	Major Histocompatibility Class II
NFkB	Nuclear factor-kappa B
nTreg	Naturally occuring T regulatory cells
PALS	Periarteriolar lymphatic sheath
pdFVIII	Plasma derived Factor FVIII
rFVIII	Recombinant Factor FVIII
TCR	T cell receptor
TF	Tissue factor
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
Th3	T helper 3
TNF-α	Tumor necrosis factor-alpha
Tr1	T regulatory 1
Treg	T regulatory cells
VWF	von Willebrand factor

Chapter 1

Hemophilia A Treatment Options and Complications

Hemophilia Introduction

Hemophilia is an inherited bleeding disorder in which patients suffer from recurrent spontaneous bleeding episodes. In 1952, two distinct types of hemophilia, A and B were recognized [1]. Both hemophilia A and B are X-linked recessive genetic diseases that result from deficiencies or dysfunction of coagulation factor VIII (FVIII) and factor IX (FIX) respectively [2]. The FVIII protein is a cofactor in the activation of factor X by FIXa. Hemophilia A affects 1 in 5000-10000 males and is characterized by recurrent spontaneous bleeding [3]. Hemophilic patients are grouped according to the severity of their clinical phenotypes into severe, moderate or mild. Severe hemophilia A patients have less than 1% FVIII, moderate hemophilia A patients have 1-5% FVIII, and mild hemophilia A patients have 5-45% FVIII [4]. Hemophilic patients and physicians face many challenges, such as the availability of safe medical products, excessive joint bleeding, life-threatening hemorrhages, and immunological responses including antibody production (FVIII inhibitors). Furthermore, treatment options become more complicated in developing countries due to the limited availability of safe, effective and affordable products.

The Hemostatic Response

The hemostatic response is divided into four stages. First, is the vascular response, second the formation of a platelet plug, third the activation of the coagulation protein cascade and formation of a clot, and the final step involves the activation of the fibrinolytic cascade and remodeling of the fibrin clot [5].

Upon blood vessel injury, blood vessels vasoconstrict in order to decrease blood flow to the injured site [6]. At the site of vascular injury, platelets rolling and binding to the sub-endothelium initiate the formation of a platelet plug allowing the platelets to adhere to the exposed collagen in the sub-endothelial matrix via von Willebrand factor (VWF) [6]. This initiates the activation of platelets and allows the binding between glycoprotein GPIb receptors on the platelet surface and VWF molecules. Additional binding between VWF and platelets will enhance platelet cross-linking, resulting in the exposure of the GPIIb/IIIa integrin receptor. The GPIIb/IIIa receptor enhances platelet aggregation allowing the platelets to form a platelet plug [6]. The binding of VWF to the platelets will cause the release of platelet cytoplasmic contents such as ADP, 5-hydroxytryptamine, serotonin and calcium. The formed platelet plug will act as a bed for the coagulation factors to form a fibrin clot.

The coagulation cascade, shown in Figure 1.1, is divided into two pathways: the extrinsic and intrinsic [7]. In the extrinsic pathway, the coagulation process is initiated after tissue injury. Injured sub-endothelial cells will release tissue factor (TF), which functions as a cofactor and activator for FVII [8]. The TF-FVII complex will further activate FVII molecules, as well as FIX [9]. These activated proteins, including FVIII, will activate FX, resulting in the formation of sufficient thrombin levels (FIIa) that will trigger the activation of the intrinsic pathway [8]. In the intrinsic pathway, the available thrombin will activate FXI, FIX and FVIII. On the platelet surface, thrombin will activate FXI, which will then activate FIX. At each step there is an amplification in the amount of product formed. Finally, thrombin will

Figure 1.1 Representation of the blood coagulation system. The cascade is divided into an extrinsic and intrinsic system. The interactions between the coagulation factors result in the activation of thrombin and the formation of a hemostatic plug. This cascade is regulated by anticoagulant proteins that prevent excessive hemostatic plug formation.

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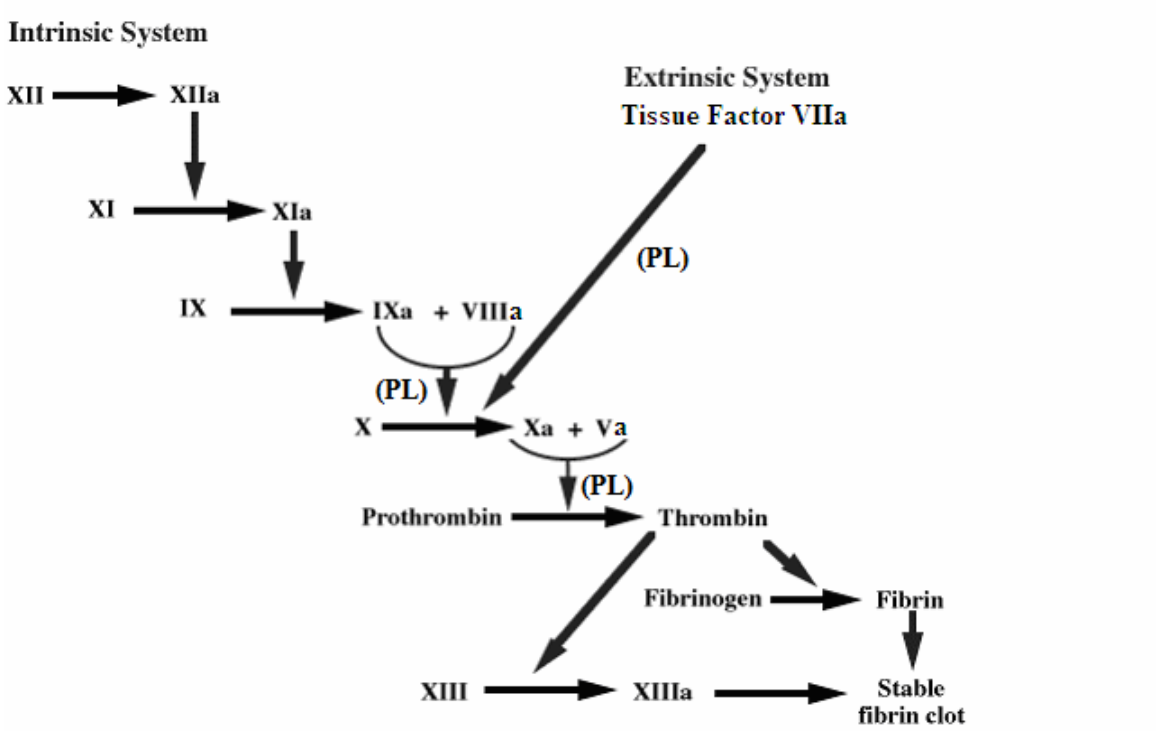

Intrinsic System
XII
XIIa
XI
XIa
IX
IXa + VIIIa
(PL)
X
Xa + Va
(PL)
Prothrombin
Thrombin
Extrinsic System
Tissue Factor VIIa
(PL)
Fibrinogen
Fibrin
XIII
XIIIa
Stable
fibrin clot

transform soluble fibrinogen into the insoluble fibrin clot [8]. Activated FXIII is responsible for covalently cross-linking the fibrin monomers to form a more stable fibrin clot.

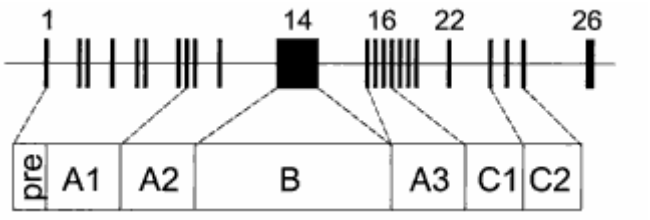
FVIII Gene and Protein

The FVIII gene is situated on the long arm of the X chromosome, band Xq28. The gene is 186,000 base-pairs (bp) long and it consists of 26 exons (Figure 1.2 A). The exon sizes range from 69 to 3,106 bp. The mutations in hemophilia A patients are due to insertions, deletions, duplications and point mutations in the FVIII gene [10]. The most frequent mutation causing severe FVIII deficiency is an inversion mutation affecting intron 22. This arises from a recombination event between homologous sequences in intron 22 and the terminal region of the long arm of the X chromosome [11]. This inversion mutation is responsible for 45% of the cases of severe hemophilia A [11]. Patients with the intron 22 inversion have no intact FVIII protein in their circulation.

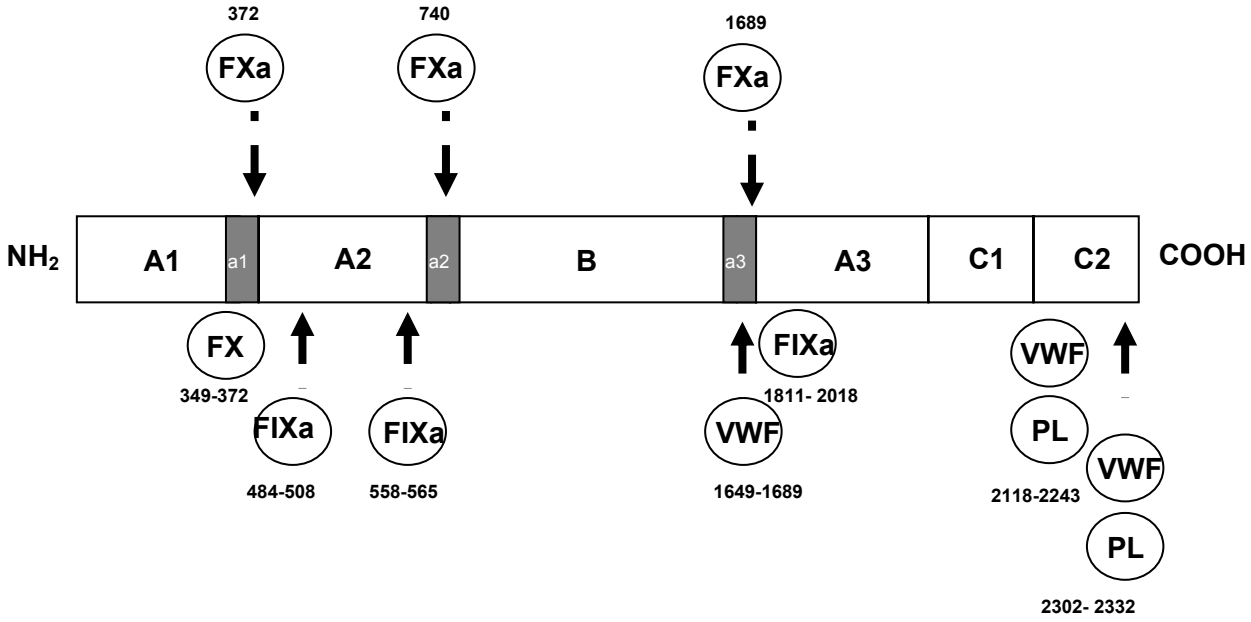
The cellular site of FVIII synthesis is controversial. Liver transplants for hemophiliac patients have been shown to increase FVIII blood levels and cure the disorder [12]. The FVIII protein consists of 2332 amino acids (AA) organized into 6 domains, A1-a1-A2-a2B-a3A3-C1-C2 (Figure 1.2 B). The A domains of FVIII contain binding sites for FIXa, FX and VWF. The function of the B domain of FVIII is not fully understood, but it seems likely to be involved in the intracellular trafficking of the protein. The C domains of FVIII contain binding sites for phospholipids and von Willebrand Factor (VWF). The protein also has three acidic regions which are cleaved by thrombin, a1, a2 and a3.

Figure 1.2 FVIII gene and protein (A) Representation of the FVIII gene exons. (B) FVIII protein domains and major protein binding sites. The von Willebrand factor (VWF), factor X (FX), factor IX (IX), activated factor Xa (FXa) and phospholipids (PL) bind to the C2, A1, A2 and C2 domains respectively. Thrombin will cleave FVIII at the three sites indicated with arrows, resulting in the active cofactor FVIIIa

A)


1
14
16
22
26
pre
A1
A2
B
A3
C1
C2

B)


372
740
1689
FXa
FXa
FXa
NH2
A1
a1
A2
a2
B
a3
A3
C1
C2
COOH
FX
349-372
FIXa
484-508
FIXa
558-565
FIXa
1811- 2018
VWF
1649-1689
VWF
PL
2118-2243
VWF
PL
2302- 2332

The acidic regions are positioned between the A1 and A2 (a1), the A2 and B domain (a2), and the B and A3 domain (a3). The FVIII protein is proteolytically processed before secretion. This results in a heterodimer of an N-terminal heavy chain (200KDa) and a C-terminal light chain (80KDa). The inactive FVIII binds VWF in the circulation; however, upon the activation of FVIII, VWF will release FVIII and the B domain is removed.

Development of FVIII Antibodies in Hemophilic Patients

The current standard of care for hemophilia A patients is either on-demand, or prophylactic treatment with either recombinant or plasma-derived FVIII concentrates. One would expect that if FVIII is infused into hemophilic patients, the effects of the disease would be reversed. This however is not always true, since 30% of hemophilia A patients develop functional neutralizing antibodies (inhibitors) against the infused FVIII, thus rendering the treatment ineffective [13].

During the 1980s, hemophilia A patients were treated with FVIII products that contained active Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) particles. However, through the use of donor screening protocols, virus inactivation procedures during the purification of FVIII, and through the increasing use of recombinant FVIII, the most serious challenge in the treatment of hemophilia A patients is now the formation of anti-FVIII antibodies. These antibodies develop in the patient because the infused factor VIII is viewed as a foreign antigen. This elicits an immune response against FVIII and renders the treatment ineffective [14]. The levels of inhibitors can be monitored by performing a

Bethesda assay. The inhibitor levels are presented in terms of Bethesda units [13], where one Bethesda Unit is defined as the inhibitory titer needed to inactivate 50 percent of the FVIII present in normal plasma within a 2-hour incubation period at 37°C.

The immunoglobulin responses toward FVIII epitopes are polyclonal in nature and most of the FVIII epitopes have been identified and mapped [15] (Figure 1.2 B). The alloantibodies, which develop in hemophiliacs following exposure to FVIII infusions, are usually directed towards the A2 and C2 domains [4,14]. The antibodies against FVIII are grouped into non-functional and functional antibodies. The non-functional antibodies, detected by ELISA, bind to FVIII causing opsonization and immune complex formation resulting in the enhanced clearance of FVIII from the circulation. In contrast, functional antibodies (FVIII inhibitors) bind to FVIII and directly inhibit the functional cofactor activity of FVIII. In this way, functional FVIII inhibitors sterically hinder the specific interactions between FVIII and other proteins, affecting the coagulation cascade. The most frequently affected interactions are those between FVIII-VWF and FVIII-FIXa [16]. The FVIII inhibitors that develop in hemophilia A patients predominantly belong to IgG_4 and sometimes IgG_1 immunoglobulin isotypes [17].

Inhibitors Against The Protein Binding Sites on The C2 Domain of FVIII

The VWF protein functions as a carrier of FVIII in the plasma via a non-covalent interaction. The majority of VWF binding sites are found on the C2 domain of FVIII. It has been shown during FVIII epitope mapping studies that the same VWF binding sites are also epitopes for anti-FVIII antibodies. Therefore, these sites behave as functional epitopes. Many reports suggest that VWF protects and covers the C2 domain epitopes from being exposed to the immune system [14,18]. It has been reported that almost 50% of FVIII inhibitors are directed toward the C2 domain of FVIII [4]. The C2 domain not only contains the binding site for VWF, but also the binding site for phospholipids. These binding sites are essential for FVIII trafficking and activating the coagulation cascade. Therefore, VWF has an important role in protecting the exposure of the functional epitopes of FVIII to the immune system.

Current Known Risk Factors That are Associated With Inhibitor Development in Hemophiliacs

There are several genetic and non-genetic risk factors that are associated with inhibitor development. Goudemand et al showed that the ethnic origin and family history of inhibitors are risk factors that are associated with the development of FVIII antibodies [19]. Furthermore, White and Hay reported that some HLA genes are associated with inhibitor development in hemophilic patients [20,21]. Moreover, Astermark et. al. showed that certain polymorphic genotypes at the IL10 and TNF-α loci are linked to FVIII inhibitor development in hemophiliacs [22,23]. Also,

there are a few reports which show a correlation between FVIII inhibitor development and age of exposure to FVIII. Santagostino et al showed that children treated at a young age (< 11 years) have higher risk of FVIII inhibitor formation [24]. Furthermore, Lee at al reported that the type of FVIII concentrate and route of FVIII administration are also associated with inhibitor development [25]. Finally and most importantly, the type of FVIII mutation has an influence of FVIII inhibitor development. It is been reported that large deletions, nonsense mutations, intron-22 inversion and no endogenous FVIII synthesis have a greater risk of inhibitor development than patients with milder molecular gene defects such as missense mutations, small deletions and splice site mutations [26].

Treatment of Hemophilic Patients with Inhibitors

The available treatments for hemophilia A patients who develop antibodies after FVIII infusion include: bypassing the need for FVIII using recombinant FVIIa, immunoadsorption of factor VIII antibodies [17], the use of alternate less immunogenic products such as porcine FVIII [17] and immune tolerance induction to FVIII (ITI) [27].

Bypassing the need of FVIII is usually successful in treating patients with inhibitors; however, this approach does not resolve the inhibitor problem and is often prohibitively expensive. In contrast, after successful ITI treatment, the FVIII inhibitors will be abolished. Therefore, ITI is usually the first treatment considered to overcome the FVIII inhibitor complication. The first protocol for ITI was described in 1977, and ever since new treatment regimens have been introduced

[28]. The most used protocols are the Bonn and Van Creveld protocols [29,30]. The Bonn protocol (high dose FVIII ITI) is divided into two phases. Phase one involves the infusion of 100 IU/kg FVIII twice daily, and 100 IU/kg of the bypassing product Factor VIII Inhibitor Bypassing Activity (FEIBA) twice daily. Phase two involves a gradual reduction in FVIII treatment over 3 months after achieving a normal FVIII half-life. The Van Creveld regimen (low dose FVIII ITI) includes a neutralizing phase during which patients are treated with 25–50 IU/kg FVIII twice daily for 1–2 weeks, followed by a tolerising phase in which patients are treated with 25 IU/kg FVIII every second day. The success rate of these ITI protocols is 87% [29], however these approaches can be very costly and practically challenging, especially in young children in whom repeated intravenous access often requires the insertion of a central venous catheter. Therefore, other less expensive and more convenient means of ITI are currently being investigated.

There are currently few research centers investigating the influence of FVIII products containing VWF on the outcomes of ITI. Kreuz at al showed a significant reduction in the success rate of ITI when rFVIII products are used instead of plasma derived FVIII products [13]. Furthermore, the authors showed that when rFVIII products are substituted with FVIII-VWF, the successful rate of ITI went up to 90% [13]. Also, an Italian group demonstrated that FVIII-VWF products are effective in ITI and even in patients who have a risk high of ITI failure [31]. Finally, the retrospective German and French studies showed that the

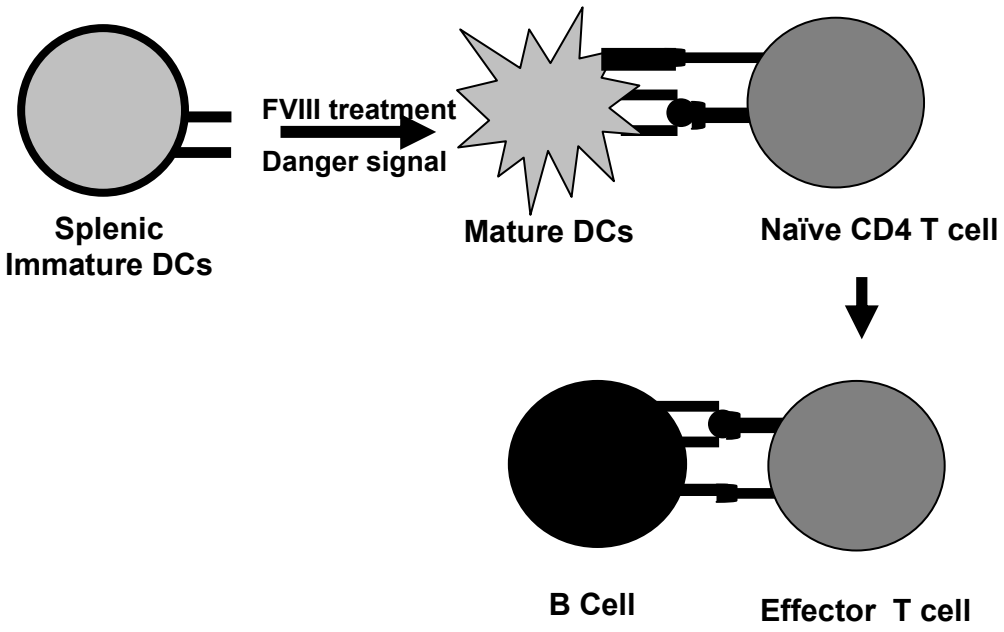
use of FVIII-VWF products result in higher ITI success rate than the ITI treatments which use rFVIII products [32].

These studies show a direct relationship between the outcomes of ITI treatment and the type FVIII product used. We currently do not understand the reasons behind this finding and more studies are being conducted in order to identify the mechanisms responsible for this observation.

FVIII Infusions and Activation of The Immune System in Hemophilic Patients

Severe hemophilia A patients with inhibitors have either no functional FVIII or a truncated protein, thus, the infused FVIII is viewed as a foreign protein. After FVIII infusions, a series of interactions occur resulting in the activation of the immune system and the formation of inhibitors. In patients with inhibitors, the following mechanism is expected to occur (Figure 1.3). First, the infused FVIII will be sampled by a specialized subset of cells, called antigen-presenting cells (APCs). These cells have the ability to internalize, process and present FVIII to T cells [33]. The antigenic presentation of FVIII is in the context of specialized protein molecules called Major Histocompatibility Class II (MHC-II) proteins [34]. Second, the antigenic presentation of FVIII will activate FVIII-specific CD4+ T helper cells. This causes the T cells to clonally expand and release cytokines that will recruit B cells. Finally, additional interactions will occur between CD4+ T helper cells and FVIII-specific B cells in order to permit B cell activation, cellular

Figure 1.3 Activation of B cells and antibody production. The activation of B cells requires a series of interactions between APCs, T cells and B cells. APCs will internalize FVIII, mature and activate T cells causing cytokine release. The activated T-cells will then bind to B cells; thus, resulting in a strong enhancement of cytokine release resulting in B cell activation.


FVIII treatment
Danger signal
Splenic
Immature DCs
Mature DCs
Naïve CD4 T cell
B Cell
Effector T cell

differentiation and antibody release by FVIII-specific plasma cells. The released antibodies will bind to and target FVIII molecules for accelerated clearance, and/or inactivation and degradation.

FVIII Presentation by Antigen-Presenting Cells (APCs)

Professional APCs (dendritic cells, macrophages and B cells) are the only cells capable of presenting exogenous proteins. However, all nucleated cells are capable of presenting endogenous proteins in the context of MHC-I. Professional APCs are unique due to their expression of MHC-II and a group of co-stimulatory molecules (maturation markers) such as CD80, CD86 and CD40 [34]. The co-stimulatory molecules are cell surface-associated protein ligands that interact with special receptors on T cells including CD28 and CD40L. This interaction causes the formation of an immunological synapse and results in T cell activation, proliferation and cytokine release. The expression of MHC-II and co-stimulatory molecules on APC is essential for the initiation of the adaptive immune response. Dendritic cells (DCs) behave as the best professional APCs because they are the only cells that constitutively express MHC-II and co-stimulatory molecules [35]. In contrast, macrophages and B cells must first be activated in order to express these molecules. Dendritic cells originate in the bone marrow and are released into the blood stream as immature cells expressing low levels of co-stimulatory molecules. Immature dendritic cells (iDCs) are characterized by their high phagocytotic potential and their weak ability to activate T cells.

After FVIII treatment, the FVIII will get phagocytosed and processed by the iDCs in order to prepare FVIII peptides for T cell recognition [33]. After the internalization of FVIII, the FVIII molecules will be processed in the cellular lysosome into ~12 - 14 AA long peptides. These peptide fragments will then be loaded into the peptide-binding cleft of the MHC-II molecule. The peptide cleft will position the FVIII fragments in a specific orientation allowing the antigenic FVIII epitopes to be accessible to the T cell receptor (TCR). Following these initial events, DCs will over-express their maturation markers and traffic toward a secondary lymphatic system where antigen presentation occurs [35,36]. The presentation of FVIII will take place predominantly in the spleen, since most of the antigens that enter the blood will be taken up by APCs in this organ [37]. In the spleen, the DCs will enter the white pulp. Within the white pulp, the T cells and B cells are found in the periarteriolar lymphatic sheath (PALS) and germinal centers, respectively. In the PALS region, mature DCs will present FVIII to T cells resulting in the development of FVIII effector T cells. These FVIII effector T cells will interact with FVIII-specific B cells and promote B cell differentiation and the development of FVIII inhibitors.

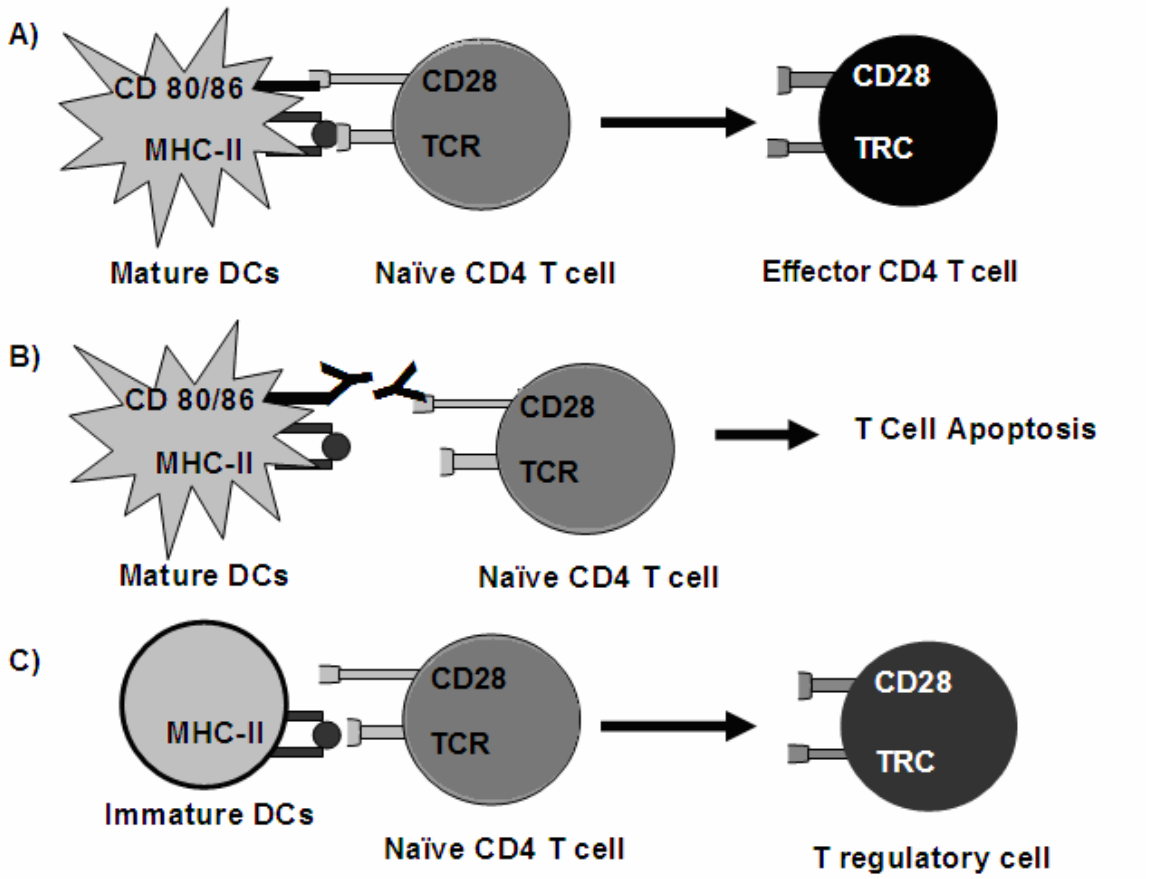
Details of The Cellular Interactions Between Dendritic Cells and T cells in Hemophilia Patients

There are 3 different signals that are required to activate FVIII-specific T cells. First, the TCR-MHC-II/FVIII peptide interaction will deliver signal 1 to the T cell [15]. Signal 1 is controlled by the phosphatidyl inositol pathway and causes the T cell

to initiate cytokine secretion. At this stage, the T cells are considered to be poised to become effector cells but are not yet fully activated. Second, to fully activate T cells, a second signal must be sent from the APC, called signal 2. Signal 2 is derived from the interaction between the co-stimulatory molecules found on the APC and their corresponding receptors on the T cell [15]. The principal co-stimulatory molecules are B7.1 (CD80)/ B7.2 (CD86) and CD40. CD80/CD86 and CD40 are all positive co-stimulatory molecules that bind CD28 and CD40L, respectively and enhance T cell activation and cytokine secretion. Finally, to enhance the process of T cell activation, a third signal, signal 3, has been recognized to play a key role in the APC-T cell interactive process. Signal 3 refers to the cytokine environment provided by the innate immune cells in the vicinity of the APC-T cell synapse (Figure 1.4 A). If an antigen was presented to T cells in the presence of IL12 cytokine, then the T cell will differentiate in to Th1 T cell. However, if the antigen was presented to T cells in the presence of IL4 cytokine, then the T cell will differentiate in to Th2 T cell.

It has been reported that when signal 2 is blocked, T cell anergy and apoptosis will occur [15] (Figure 1.4 B). In addition, an inhibitory co-stimulatory signal can develop in which CD80/CD86 can bind CD152 (CTLA-4) instead of CD28 and result in the generation of an inhibitory signal within T-cells that in turn causes the down regulation of cytokine secretion.

Figure 1.4 Importance of the co-stimulatory molecules. A) In a normal immune response, signal 1 and 2 will be delivered to the T-cell causing T cell activation. B) Blocking these signals via antibodies will prevent the activation of T cells resulting in T cell apoptosis. C) Immature dendritic cells which lack the co-stimulatory molecules will induce the development of T-regulatory cells.


A)
CD 80/86
MHC-II
CD28
TCR
CD28
TRC
Mature DCs
Naïve CD4 T cell
Effector CD4 T cell
B)
CD 80/86
MHC-II
CD28
TCR
T Cell Apoptosis
Mature DCs
Naïve CD4 T cell
C)
MHC-II
CD28
TCR
CD28
TRC
Immature DCs
Naïve CD4 T cell
T regulatory cell

Influences of Dendritic Cells on T cell Differentiation

In normal CD4+ T cell activation, the mature DCs (mDCs) must first bind to the T cell via adhesion molecules, such as ICAM-1 and LFA-1. These molecules will allow the interaction between MHC-II and TCR, resulting in the transmission of signal 1. This is followed by interactions between the co-stimulatory molecules and their appropriate receptors on T cell, to provide signal 2. This will result in the differentiation of naïve T cells into effector T cells that will secrete cytokines and induce antibody secretion. In contrast, immature DCs (iDCs) can also interact with T cells via MHC-II and TCR, with very low levels of co-stimulatory molecules, to induce the differentiation of T regulatory cells (Treg) as shown in (Figure 1.4 C) [38]. T-regulatory cells are a specialized subset of T cells that suppress the immune response and down regulate the activation of effector T cells, resulting in a state of immunologic tolerance [39].

Role of Dendritic Cells in Inducing Tolerance

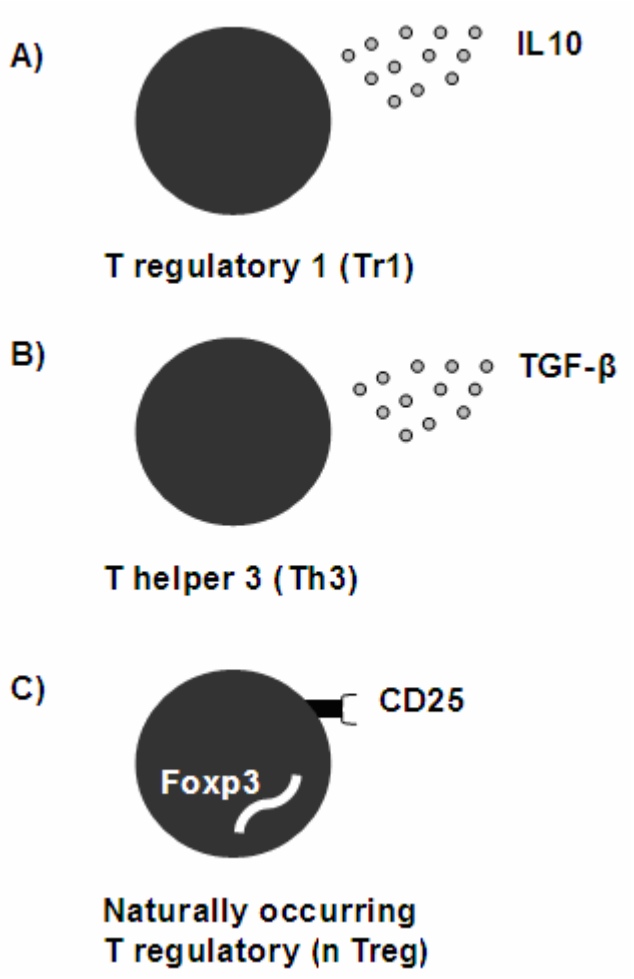
There are two main types of immunologic tolerance [39]. The first form involves central tolerance, which occurs early in life during the ontogeny of T cells. The second form of tolerance is peripheral tolerance, which occurs throughout the life span of an individual. Central tolerance is known to occur in the thymus and results in the deletion of self-reactive T cells. However, some T cells can escape this selection process. In this case, the Hassall's corpuscles in the thymus recruit immature dendritic cells to induce the differentiation of Treg cells that can in turn suppress the activity of self-reactive T cells [40]. Peripheral tolerance is also

maintained by Treg cells, which suppress the activation and clonal expansion of antigen-specific self-reactive CD4+ T cells [41]. Continuous Treg activation by the host system is not always beneficial, however, the absence of Tregs can lead to serious autoimmune diseases.

T-Regulatory Cell Involvement inThe Induction of Immune Tolerance

There are several different Treg cell populations described in the literature. Tregs are divided into different classes depending on their mechanism of tolerance induction. These classes include the IL-10 producing CD4+ Tr1 cells (Figure 1.5 A), the TGF-β producing CD4+ Th3 cells (Figure 1.5 B), and the naturally occurring $CD25^{+}Foxp3^{+}$ cells (Figure 1.5 C). The Tr1 and Th3 Tregs mediate their suppressive activities via the production of immunomodulatory cytokines. In contrast, the naturally occurring $CD25^{+}Foxp3^{+}$ T regulatory cell (nTreg) cells constitutively express CD25 and the forkhead transcription factor (Foxp3). These nTregs mediate their suppressive effect through direct cell-to-cell contact and do not necessarily require IL-10 or TGF-β production. Furthermore, nTreg cells develop in the thymus and make up 5-10% of the peripheral naïve CD4+ T lymphocyte pool in normal mice and humans. Interestingly, iDCs are known to expand the nTreg population [42]. These nTreg cells have been shown to be involved in inducing tolerance to the immunogenic A2 and C2 domains of FVIII[43,44].

Figure 1.5 The different subsets of T regulatory cells (Treg). (A) T regulatory 1 (Tr1) cells which releases interleukin 10 (IL10). IL10 is an immuno-modulatory cytokine which suppress the immune system. (B) T helper 3 (Th3) cells which releases Transforming Growth Factor Beta (TGF-β). TGF-β is an immuno-modulatory cytokine will suppress the immune system. (C) Naturally occurring CD25Foxp3 T regulatory cells (nTreg). CD25Foxp3 T regulatory cells suppress the specific CD4 effector T cells through direct cellular contact.


A)
IL10
T regulatory 1 (Tr1)
B)
TGF-β
T helper 3 (Th3)
C)
CD25
Foxp3
Naturally occurring
T regulatory (n Treg)

Modulation of The Immune System Through Manipulations of The Cytokine Microenvironment

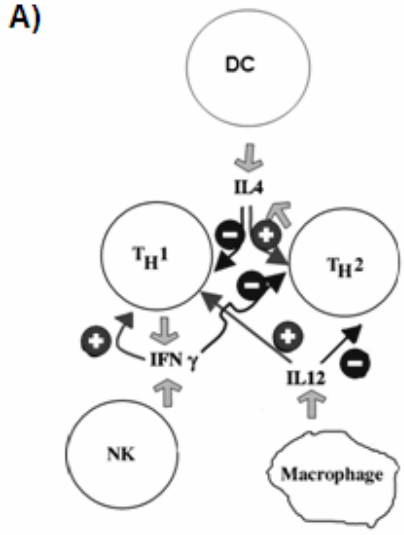
There is growing evidence that the cytokine microenvironment is a key contributor to the regulation of the immune system. Cells of the innate immune system will release cytokines that will promote the differentiation of CD4+ T cells into either T helper 1 cells (Th1), T helper 2 cells (Th2) or T regulatory cells [45,46] (Figure 1.6 A).

The presence of a “danger” signal induces the release of inflammatory cytokines directing the immune system towards an immunogenic response. The scavenger cells of the immune system will identify foreign molecules and initiate an inflammatory immune response. Pro-inflammatory cytokines such interferon gamma (IFN-γ), will activate the innate immune system and promote the activation of Th1 T cells. Th1 T cells release the essential cytokines needed for cytotoxic T cell immunity. The cytokine profile of Th1 T cells includes IL1, IL2, IL12 and IFN-γ (Figure 1.6 B). Th1 cytokines result in the activation of macrophages and the activation/proliferation of T cells. Furthermore, the Th1 cytokines will inhibit the activity of Th2 T cells. Th2 T cells are important in the generation of a humoral immune response. The cytokine profile of Th2 T cells includes IL4, IL5 and IL10 (Figure 1.6 B). These cytokines are important for B cell proliferation, differentiation and immunoglobulin isotype switching.

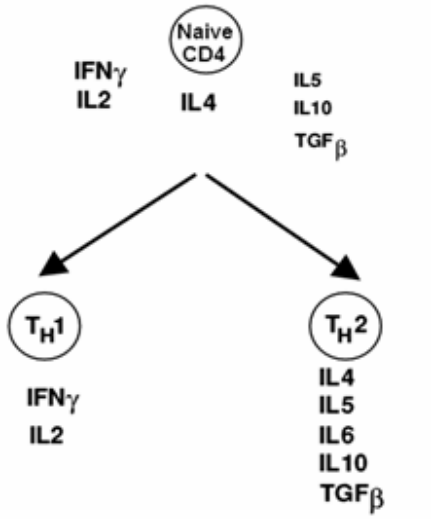
As mentioned earlier, the cytokine microenvironment controls the differentiation of CD4+ T cells into either Th1 or Th2 cells (signal 3). Signal 3 is initiated by the DCs and innate immunity. If IL12 is present during the antigenic

Figure 1.6 T helper 1 and T helper 2 CD4 T cells. (A) The influence of innate immunity on the differentiation of T cell. Dendritic cells (DCs) release IL 4 which promotes the development of Th2 and inhibits Th1. Natural Killer cells (NK) and Macrophage release IFN-γ and IL 12 which promotes the development of Th1 and inhibits Th2. (B) The cytokine profile of Th1 and Th2 T cells. Th1 are involved in inflammation and assisting cytotoxic T cells. Th2 are involved in the anti-inflammatory immune response. Th2 T cells are essential for the humoral immune system.

A)


DC
IL4
T_H1
T_H2
IFN γ
IL12
NK
Macrophage

B)


Naive CD4
IFNγ
IL2
IL4
IL5
IL10
TGFβ
T_H1
T_H2
IFNγ
IL2
IL4
IL5
IL6
IL10
TGFβ

presentation, then the CD4+ T cells will differentiate into Th1 T cells, resulting in a pro-inflammatory response. However, if IL4 is present during the antigenic presentation, the CD4+ T cells will differentiate into Th2 T cells resulting in an anti-inflammatory immune response. Therefore, proteins presented in the absence of an inflammatory "danger" signal will not cause the activation of the immune system, but will result in a state of tolerance.

The Objectives of This Thesis are:

1. To investigate the in vivo behavior of FVIII in different strains of hemophilia A mice. This will be achieved by identifying the differences in the activation of the immune response due to FVIII in each strain.
2. To study the reduction of the immunological response towards FVIII by the tolerogenic presentation of FVIII to the immune system. This will be achieved through studying the tolerogenic abilities of immature dendritic cells in inducing immune tolerance towards FVIII in naïve hemophilia A mice.
3. To investigate the incidence of FVIII inhibitor development in mice treated with either recombinant or plasma-derived FVIII products. This will include characterization of the splenic microenvironment in mice after the various FVIII treatments.

The results and knowledge derived from this thesis will further contribute to our understanding of the mechanisms involved in the immune response to FVIII and may help us develop improved treatments for hemophilic patients with FVIII inhibitors.

Chapter 2

Immunoglobulin Isotypes and Functional Fnti-FVIII Antibodies in Response to FVIII Treatment in Two Strains ofHemophilia A Mice

I performed all experiments. All animal techniques were performed by Erin Burnett and Andera Labelle

Abstract

Previous studies have demonstrated that genetic factors play an important role in determining the likelihood of formation of anti-FVIII antibodies in hemophilia A patients. We were interested in characterizing the spectrum of FVIII antibody responses after FVIII treatment in two different exon 16-disrupted hemophilia A mouse strains, Balb/c and C57BL/6. We were also interested in studying the primary and secondary cytokine responses towards FVIII in these mice. FVIII antibodies but not functional inhibitors were detectable one week after the first FVIII treatment in both strains. These antibodies mainly belonged to the IgM and IgA isotypes. After the fourth FVIII treatment, neutralizing anti-FVIII antibodies were detected in both mouse strains: Balb/c (mean inhibitory titer 68 BU) and C57BL/6 (mean inhibitory titer 82 BU). IgG1 levels were similar in both strains but the IgG2A and IgG2B subclasses were higher in C57BL/6 mice. The results of intracellular cytokine staining of T cells indicated that the FVIII-treated C57BL/6 mice produced more IL10 and Th1 cytokines than the FVIII-treated Balb/c mice. In summary, we expect the FVIII neutralizing antibodies in hemophilia A mice belong to IgG1 (Th2 dependent) and IgG2A (Th1 dependent) subclasses. These studies confirm the role of immunogenotypic determinants in the regulation of FVIII immunogenicity, and emphasize that strain-dependent differences in the immune response toward FVIII should be considered when evaluating immunological outcomes in mouse models of hemophilia A.

Introduction

Among the complications of current treatments for hemophilia A, the development of anti-FVIII antibodies including “FVIII inhibitors” remains the major clinical problem in treating these patients. FVIII inhibitors produce their effect through neutralizing the coagulation cofactor activity of the infused FVIII and preventing the restoration of normal hemostasis. An accepted estimate of the incidence of inhibitors is 25-30% of hemophilia A patients. Furthermore, anti-FVIII non-neutralizing antibodies can develop in patients treated with FVIII[47]. These antibodies will not inhibit the activity of FVIII, but they will shorten its half-life in the circulation[48]. Reding et al demonstrated the presence of different anti-FVIII isotypes in hemophilic patients. The authors observed mainly IgG1, IgG2 and IgG4 anti-FVIII in hemophilic patients[49]. However, It has been reported that humans and mice do not have one-to-one correspondence in terms of IgG subclasses[50]. Therefore, we aimed to learn more about the anti-FVIII isotypes in the E16-hemophilic mouse models.

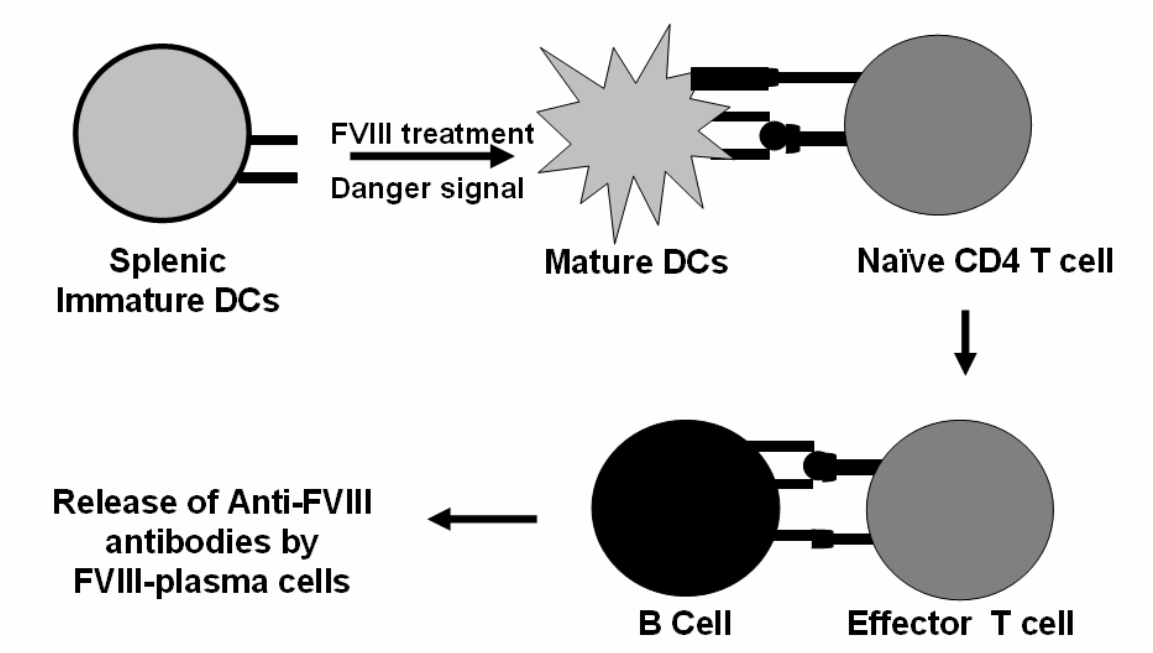
The range of FVIII antibody titer in hemophilic patients varies between different studies. It has been reported that the formation of FVIII antibodies depends on an assortment of variables including genetic and non-genetic factors. One approach to studying the genetic influence on FVIII antibody formation, takes advantage of the genetic homogeneity of inbred mouse models of hemophilia A.

Currently, there are two available hemophilia A mouse strains: Balb/c and C57BL/6. Each mouse strain develops a different pattern of immunological responses to antigens. Unlike C57BL/6 mice that are known to develop Th1

responses, Balb/c mice are known to develop Th2 responses. Furthermore, Balb/c and C57BL/6 mice have different MHC-II haplotypes; $H2^D$ and $H2^B$ respectively. This difference in MHC-II has been shown to have an influence on the cytokine production by T cells. Whereas, C57BL/6 are resistant to *Leishmania* infection due to their ability to produce IL12 and IFN-γ, Balb/c mice are known to be susceptible to this pathogen[51]. In contrast, Balb/c mice are resistant to *Toxoplasma gondii* infection due to their ability to produce IL-4, while C57BL/6 mice are more susceptible to infection due to the reduced production of IL4[52,53,54]. Moreover, it has been previously shown that the involvement of cells of the innate immune system, that regulate the differentiation of Th1 or Th2 T cells, are different in the two mouse strains and that this results in a distinct T cell immune response in each strain[55].

The activation of the immune system towards FVIII depends on the successful presentation of FVIII by antigen presenting cells (APC) such as dendritic cells. The presentation of FVIII on the MHC-II molecules of DCs and the interaction of the DC maturation molecules (such as CD40 and CD80) with their cognate receptors on T cells results in the activation of FVIII-specific CD4+ T cells. These activated FVIII-specific T cells will then interact with FVIII-specific B cells, inducing B cell differentiation and anti-FVIII antibody production by plasma cells as shown in Figure 2.1. Therefore, this sequential pathway represents several steps that are required for the development of FVIII antibodies. Also, it is very likely that the regulation of these steps is different in each mouse strain, resulting in a different immunological response.

Figure 2.1 The cellular interactions between dendritic cells, T cells and B cells which are essential for the formation of FVIII antibodies in hemophilic mice. First, DCs will present FVIII to FVIII-specific $CD4^+$ T cells resulting in T cell activation. Second, $CD4^+$ effector T cells will activate FVIII-specific B cells resulting in B cell differentiation and anti-FVIII antibody production by plasma cells


FVIII treatment
Danger signal
Splenic
Immature DCs
Mature DCs
Naïve CD4 T cell
Release of Anti-FVIII
antibodies by
FVIII-plasma cells
B Cell
Effector T cell

In this study, we investigated the immunological responses toward FVIII in E-16 hemophilia A Balb/c and C57BL/6 mice via treatment of these mice with a clinically relevant dose of FVIII. We believe that through characterizing genetic factors important in the formation of FVIII antibodies, we may have a better understanding of the pathophysiological mechanisms that govern this response. Two specific areas, the T cell cytokines and co-stimulatory markers on B cells, will be investigated in each mouse strain in order to determine their influence on the formation of FVIII antibodies. These results will add to the current knowledge concerning genetic factors influencing FVIII antibody formation in hemophilic mice and may provide insights into this problem in hemophilic patients.

Materials and Methods

Mouse Models

Male and female C57BL/6 E16-FVIIIKO ($H\text{-}2^B$) and Balb/c E16-FVIIIKO ($H\text{-}2^D$) mice were used throughout the study. These hemophilic mice were generated as previously described by Bi et al[56]. All mice were repeatedly assessed for the FVIII exon 16 deletion by polymerase chain reaction (PCR) on genomic DNA as previously described by Connelly et al[57]. All mouse experiments were performed in accordance with the Canadian Council for Animal Care and the Queen's University Animal Care Committee approved all animal protocols.

FVIII Treatment Regimen in Hemophilic Mice

Mice used in this experiment were 6-10 week-old male and female C57BL/6 and BALB/c E16-hemophilia A mice. All C57BL/6 and BALB/c hemophilic mice were treated intravenously via tail vein with 2 IU (~80 IU/kg: 200 ng) of human recombinant FVIII (rFVIII) (Kogonate-FS®) diluted in HBSS, in weeks (0, 2, 4, and 6). The mice were sampled in weeks (1, 3, 5, and 7). The negative control mice for this experiment received only 4 intravenous injections of 200 µL of HBSS in weeks (0, 2, 4, and 6).

Blood Sampling

Mice were anesthetized with 0.2cc hypnorm/water/midazolam (1:2:1) and blood samples were obtained using uncoated microhematocrit capillary tubes (Fisher Scientific Pittsburgh, PA, USA) via the retro-orbital plexus. Blood was mixed with 1/10 volume of 3.2% sodium citrate. Plasma was prepared by centrifugation of

blood samples at 10,000 RPM for five minutes at 4°C, frozen on dry ice and stored at -70°C until tested.

Measurement of FVIII Antibodies and FVIII Inhibitors in Mouse Plasma

Total anti-FVIII antibody titers were measured by ELISA. In brief, ELISA plates were coated over night at 4 °C with 10 IU/mL human recombinant FVIII (Kogenate-FS®). Microtiter plate wells were blocked using 10% PBS-BSA. Plasma samples were diluted in HBS-BSA buffer. The anti-FVIII antibody isotypes (IgA, IgM, IgG1, IgG2A and IgG2B) were studied using the clonotype kit (Southern Biotech, Birmingham, Alabama USA). Furthermore, plasma samples were analyzed for FVIII inhibitors using a one-stage FVIII clotting assay. In brief, plasma samples were diluted in HBS-BSA and mixed with human pooled plasma in a 1:1 ratio and incubated at 37°C for 2 hours. Samples were then assayed for residual FVIII activity using APTT reagent (Organon Teknika; Durham, NC, USA) in an automated coagulometer (General Diagnostics Coag-A-Mate, Toronto, Canada) following the manufacturer's protocol. Inhibitor titers (Bethesda Units) were calculated as detailed in the standard Bethesda protocol[58].

***In-vitro* Assessment of The Primary Immune Response**

In order to assess the primary immune response to FVIII in the two strains of hemophilic mice, cytokines released from splenocytes co-cultured with FVIII were studied as follows. Spleens were isolated from naïve hemophilic C57BL/6 and Balb/c mice between 6 and 10 weeks of age and splenocytces were cultured in RPMI 1640 (Invitogen Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Burlington, Ontario, Canada), 100 U/mL

penicillin/streptomycin (Invitrogen, Burlington, Ontario, Canada) and 50 μM 2 mercaptoethanol (Sigma-Aldrich St. Louis, MO USA) and 10 IU/mL of rFVIII. The cytokines were measured in the supernatant after 120 hours of culture. Culture media was studied for the presence of IL2, IL4, IL5, IL10 and IFN-γ (eBiosciences, San Diego, CA, USA) via ELISA according to the manufacturer's protocol.

***In-vivo* Assessment of The Secondary Immune Response**

In order to assess the secondary immune response to FVIII in hemophilic mice, splenic T cells isolated from FVIII immunized C57BL/6 and Balb/c mice were studied as follows. Hemophilic mice that were previously immunized with rFVIII were challenged intravenously with 2 IU rFVIII 4 months following their last FVIII treatment. Twenty four hours after the FVIII challenge, T cells were isolated from the spleens using the CD4 negative selection isolation kit (Miltenyi Biotec Bergisch Gladbach, Germany) following the manufacturer's protocol. All isolated T cell populations were >90% FITC-CD4+ as shown by flow cytometry assessment. The CD4+ cells were stained with specific antibodies against the intracellular cytokines IL2, IL4, IL10 and IFN-γ as described by the manufacturer (BD Biosciences, San Diego CA, USA). The cells were fixed using the Cytofix/Cytoperm fixation kit (BD Biosciences, San Diego CA, USA) and analyzed by flow cytometry within 12 hours.

Phenotypic Characterization of The *in-vivo* FVIII-Activated B Cells

In order to study the phenotype of B-cells in FVIII-treated hemophilic mice, mice that were previously immunized with rFVIII were challenged with 2 IU rFVIII 4

months after their last FVIII treatment. Twenty four hours following the FVIII challenge, the CD19+ B cells were isolated from the spleens using magnetic beads coated with anti-CD19 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's guidelines. All isolated B cell populations were >90% FITC-CD19+ as shown by flow cytometry assessment. The co-stimulatory molecules CD40 and CD80 (BD Biosciences, San Diego CA, USA) were fluorescently labeled with PE in order to identify the activation stage of B cells. The cells were fixed using the Cytofix/Cytoperm fixation kit (BD Biosciences, San Diego CA, USA) and were analyzed by flow cytometry within 12 hours.

Results

FVIII Antibodies and Inhibitors in FVIII-Treated Balb/c and C57BL/6 Hemophilic Mice

The levels of FVIII antibodies and inhibitors were measured after treating hemophilic mice with four biweekly intravenous infusions of 2 IU FVIII. Interestingly, after the first and second FVIII treatments, both mouse strains developed IgM and IgA FVIII antibodies as summarized in table 1. However, the mice at that time had no evidence of FVIII inhibitory antibodies. These data suggest that the anti-FVIII IgM and IgA antibodies represent non-inhibitory antibodies. However, after the 3rd and 4th FVIII treatments, our results demonstrate that C57BL/6 mice produce higher FVIII inhibitory and total antibody titers than the Balb/c mice Table 2.1 and Figure 2.2 A. This finding indicates that there is an association between the genetic background of E16 hemophilic mice and FVIII antibody formation. Interestingly, after the four FVIII treatments in each of the C57BL/6 and Balb/c mice, there was a direct relationship between FVIII inhibitor formation and the IgG1 subclass (Th2 predominant) and as well as the IgG2A and IgG2B subclasses (Th1 predominant). Moreover, in the C57BL/6 mice we observed an increase in inhibitor formation as the IgG2A/IgG1 and IgG2B/IgG1 ratios increase. This finding indicates that there is an association between the Th1-dependent IgG subclasses and FVIII inhibitors. In contrast, the FVIII inhibitory antibodies in humans belong to IgG1 and IgG4 subclasses[17]. Our studies confirm the results obtained by Rossi et al. and are consistent with the

association of FVIII inhibitory activity in IgG1, IgG2A and IgG2B subclasses in hemophilic mice[59].

Table 1. The development of FVIII antibodies (ng/mL) and inhibitors (BU) in Balb/c (A) and C57BL/6 (B) hemophilic mice after biweekly FVIII treatments. Mice were sampled 1 week after each FVIII treatment and the anti-FVIII isotypes were measured via ELISA. The amount of IgA, IgM, IgG2A and IgG2B were quantified by the ELISA standard curve

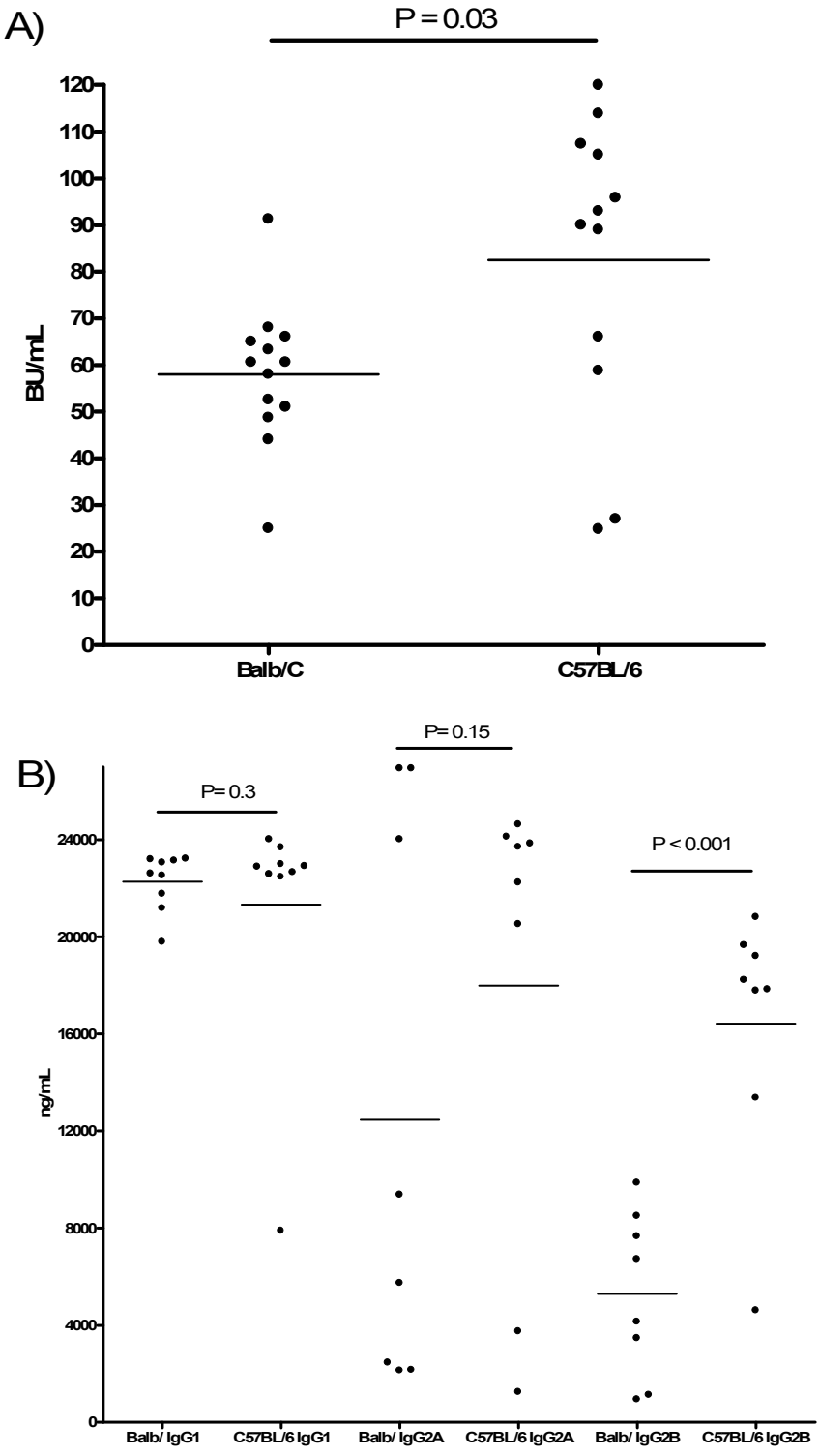
A) FVIII antibody and inhibitor titer (ng/mL) in Balb/c mice after 1st, 2nd and 3rd FVIII treatments:

	IgA	IgM	IgG1	IgG2A	IgG2B	BU
1st treatment	231 ±10	2788 ± 50	0	0	0	0
2nd dose	717 ±12	5200 ± 53	0	0	0	0
3rd dose	2800 ± 198	8060 ± 278	14800 ± 9000	700 ± 300	1435 ± 500	22

B) FVIII antibody and inhibitor titer (ng/mL) in C57BL/6 mice after 1st, 2nd and 3rd FVIII treatments:

C57BL/6	IgA	IgM	IgG1	IgG2A	IgG2B	BU
1st treatment	130 ± 70	1089 ± 172	0	0	0	0
2nd treatment	400 ± 21	2440 ± 97	0	0	0	0
3rd treatment	2390 ± 345	10760 ± 3546	18211 ± 5046	1527.8 ± 550	2494.1 ± 130	28

Figure 2.2 Comparison of FVIII inhibitors (A) and anti-FVIII IgG subclasses (B) in C57BL/6 and Balb/c hemophilia A mice after four FVIII intravenous treatments. Naïve hemophilia A Balb/c and C57BL/6 mice were treated with four biweekly FVIII infusions. Mice were sampled 1 week after the fourth FVIII treatment. The horizontal bars represent the mean antibody titers for 13 mice.


A)
P = 0.03
BU/mL
120
110
100
90
80
70
60
50
40
30
20
10
0
Balb/C
C57BL/6
B)
P= 0.3
P= 0.15
P < 0.001
ng/mL
24000
20000
16000
12000
8000
4000
0
Balb/ IgG1
C57BL/6 IgG1
Balb/ IgG2A
C57BL/6 IgG2A
Balb/ IgG2B
C57BL/6 IgG2B

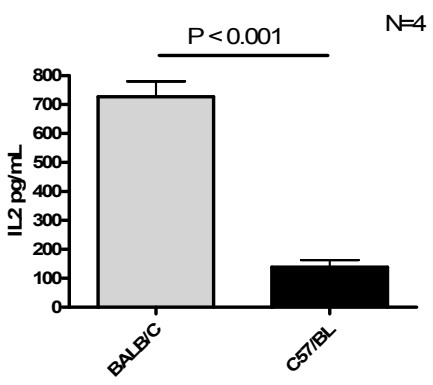
Cytokine Profile in Splenocytes Isolated From Naïve Hemophilic Balb/c and C57BL/6 Mice

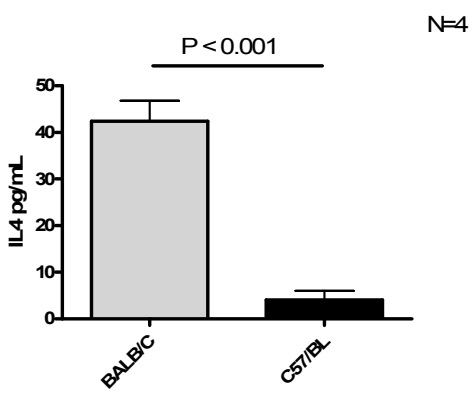
The primary immune response to FVIII was studied in the two different mouse strains. Splenocytes isolated from both strains of mice released cytokines following infusion of FVIII. However, based on the cytokine profiles, our results show that the Balb/c mice had a stronger Th2 and Th1 response than the C57BL/6 mice (Figure 2.3). Interestingly, we were not able to detect IL10 in either set of splenocyte cultures. These studies indicate that the Balb/c mice have a stronger primary immune response to FVIII than the C57BL/6 mice and that this response is not dependent on IL10. However, since total splenocytes were used, we were not able to identify which cell(s) released these cytokines.

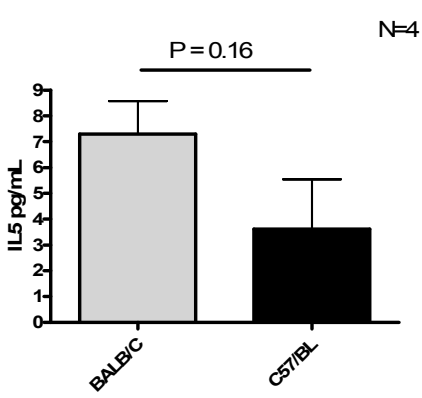
Cytokine Profile in CD4 T cells Isolated From FVIII-Treated Hemophilic Balb/c and C57BL/6 Mice

The secondary immune response due to FVIII was studied in each mouse strain. In order to investigate the cytokine release by T cells due to FVIII, we stained CD4 T cells for the presence of intracellular cytokines. The Th1 and Th2 cytokines were examined in vivo after challenging the FVIII immunized mice with FVIII. We observed that both mouse strains produced Th1 and Th2 cytokines as shown in (Figure 2.4). In the secondary immune response, we observed more of the Th1 and Th2 cytokines in the C57BL/6 than Balb/c mice. These studies

Figure 2.3 Detection of Th1 and Th2 cytokines released by splenocytes isolated from C57BL/6 and Balb/c hemophilia A mice after 120 hrs of culture in the presence of 1ug/mL of FVIII. The Th1 cytokines IL2 and IFNγ, and the Th2 cytokines IL4 and IL5 were quantified by ELISA. An IL10 ELISA was also performed but IL10 was not detected in either set of cultures. The error bars represent the standard error of the mean for four mice.


N=4
P < 0.001
IL2 pg/mL
800
700
600
500
400
300
200
100
0
BALB/C
C57/BL


N=4
P < 0.001
IL4 pg/mL
50
40
30
20
10
0
BALB/C
C57/BL


N=4
P = 0.16
IL5 pg/mL
9
8
7
6
5
4
3
2
1
0
BALB/C
C57/BL

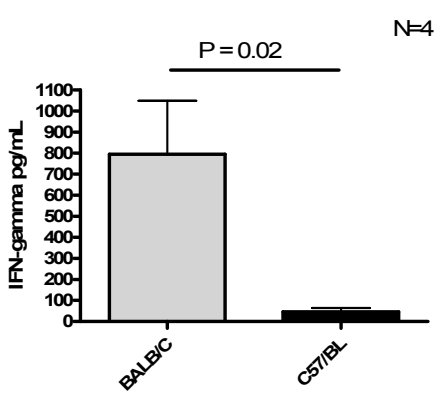
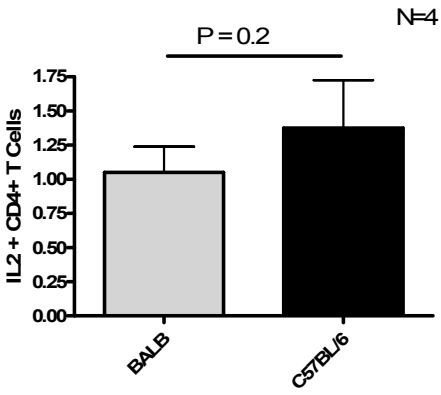
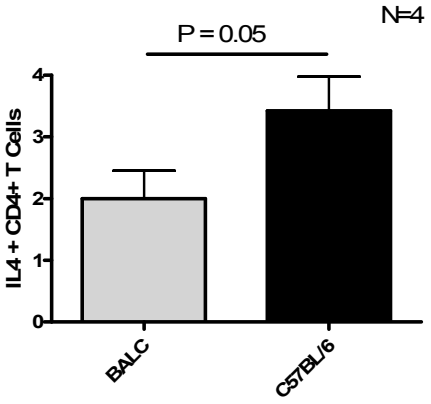
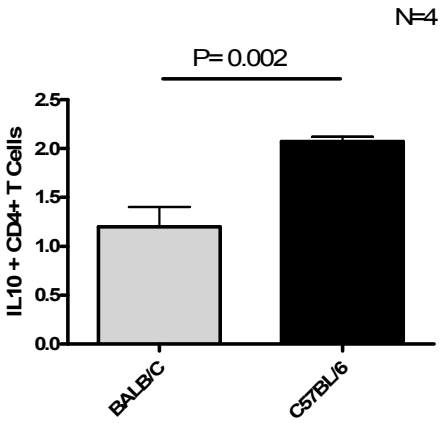
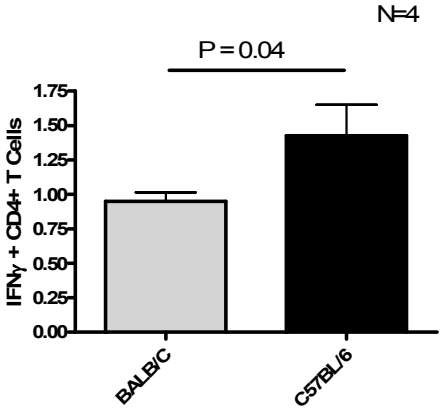

N=4
P = 0.02
IFN-gamma pg/mL
1100
1000
900
800
700
600
500
400
300
200
100
0
BALB/C
C57/BL

Figure 2.4 Detection of intracellular Th1 and Th2 cytokines produced by CD4+ T cells isolated from C57BL/6 and Balb/c mice that had previously been immunized with intravenous FVIII. Mice were immunized with four intravenous FVIII infusions; Four months later, mice were re-challenged with 2 IU FVIII and the CD4+ T cells were purified from the spleens. The Th1 cytokines IL2 and IFNγ, and the Th2 cytokines IL4 and IL10 were quantified by flow cytometry. The error bars represent the standard error of the mean for four mice.


N=4
P = 0.2
IL2 + CD4+ T Cells
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
BALB
C57BL/6


N=4
P = 0.05
IL4 + CD4+ T Cells
4
3
2
1
0
BALC
C57BL/6


N=4
P= 0.002
IL10 + CD4+ T Cells
2.5
2.0
1.5
1.0
0.5
0.0
BALB/C
C57BL/6

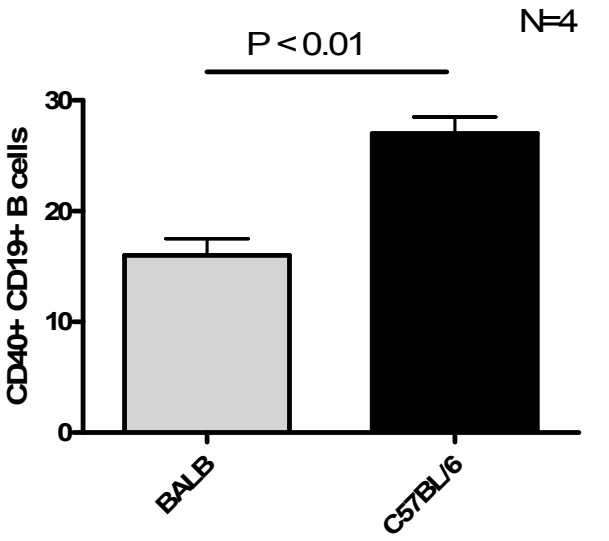

N=4
P = 0.04
IFNγ + CD4+ T Cells
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
BALB/C
C57BL/6

demonstrate that the C57BL/6 mice have a stronger Th1 and Th2 secondary immune response to FVIII than the Balb/c mice

B cell Maturation in FVIII-Immunized C57BL/6 and Balb/c Mice

The maturation state of B cells due to FVIII exposure was studied in each mouse strain. The B cells were isolated from FVIII-treated and challenged mice. The cells were then stained for CD40 and CD80. It was observed that the B cells isolated from the C57BL/6 mouse strain had higher expression of the CD40 maturation molecule than those isolated from Balb/c mice (Figure 2.5). Regarding the CD80 maturation marker on B cells, even though the expression was higher on C57BL/6 B cells than those of Balb/c mice, it was not statistically significant. These results show that FVIII challenge causes a higher expression of CD40 on B cells obtained from C57BL/6 than in Balb/c mice.

Figure 2.5 Assessment of B cell maturation after a FVIII re-challenge in C57BL/6 and Balb/c hemophilic mice that had been previously immunized with FVIII. Mice were immunized with four FVIII intravenous infusions; 4 months later, mice were re-challenged with 2 IU FVIII and CD19+ B cells were purified from the spleens. CD80 and CD40 maturation markers were quantified by flow cytometry. The error bars represent the standard error of the mean for four mice.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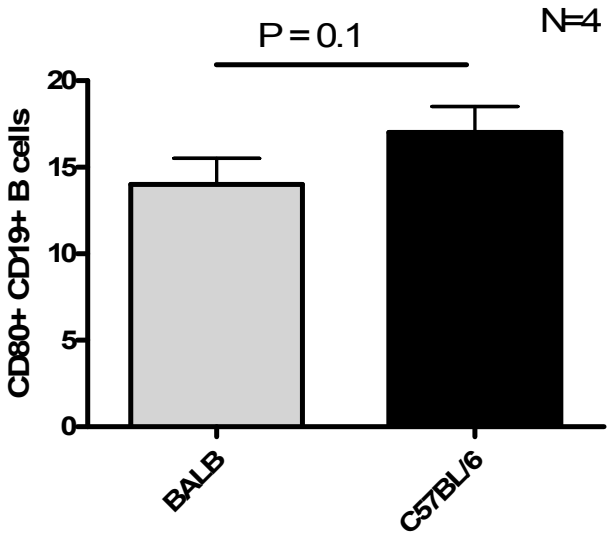
P < 0.01
CD40+ CD19+ B cells
30
20
10
0
BALB
C57BL/6


N=4
P = 0.1
CD80+ CD19+ B cells
20
15
10
5
0
BALB
C57BL/6

Discussion

The availability of hemophilia A mouse models has contributed significantly to the current understanding of the immunology of FVIII. Studying the immunogenicity of FVIII in different mouse strains is important since each model has a different MHC-II haplotype and a distinct global immunophenotype. These genetic factors have been studied previously in human hemophiliacs and a weak association has been found between the development of FVIII antibodies and the HLA haplotype in the patients[60]. Therefore, gaining more knowledge about the influence of the genetic background on the immunogenicity of FVIII will help us gain an improved understanding of the mechanisms responsible for the development of FVIII antibody responses.

The balance between Th1 and Th2 immune responses has been previously associated with the FVIII antibody formation in E17-hemophilic mouse model [61]. We used two hemophilic mouse strains the Balb/c mice (Th2 prone) and the C57BL/6 mice (Th1 prone) in order to investigate the importance of the genetic background on FVIII antibody formation in E16 hemophilic mice. The studies performed on total splenocytes isolated from C57BL/6 and Balb/c mice cultured in the presence of FVIII showed higher Th1 and Th2 primary immune cytokine profiles in the Balb/c mice (Figure 2.3). Therefore, we expect that the increased release of Th1 and Th2 cytokines in the primary immune response in Balb/c mice is responsible for the observed higher titers of the IgM and IgA isotypes after the first FVIII treatment[62,63]. It was previously reported that after antigen stimulation,

naïve splenocytes isolated from Balb/c mice produced more IFN-γ and other cytokines than the C57BL/6 splenocytes[64]. The mechanisms responsible for this finding are not fully understood. Differences in the innate immune response to FVIII between the mouse strains may play an important role in this difference. The natural killer cell responses in Balb/c mice are more rapid and pronounced than those seen in C57BL/6 mice[65]. Also, there are differences in Toll Like Receptor (TLR) expression patterns between the mouse strains[66]. These differences in TLR expression and other pathogenic recognition receptors (PRR) expression were reported to influence the cytokine release in splenocyte cultures of Balb/c and C57BL/6 after antigen or LPS stimulation[67].

For the secondary immune response, our results show that the anti-FVIII antibodies detected after the 4th FVIII treatment, mainly belonged to the IgG1, IgG2A and IgG2B subclasses Figure 2.2 (B). Moreover, we observed that the Balb/c mice had lower (IgG2A/IgG1) and (IgG2B/IgG1) ratios than the C57BL/6 mice. These results indicate that in Balb/c mice, the IgG1 subclass is the major inhibitory isotype, whereas, in C57BL/6 mice, the IgG1, IgG2A and IgG2B subclasses all appear to inhibit FVIII activity. From the Ig ratio calculations, it appears that IgG2A and IgG2B have a more significant role in inhibiting FVIII's activity in C57BL/6 than in Balb/c mice. Interestingly, the secondary cytokine response towards FVIII was also different for the two mouse strains. The C57BL/6 mice had more T cells producing Th1 and Th2 cytokines than was

documented in the Balb/c mice (Figure 2.4). This phenomenon likely contributes to the higher FVIII antibody titers observed in C57BL/6 mice[61,68,69].

It has been previously suggested that the increased incidence of FVIII inhibitors seen in mice might be due, at least in part, to the increased production of IFN-γ in these animals[68]. This is supported by our data showing that the FVIII inhibitory antibodies mainly belonged to the IFN-γ dependent IgG2A subclass. Our findings confirm the data obtained by Sasgary and Rossi et al. and support the involvement of Th1 cytokines in FVIII antibody generation. Furthermore, expression of the CD40 co-stimulatory molecule on B cells also plays a role in regulating the immune system[70]. The CD40 molecule on APCs binds CD40L on T cells and facilitates the activation of effector cells. It has been previously reported that CD40 regulates the production of type I IFN cytokines, resulting in the regulation of the immune system[70]. Since the B cells isolated from C57BL/6 FVIII-treated mice had higher levels of CD40 expression, we expect that these cells will generate increased IFN cytokine release and higher FVIII antibody formation.

The present mouse study agrees with the course of immune response effects documented in hemophilic patients. In both instances, FVIII increases the production of IFN-γ and IL4 by hemophilic CD4+ T cells[69]. In a previous computerized study, White et al reported that specific motifs of FVIII are recognized by particular HLA-DR molecules[20]. Moreover, Hay et al reported an

association between the HLA DRB1*1501 molecule and inhibitor formation in hemophilia A patients[21]. We have also observed an effect of the background immunogenotype in this study of two inbred hemophilic mouse strains. The C57BL/6 (MHCII-$H2^B$) had a higher FVIII antibody and inhibitor titer than the Balb/c (MHCII-$H2^D$) mice. We hypothesize that more T cell epitopes are presented by the $H2^B$ haplotype in the C57BL/6 mice resulting in the activation of more CD4+ T cells which release IFN-γ and other essential factors for the generation of FVIII antibodies in the C57BL/6 strain.

In summary, we have shown that the genetic background of hemophilic mice influences the formation of FVIII antibodies; after four intravenous FVIII treatments, the Th1 prone C57BL/6 mouse strain releases more IFN-γ, resulting in higher anti-FVIII inhibitory titers in comparison to the Th2 prone Balb/c mice. Also, we have shown that FVIII inhibitors mainly belong to the IgG1, IgG2A and IgG2B antibody subclasses in the E16 hemophilic mice and that these inhibitors will only be seen after the third FVIII challenge. These studies confirm the complex role of immunogenotypic factors on the likelihood for FVIII antibody generation. They also emphasize that the background strain of the hemophilic mice must be taken into account when results of FVIII immunogenicity are being evaluated.

Chapter 3

Reduction of The ImmuneResponse to Factor VIII Mediated Through Tolerogenic Factor VIII Presentation by Immature Dendritic Cells.

This chapter has been submitted for publication in the journal of thrombosis and haemostasis

I performed all experiments. Kirsten Walker helped me with the Andrographolide studies. All animal techniques were performed by Andera Labelle

Abstract

Development of neutralizing antibodies to factor VIII (FVIII) represents the most serious treatment complication of hemophilia A. We have explored, for the first time, the potential of using immature dendritic cells (iDCs) to present factor VIII in a tolerogenic manner to T cells. iDCs were isolated from hemophilic murine bone marrow and pulsed with canine FVIII (cFVIII-iDCs) in the presence or absence of the NFκB pathway blocking compound andrographolide (Andro-cFVIII-iDCs). We have shown that cFVIII in the presence or absence of Andro, is efficiently taken up by iDCs and that this process does not result in the maturation of DCs or the activation of co-cultured T cells. Three weekly intravenous infusions of one million FVIII pulsed-iDCs were administered to a group of five hemophilic Balb/c mice. Anti-FVIII antibody levels were monitored by functional Bethesda assay after four weekly IV challenges with 2IU of cFVIII. Following repeated infusion of the cFVIII-iDCs and Andro-cFVIII-iDCs into hemophilic mice that were subsequently challenged with cFVIII, long-term reductions of FVIII inhibitors of 25 and 40% respectively were documented. Studies of cytokine release and T cell phenotypes indicate that the mechanisms responsible for reducing immunologic responsiveness to FVIII appear to involve an expansion of Foxp3 T regulatory cells in the case of cFVIII-iDC infusion and the elaboration of the immunosuppressive cytokines IL-10 and TGF-β following andrographolide-treated cFVIII-iDCs. This study shows that tolerogenic presentation of FVIII to the immune system can significantly reduce immunogenicity of the protein.

Introduction:

Hemophilia A is the most common severe inherited bleeding disorder. Bleeding episodes in hemophilic patients are treated with the infusion of factor VIII (FVIII) concentrates. The current most significant challenge in the clinical care of hemophilia A patients is the generation of adverse immunological responses to the infused therapeutic FVIII concentrates. The available treatments for hemophilia A patients who develop anti-FVIII antibodies (also referred to as inhibitors) include: immune tolerance induction using high concentrations of FVIII [27], immunoadsorption of the factor VIII antibodies [17], the use of less immunogenic products such as porcine FVIII [17] and FVIII bypassing agents such as recombinant FVIIa and FEIBA. However, these approaches are not always effective and they are all expensive. The induction of immunological tolerance to FVIII prior to the initiation of replacement therapy would represent a novel strategy to prevent FVIII inhibitor development.

Severe hemophilia A patients have no circulating functional FVIII, and thus infused FVIII may be viewed as a foreign protein. After a number of FVIII infusions, approximately 25% of patients experience activation of the immune system and the formation of neutralizing anti-FVIII inhibitors. In patients with inhibitors, the infused FVIII will be sampled by a specialized subset of cells, called antigen presenting cells (APCs). Through receptor-mediated endocytosis, these cells have the ability to process and present antigens such as infused FVIII to T cells in the context of Major Histocompatibility Class II (MHC-II) molecules

[14,33]. In the appropriate microenvironment, this antigen presentation will activate CD4+ T helper cells causing the T cells to clonally expand and release cytokines that will recruit B cells. Additional interactions then occur between CD4+ T helper cells and B cells in order to permit B cell activation, clonal expansion and cellular differentiation into FVIII antibody-producing plasma cells. The released antibodies will bind to FVIII molecules to neutralize their cofactor function and, in some instances, accelerate FVIII clearance from the circulation.

Dendritic cells (DCs) are professional antigen presenting cells that play a central role in directing T cells towards either immunity or tolerance. These cells have the ability to engulf and present antigens to CD4+ T cells in the context of MHC-II molecules resulting in T cell activation and cytokine release. Dendritic cells originate in the bone marrow and are released into the blood stream as immature cells expressing low levels of cell surface co-stimulatory molecules. Immature DCs (iDCs) are characterized by their ability to sample antigens, however, in the absence of inflammatory “danger” signals, antigen presentation by iDCs does not result in effector T cell activation. In contrast, mature dendritic cells have the ability to present antigens to T cells in an immunogenic fashion with subsequent T cell activation.

If antigens such as FVIII are internalized by iDCs in the absence of an inflammatory response, immunologic tolerance towards the presented antigen can be mediated by a number of mechanisms that include the development of T

cell anergy (functional inhibition), T cell apoptosis and, perhaps most importantly, the generation of T-regulatory cells (Tregs) [42,71,72]. This lack of an immunological response is thought to depend principally on the development of Foxp3+ Tregs since iDCs are known to expand this naturally occurring Treg (nTreg) population [42]. Recently, it has been demonstrated that nTregs are involved in inducing tolerance to the A2 and C2 domains of FVIII [43,44].

The conditions under which DCs are cultured have a significant impact on the behavior of DCs in promoting either immunity or tolerance. For example, if DCs are cultured with pro-inflammatory mediators such as interferon or lipopolysaccharide (LPS), they will mature and increase their expression of the co-stimulatory molecules CD80/CD86 [73]. In contrast, if DCs are cultured in sterile/non-inflammatory conditions, they will maintain their immature phenotype and their tolerogenic potential will be enhanced [74]. Anti-inflammatory agents such as the Chinese herb extract NFκB pathway inhibitor (Andrographolide) have been described to enhance the tolerogenic abilities of DCs by inhibiting their maturation. Andrographolide (Andro) covalently modifies NFκB, inhibiting its binding to its DNA target sequence [75]. In this study, we have evaluated the tolerogenic potential of iDCs to modulate the immune response to FVIII in hemophilia A mice.

Materials and Methods:

Animals

Male and female Balb/c hemophilia A mice, 6-10 weeks old were used in all experiments. Mouse genotype was assessed for the deletion of FVIII exon 16 by polymerase chain reaction (PCR) using genomic DNA isolated from tail clips as described by Connelly et al [57]. All mouse experiments were performed in accordance with the Canadian Council for Animal Care and the Queen's University Animal Care Committee approved all animal protocols.

Generation of Bone Marrow DCs.

Hematopoietic cells were isolated as described by Inaba et al [76] with minor modifications. Briefly femurs from hemophilic Balb/c mice were isolated and flushed with RPMI 1640 (Invitogen Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Burlington, Ontario, Canada), 100U/mL penicillin/streptomycin (Invitrogen, Burlington, Ontario, Canada) and 50 μM 2 mercaptoethanol (Sigma-Aldrich St. Louis, MO USA). Bone marrow hematopoietic cells at a concentration of 1 million cells/mL were cultured at 37°C in media containing a cytokine environment of 1000 IU/mL of each of interleukin 4 (IL-4) (BD Biosciences, San Diego CA, USA) and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (BD Biosciences, San Diego CA, USA) for 5 days. The culture regimen was designed so that the cells were fed on days 0, 2 and 4. On day 5, loosely-adherent cells were aspirated and washed four times with HBSS (GIBCO BRL, Rockville, MD). DCs were isolated using magnetic

beads coated with anti-CD11c antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's guidelines. All isolated DC populations were >90% CD11c+ by flow cytometry assessment.

Phenotypic Characterization of Immature Dendritic Cells by Flow Cytometry.

DCs harvested on day 5 of culture were stained with specific antibodies against the DC marker, PE-CY7 -CD11c (BD Biosciences San Diego CA, USA). Cell surface proteins such as MHC-II and the co-stimulatory molecules CD86, CD80 and CD40 (BD Biosciences, San Diego CA, USA) were fluorescently labeled with PE or FITC in order to identify the maturation stage of dendritic cells. The cells were fixed with 1% paraformaldehyde (Sigma-Aldrich St. Louis, MO USA) and analyzed by flow cytometry.

Internalization of a FVIII-GFP Fusion Protein.

The internalization of FVIII by iDCs was evaluated using a FVIII-GFP fusion protein. Baby hamster kidney cells transfected with FVIII-GFP were generously provided by Dr. Pete Lollar. The FVIII-GFP was isolated as described by Lin et al. with minor modifications [77]. Briefly, the FVIII-GFP transfected cells were cultured in DMEM:F-12 containing 1.5mg/mL AlbuMAX I (lipid-rich bovine serum albumin) (Invitrogen, Auckland, New Zealand). The media was collected every 24 hours and replaced with fresh media for three consecutive days The media was centrifuged at 350 x g to remove cellular debris, and the media was immediately

loaded onto a SP-Sepharose Fast Flow column (GE Healthcare Bio-Sciences, Uppsala, Sweden) to purify the GFP-FVIII. The final purified eGFP-hFVIII was also analyzed by chromogenic assay (Coamatic, Chromogenix/Diapharma Group, West Chester, OH), Bradford assay and SDS-10% PAGE. One million iDCs were pulsed with 50IU FVIII-GFP for 2 hours. The pulsed cells were washed extensively and examined for the presence of intracellular GFP by flow cytometry.

In-vitro Pulsing of DCs With Canine Factor VIII (cFVIII)

The DCs were pulsed with canine FVIII. The cFVIII was prepared as follows. 293T cells expressing cFVIII gene were grown with D 10 medium. The media was collected every 24 hours and replaced with fresh media for three consecutive days. Subsequently, the media was centrifuged at 350 x g to remove cellular debris. The cFVIII protein was purified using a HiTrap Chelating HP column as previously described by Shibata et al [78]. The final purified cFVIII was analyzed by chromogenic assay (Coamatic, Chromogenix/Diapharma Group, West Chester, OH), Bradford assay and SDS-10% PAGE. One million DCs were pulsed with canine FVIII by culturing the iDCs with 10µg/mL cFVIII for 12-14 hours (cFVIII-iDCs). Mature, pulsed dendritic cells (mDCs) were generated by culturing the iDCs with 0.1µg/mL LPS (Sigma-Aldrich St. Louis, MO USA) and 10µg/mL cFVIII for 12-14 hours (cFVIII-mDCs). In some experiments, iDCs were pre-treated with 10 µM andrographolide [79] (Sigma-Aldrich St. Louis,

MO USA) for 12 hours followed by pulsing with 10μg/mL cFVIII for 12-14 hours (Andro-cFVIII-iDCs).

In-vitro Assessment of T cell Activation by iDCs.

The tolerogenic ability of DCs was studied in vitro via cytokine assays in T cell-iDC co-cultures. CD4+ T cells were isolated from naïve hemophilia A mouse spleens using CD4 negative selection magnetic beads (Miltenyi Biotec Bergisch Gladbach, Germany) according to the manufacturer's protocol. Unprimed, syngeneic CD4+ T cells were co-cultured with cFVIII-pulsed iDCs in a ratio of 10:1 (T cells: cFVIII-iDCs)[74] for 72 hours in X-Vivo serum- free medium (Cambrex Bio Science, Walkersville, Inc.; MD USA). The supernatant was collected after 24 hours to detect IL-4 and IL-6, after 48 hours to detect IL-10 and IFN-γ, and after 72 hours to detect TGF-β. The positive and negative controls for this experiment included CD4+ T cells co-cultured with cFVIII-mDCs and unpulsed iDCs, respectively. Culture supernatants were analyzed by ELISA for the presence of IL-6, IFN-γ and IL-10 (eBiosciences, San Diego, CA, USA) and IL-4 and TGF-β (R&D systems Minneapolis, USA) according to the manufacturer's protocol.

Injection of DCs into Hemophilia A Mice and In vivo Challenge with cFVIII.

cFVIII-iDCs or Andro-cFVIII-iDCs were isolated and washed extensively to remove excess cFVIII. Cells were counted and re-suspended in HBSS to obtain a concentration of one million cells per 200μL. One million cFVIII-iDCs or Andro-cFVIII-iDCs were administered via tail vein injection to a group of 5 mice, weekly

for 3 weeks. As a negative control, an independent group of 5 mice received 3 weekly infusions of one million unpulsed iDCs.

To assess the induction of immune tolerance toward FVIII by FVIII-iDCs or Andro-cFVIII-iDCs, hemophilic mice were challenged weekly for 4 weeks or 8 weeks ,respectively, with 2 IU (200 ng: equivalent to 100 IU/kg) cFVIII via tail vein injections (the Andro-cFVIII-iDC mice received a different batch of cFVIII that required 8 weekly IV infusions to generate cFVIII inhibitors). Alternatively, as a positive control, one million cFVIII-mDCs were injected via the tail vein into 5 hemophilic mice in order to assess the ability of cFVIII-mDCs to activate the immune system. The negative control mice for this experiment received 4 intravenous injections of 200 µL of HBSS.

Blood Sampling from Mice*.*

Mice were anesthetized with 0.2cc hypnorm/water/midazolam (1:2:1) and blood samples were obtained using uncoated microhematocrit capillary tubes (Fisher Scientific Pittsburgh, PA, USA) via the retro-orbital plexus. Blood was mixed with 1/10 volume of 3.2% sodium citrate. Plasma samples were isolated by centrifugation of blood samples at 10,000 RPM for three minutes at 4°C, frozen on dry ice and stored at -70°C until tested.

Measurement of cFVIII Inhibitors in Mouse Blood Samples*.*

Plasma samples were analyzed for cFVIII inhibitors using a one-stage FVIII clotting assay. In brief, plasma samples were diluted in HBS-BSA and mixed with

canine pool plasma in a 1:1 ratio and incubated at 37°C for 2 hours. Samples were then assayed for residual FVIII activity using APTT reagent (Organon Teknika; Durham, NC, USA) in an automated coagulometer (General Diagnostics Coag-A-Mate, Toronto, Canada) following the manufacturer's protocol. Inhibitor titers (Bethesda Units) were calculated as detailed in the standard Bethesda protocol [58].

Phenotypic Characterization of the Naturally Occurring T Regulatory Cells.

T cells were isolated from hemophilic mouse spleens via the use of a CD4 negative selection isolation kit (Miltenyi Biotec Bergisch Gladbach, Germany) following the manufacturer's protocol. The T cells were stained with specific antibodies against the T regulatory cell markers CD4, CD25 and Foxp3 as described by the manufacturer (eBiosciences, San Diego, CA, USA).

Statistical Analysis.

All data are presented as mean ± standard error of the mean (SEM). Statistical comparisons of experimental groups were evaluated with a Student T test. A p value of less than 0.05 was considered statistically significant and indicated by *.

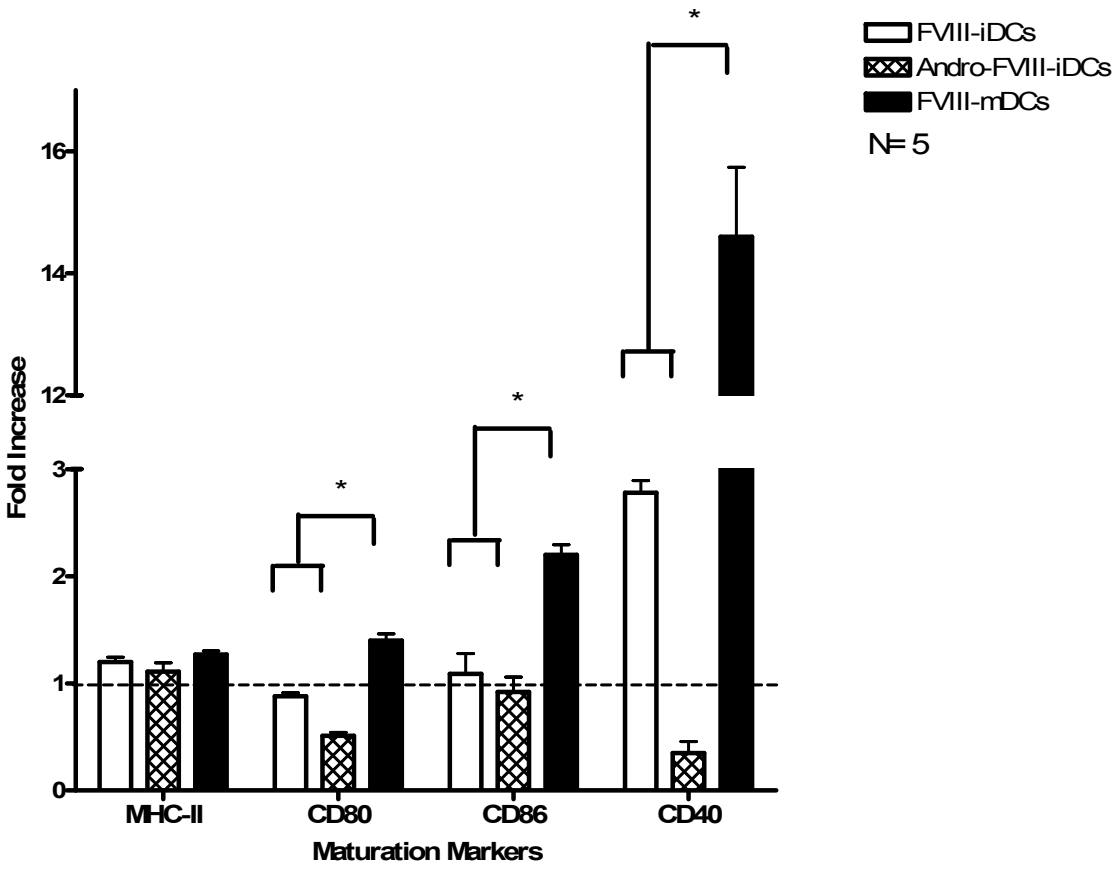
Results:

Dendritic Cells Can Internalize FVIII for Tolerogenic Presentation.

To confirm that DCs can internalize FVIII, iDCs were cultured in the presence of a FVIII-GFP fusion protein. Flow cytometry showed intracellular FVIII-GFP in 95% of DCs within 2 hours (data not shown); thus, confirming that the iDCs were able to internalize the FVIII-GFP fusion protein in the presence and absence of Andro or LPS.

Next, we determined if internalization of cFVIII causes maturation of the iDCs. After 5 days of culture with or without Andrographolide (Andro), the non-adherent iDCs were isolated and pulsed with cFVIII alone, or with cFVIII in the presence of LPS. The results presented in (Figure 3.1) show that pulsing iDCs with cFVIII ± Andro in the absence of LPS does not induce increased expression of CD80 and CD86. Levels of MHC-II expression also did not change significantly while the CD40 expression increased slightly on the cFVIII-iDCs following pulsing with cFVIII. In contrast, iDCs pulsed with cFVIII in the presence of LPS showed a marked increase in the expression of all 3 of the co-stimulatory maturation markers. These results indicate that in vitro, using the conditions employed in these studies, pulsing with cFVIII does not induce DC maturation and that the pulsed-iDCs are less likely to present cFVIII to the immune system in an immunogenic fashion.

Figure 3.1 Dendritic cells retain their immature phenotype after being pulsed with cFVIII. Flow cytometry studies on the expression of MHC-II and the maturation markers CD80, CD86 and CD40 on cFVIII-iDCs/±Andro and cFVIII-mDCs. The immature DCs were isolated and cultured in media containing 10μg/ml of cFVIII for 12 hours (cFVIII-iDCs) or pretreated with Andro for 12 hours before the pulsing process (Andro-cFVIII-iDCs), while the cFVIII-mDCs were prepared by culturing day 5 immature DCs with 10μg/ml cFVIII and 0.1 μg/ml LPS for 12 hours. The dashed line represents the protein expression on the unpulsed immature DCs isolated on day 5. Co-stimulatory protein expression for immature/mature DCs is presented in terms of fold increase relative to the unpulsed immature dendritic cells. The pulsed immature DCs did not induce the expression of the maturation markers. Error bars represent the standard error of the mean of 5 separate experiments: * designates a p value <0.05.


FVIII-iDCs
Andro-FVIII-iDCs
FVIII-mDCs
N= 5
Fold Increase
16
14
12
3
2
1
0
*
*
*
MHC-II
CD80
CD86
CD40
Maturation Markers

cFVIII-Pulsed iDCs Do Not Induce T Cell Activation.

To assess the tolerogenic potential of cFVIII-iDCs and Andro-cFVIII-iDCs in vitro, antigen-specific cytokine release assays were employed. cFVIII-pulsed iDCs were co-cultured with CD4+ T cells isolated from naïve hemophilia A mice and cytokine levels were assayed in the culture supernatant. The results (Figure 3.2) indicate that the unpulsed-iDCs, cFVIII-iDCs and Andro-cFVIII-iDCs do not induce the secretion of pro-inflammatory cytokines (IFN-γ and IL-6) or the B cell-stimulating factor IL-4, indicating that the cFVIII-pulsed iDCs are weak activators of T cells. In contrast, upon stimulating T cells with cFVIII-mDCs, T-cells were activated as indicated by a significant increase in the secretion of IL-4, IL-6, IFN-γ, IL-10 and TGF-β. These studies indicate that the cFVIII-mDCs were able to interact with and activate T cells. This finding confirms the results previously reported by Pfistershammer et al. [80]. Of additional note, T cells co-cultured with Andro-cFVIII-iDCs were documented to secrete more of the immunosuppressive cytokines, IL-10 and TGF-β than cFVIII-iDCs.

In vivo Infusions of cFVIII-Pulsed iDCs Do Not Incite cFVIII Inhibitor Development.

We next evaluated if in vitro cFVIII-pulsed iDCs initiate an anti-FVIII immune response in naïve hemophilia A mice. To this end, we administered 3 weekly injections of cFVIII-mDCs, cFVIII-iDCs or Andro-cFVIII-iDCs, and FVIII inhibitor development was assessed using the Bethesda assay. Our results (Figure 3.3)

Figure 3.2 cFVIII-pulsed iDCs inhibit the release of T cell inflammatory cytokines. CD4+ T cells were isolated from the spleens of naïve hemophilic mice and co-cultured with either cFVIII-iDCs, cFVIII-mDCs, Andro-cFVIII-iDCs or Andro-cFVIII-mDCs for 24 hours to detect IL-4 (A) and IL-6 (B) , 48 hours to detect IFN-γ (C) and IL-10 (D), and 72 hours to detect TGF-ß (E). All cytokines were detected by ELISA experiments. The T cells that were stimulated with cFVIII-iDCs or Andro-cFVIII-iDCs did not release any inflammatory cytokines while the T cells that were stimulated with cFVIII-mDCs or Andro-cFVIII-mDCs released high levels of inflammatory cytokines. Error bars represent the standard error of the mean of 4 separate experiments: * designates a p value <0.005.

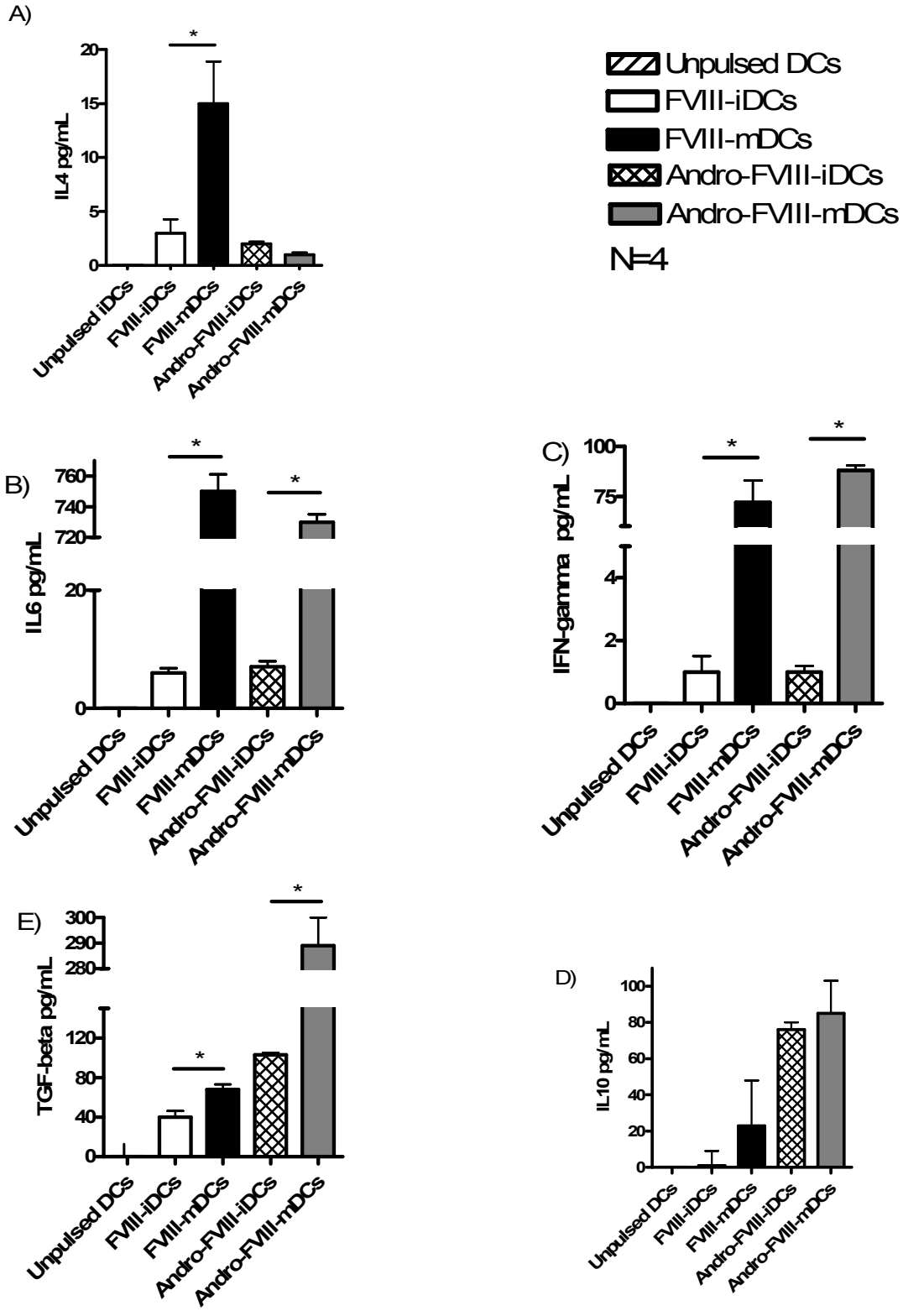
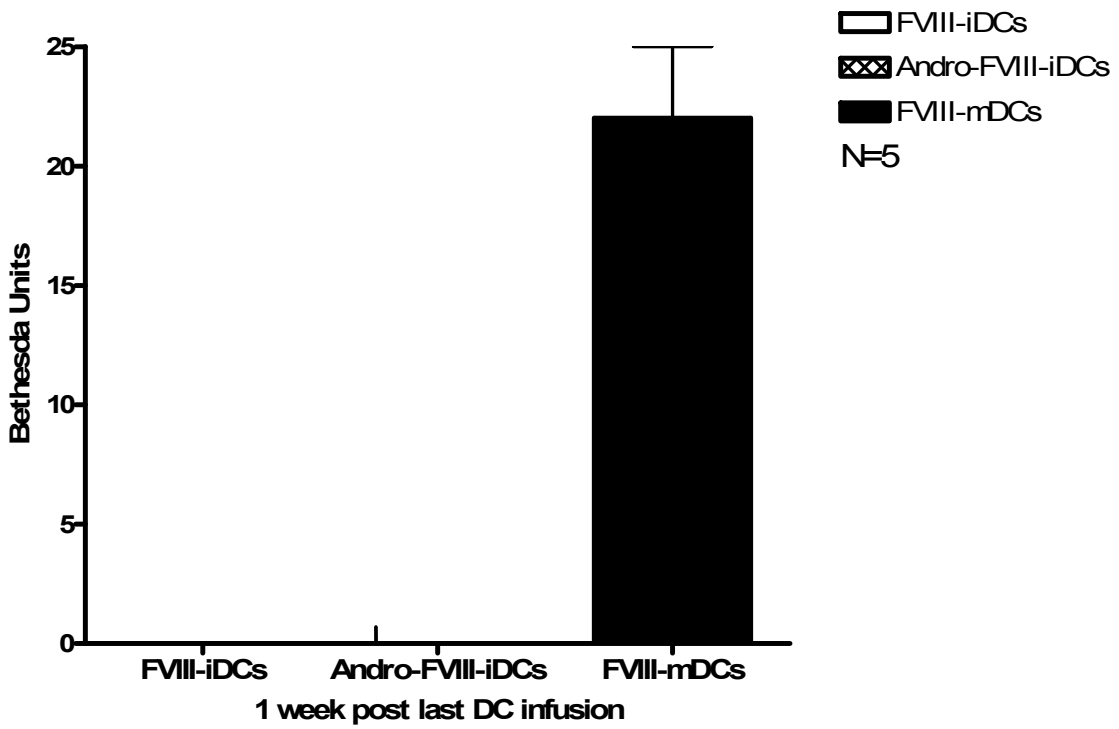

A)
IL4 pg/mL
20
15
10
5
0
*
Unpulsed iDCs
FVIII-iDCs
FVIII-mDCs
Andro-FVIII-iDCs
Andro-FVIII-mDCs
Unpulsed DCs
FVIII-iDCs
FVIII-mDCs
Andro-FVIII-iDCs
Andro-FVIII-mDCs
N=4
B)
IL6 pg/mL
760
740
720
20
0
*
*
Unpulsed DCs
FVIII-iDCs
FVIII-mDCs
Andro-FVIII-iDCs
Andro-FVIII-mDCs
C)
IFN-gamma pg/mL
100
75
4
2
0
*
*
Unpulsed DCs
FVIII-iDCs
FVIII-mDCs
Andro-FVIII-iDCs
Andro-FVIII-mDCs
E)
TGF-beta pg/mL
300
290
280
120
80
40
0
*
*
Unpulsed DCs
FVIII-iDCs
FVIII-mDCs
Andro-FVIII-iDCs
Andro-FVIII-mDCs
D)
IL10 pg/mL
100
80
60
40
20
0
Unpulsed DCs
FVIII-iDCs
FVIII-mDCs
Andro-FVIII-iDCs
Andro-FVIII-mDCs

Figure 3.3 Repetitive Infusions of cFVIII-iDCs or Andro-cFVIII-iDCs Do Not Activate The Immune System of Hemophilic Mice. Naïve hemophilic mice were infused on a weekly basis with either 1 million of cFVIII-iDCs, Andro-cFVIII-iDCs or cFVIII-mDCs for three weeks. Mice were sampled 1 week after the last DC infusion. Mice that received cFVIII-iDCs or Andro-FVIII-iDCs had no FVIII inhibitors, while mice that received cFVIII-mDCs developed high levels of FVIII inhibitors. Error bars represent the standard error of the mean for 5 mice.


FVIII-iDCs
Andro-FVIII-iDCs
FVIII-mDCs
N=5
Bethesda Units
25
20
15
10
5
0
FVIII-iDCs
Andro-FVIII-iDCs
FVIII-mDCs
1 week post last DC infusion

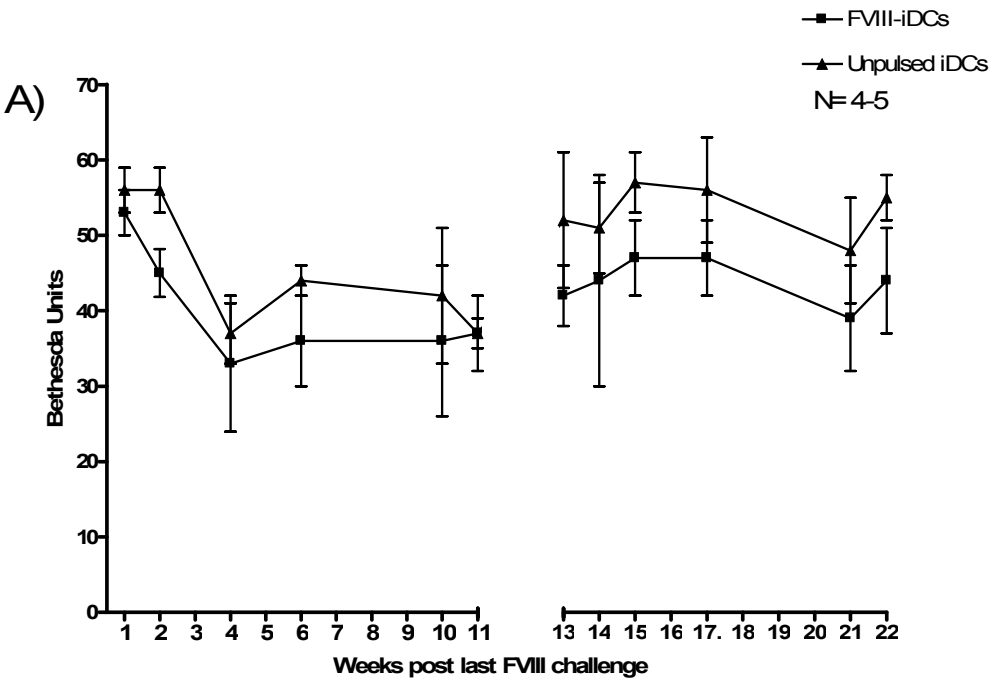
indicate that the immune system of hemophilic mice was not activated after 3 weekly infusions of in vitro cFVIII-pulsed iDCs or Andro-cFVIII-iDCs. Hemophilic mice that only received HBSS also did not develop cFVIII inhibitors. In marked contrast, inhibitors appeared after the infusions of cFVIII-mDCs as shown in (Figure 3.3) (mean Bethesda titer 22 BUs). These studies indicate that cFVIII-iDCs and Andro-cFVIII-iDCs lack the ability to present FVIII in an immunogenic manner to T cells in vivo. On the other hand, cFVIII-mDC infusion results in the immunogenic presentation of cFVIII and causes the formation of cFVIII inhibitors. Studies evaluating total anti-FVIII antibody levels with an ELISA method have shown the same outcomes.

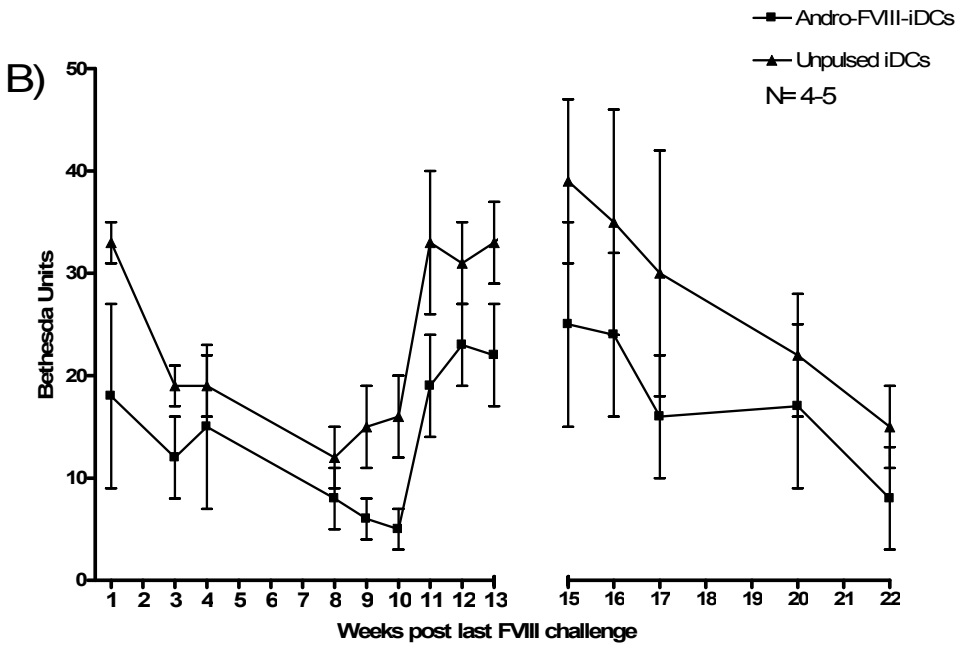
Infusion of cFVIII-Pulsed iDCs Modulates The Generation of FVIII Inhibitors.

The tolerogenic potential of cFVIII-iDCs and Andro-cFVIII-iDCs was subsequently assessed in vivo by infusing naïve hemophilia A mice with 3 weekly infusions of cFVIII-pulsed iDCs followed by 4 or 8 weekly intravenous injections of 2 IU of cFVIII. Control mice received 3 weekly injections of unpulsed iDCs followed by 4 or 8 weekly infusions of 2 IU cFVIII or 4 infusions of HBSS instead of cFVIII. FVIII inhibitors were detected by Bethesda assays and (Figure 3.4) summarizes the inhibitor response of each group over the course of the study.

The hemophilic mice that received 4 infusions of HBSS did not develop FVIII inhibitors (data not shown). From (Figure 3.4), it is shown that in comparison to

Figure 3.4 The infusion of FVIII-iDCs and Andro-FVIII-iDCs results in reduced FVIII inhibitor formation after FVIII treatment. Levels of FVIII inhibitors in mice receiving either (A) cFVIII-iDCs shown in squares, unpulsed-iDCs shown in triangles or (B) Andro-cFVIII-iDCs shown in squares, unpulsed-iDCs shown in triangles . Mice were infused on a weekly basis with either cFVIII-iDCs or unpulsed iDCs for 3 weeks followed by FVIII challenges. The X axis shows the time in weeks since the last FVIII challenge. The infusion of cFVIII-iDCs or Andro-cFVIII-iDCs into mice results in lower FVIII inhibitor titers. All mice were re-challenged with 2IU of FVIII 12 and 23 weeks after the last FVIII infusion in order to analyze the tolerogenic memory response towards FVIII and to expand the FVIII-Treg population, respectively. Error bars represent the standard error of the mean for 4-5 mice.


A)
FVIII-iDCs
Unpulsed iDCs
N= 4-5
Bethesda Units
0
10
20
30
40
50
60
70
1 2 3 4 5 6 7 8 9 10 11
13 14 15 16 17. 18 19 20 21 22
Weeks post last FVIII challenge

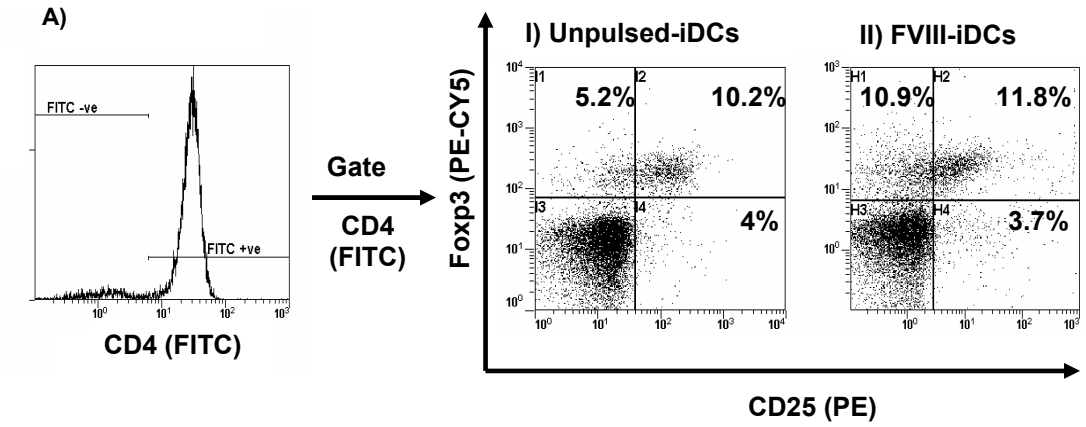

B)
Andro-FVIII-iDCs
Unpulsed iDCs
N= 4-5
Bethesda Units
0
10
20
30
40
50
1 2 3 4 5 6 7 8 9 10 11 12 13
15 16 17 18 19 20 21 22
Weeks post last FVIII challenge

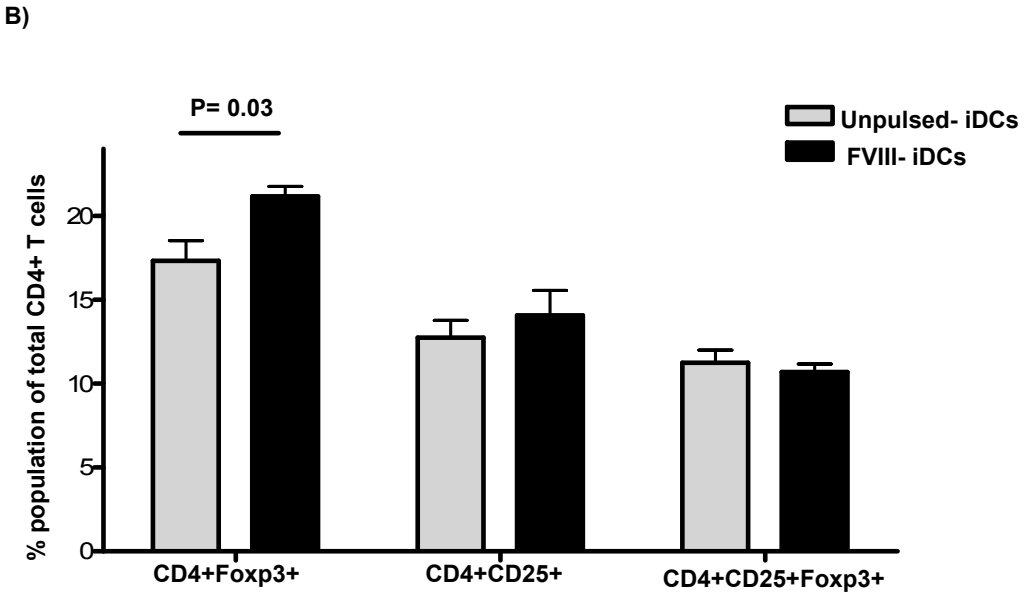
mice that received unpulsed iDCs, mice that received cFVIII-iDCs and Andro-cFVIII-iDCs had 25% and 40% lower levels of FVIII inhibitors respectively for 11 weeks. These results demonstrate that the cFVIII-pulsed iDCs were able to reduce the reactivity of the immune system towards cFVIII. The positive control and experimental mice were subsequently re-challenged with 2 IU of cFVIII 12 weeks after the last cFVIII infusion to investigate if cFVIII-pulsed iDCs were able to induce a long-term tolerogenic modulation of the immune response to FVIII. As shown in (Figure 3.4), the cFVIII-iDC and Andro-cFVIII-iDC treated mice maintained a 25 and 40% reduction in inhibitors respectively, relative to the mice that received unpulsed iDCs over the remaining 10-week duration of the experiment. These results show that the infused cFVIII-iDCs and Andro-cFVIII-iDCs induce a long-lasting negative modulation of the immunogenic response to FVIII.

cFVIII-iDC Infusion Induces an Expansion of Foxp3+ T Regulatory Cells.

We quantified the CD4+CD25+Foxp3+ Treg populations in the mice that had received the c-iDCs and Andro-cFVIII-iDCs. All mice were challenged with the intravenous infusion of 2 IU of cFVIII to expand the Foxp3+ Tregs. One week later, spleens were collected and CD4+ T cells were studied by flow cytometry. The results are presented in (Figures 3.5 A and B). These results demonstrate that after an in vivo challenge with cFVIII, the cFVIII-iDC treated mice had 5% more CD4+Foxp3+ T cells than the mice that received the unpulsed iDCs. This population of CD4+Foxp3+ T regs is probably involved in reducing the immunological response towards FVIII in the experimental mice. In contrast,

Figure 3.5 Infusion of cFVIII-iDCs increases the Foxp3 T regulatory cell population. The FITC-CD4+ T cells were gated. A) The gated CD4+ T cells were analyzed for Foxp3 and CD25 expression. Panel I represents the T regulatory CD4+ cells isolated from the control mouse (that received the unpulsed iDCs followed by the cFVIII challenges) and panel II represents the T regulatory CD4+ cells isolated from the tolerized mouse (that received the cFVIII-iDCs and subsequent cFVIII challenges) this data is representative of 4 mice. B) Quantification of the raw data (shown in A) for the unpulsed-iDCs and cFVIII-iDCs treated mice. This figure demonstrates that cFVIII-iDCs induce the formation of CD4-Foxp3 T regulatory cells in the tolerized mice.


A)
FITC -ve
FITC +ve
CD4 (FITC)
Gate
CD4
(FITC)
Foxp3 (PE-CY5)
I) Unpulsed-iDCs
5.2%
10.2%
4%
II) FVIII-iDCs
10.9%
11.8%
3.7%
CD25 (PE)


B)
P= 0.03
Unpulsed- iDCs
FVIII- iDCs
% population of total CD4+ T cells
0
5
10
15
20
CD4+Foxp3+
CD4+CD25+
CD4+CD25+Foxp3+

there was no significant difference in the Foxp3+ Treg population in the Andro-cFVIII-iDC treated mice compared to mice that received the unpulsed iDCs. These results suggest that the Andro-cFVIII-iDCs reduce the immunological response to FVIII through a mechanism that does not involve CD4+ CD25+Foxp3+ Tregs.

Discussion

The experiments described in this chapter report, for the first time, a modulatory effect of cFVIII-pulsed immature dendritic cells on the immunologic response to FVIII. The results obtained with the cFVIII-iDC and Andro-cFVIII-iDC infusions suggest that different T cell regulatory mechanisms are involved in the long-term suppression of FVIII inhibitor formation in this model.

The maturation stage of dendritic cells plays a crucial role in directing the immune system towards either immunity or tolerance [81,82]. With this in mind, we initially evaluated the phenotype of cFVIII-pulsed immature dendritic cells. We showed that in vitro, in the absence of a danger signal, cFVIII can be internalized by iDCs with no evidence of CD80 and CD86 over-expression but a minimal increase in CD40 expression. Previous studies have shown that DCs can have a semi-mature phenotype with a persistence of tolerogenic abilities [83,84]. We believe that this is the case for cFVIII-iDCs, since these cells do not induce the release of T cell inflammatory cytokines. In contrast to our findings, Pfistershammer et al showed no upregulation of CD40 after pulsing iDCs with FVIII. This might be due to differences in the source of FVIII, culturing and pulsing protocols. Therefore, in vitro, cFVIII uptake by iDCs does not generate inflammatory signals that result in iDC maturation. Pre-treating iDCs with the NFκB pathway blocking compound andrographolide, similarly enabled iDCs to retain their immature state and promoted their tolerogenic potential by inhibiting the expression of co-stimulatory molecules.

The presentation of cFVIII to T cells by iDCs was also investigated, and our results show that in vitro, whether pulsed with cFVIII alone or in the presence of andrographolide, iDCs do not induce the release of pro-inflammatory cytokines from naïve hemophilic T cells. These studies indicate that the cFVIII-iDCs and Andro-cFVIII-iDCs do not present FVIII to T cells in an immunogenic fashion, as reported by other research groups [84]. Of note, T cells co-cultured with Andro-cFVIII-iDCs secreted more immunosuppressive IL-10 and TGF-β. Finally and in marked contrast, DCs stimulated with LPS (i.e. cFVIII-mDCs) induced a significant inflammatory response via the release of pro-inflammatory cytokines including IL-6 and IFN-γ [85] and the release of IL-4. This combination of pro-inflammatory mediators would provide a powerful stimulus for the subsequent development of an adaptive immune response. A similar pattern of results has previously been reported by Pfistershammer et al [80].

Overall, these studies indicate that the cFVIII-iDCs and Andro-cFVIII-iDCs are weak stimulators of T cells while cFVIII-mDCs are strong T cell activators. It has been reported that the presence of IL-6 and IFN-γ will promptly induce a pro-inflammatory response resulting in the activation of the immune system [35]. Also of note is the fact that levels of IL-4 secreted in the Andro-cFVIII-iDC and Andro-cFVIII-mDC co-cultures were lower than the levels of IL-4 secreted in the cFVIII-iDC and cFVIII-mDC co-cultures, respectively. IL-4 is an essential contributor to adaptive immunity and antibody production and thus, reduced production of this

cytokine by T cells stimulated with Andro-cFVIII-iDCs could contribute to the reduced FVIII immune responsiveness.

Although increased secretion of the immunomodulatory cytokines IL-10 and TGF-β was observed in the T cell-Andro-cFVIII-iDC co-cultures, this was not documented with the cFVIII-iDC studies. Nevertheless, tolerogenic antigen presentation by pulsed iDCs can still occur without the induction of immunomodulatory cytokines [84]. This is due to the fact that the antigen-presenting iDCs interact with T cells to produce only "signal 1"; the absence of any additional antigen-associated signaling will induce the T cells to either undergo apoptosis [46], become anergic [86] or differentiate into an IL-10/TGF-β secretor (Tr1 and/or Th3) or non-secretor T regulatory cell (Foxp3+ T regulatory cell) [46,87]. Our results show that the infusion of cFVIII-iDCs into hemophilic mice resulted in increased levels of Foxp3+ nTregs and decreased FVIII inhibitor formation.

This latter observation is consistent with extensive evidence in the literature showing that iDCs can expand naturally occurring Foxp3+ nTregs [42,46,87]. Indeed, nTreg cells have been reported to be involved in reducing the immunological responses to FVIII [43]. Our studies have shown that cFVIII-iDCs were able to achieve their immunomodulatory activities by increasing the CD4+Foxp3+ Treg population rather than inducing the development of IL-10/TGF-β secreting T regulatory cells. These CD4+CD25-Foxp3+ T regs have

been reported to have suppressive abilities [88]. In contrast to the results with cFVIII-iDCs, T cells stimulated by Andro-cFVIII-iDCs were documented to secrete high levels of IL-10 and TGF-β in vitro. These findings suggest that the Andro-treated DCs may have induced naïve T cells to differentiate into Tr1 and Th3 T regulatory cells that exert their immunosuppressive activity by secreting IL-10 and TGF-β, respectively [89]. This may explain the reduced inhibitor levels in these mice. Studies of Foxp3 nTregs in the Andro-cFVIII-iDC treated mice did not show an expansion of these cells.

Our results suggest that induction of immunomodulatory cytokine release by Andro-cFVIII-iDCs significantly reduces the immunological response towards FVIII. Moreover, we have shown that Andro-cFVIII-iDCs are more efficient than the cFVIII-iDCs in reducing the immune response toward FVIII (40% vs 25% inhibitor reduction, respectively). This immunomodulatory enhancement might be the result of the combined reduction of co-stimulatory molecule expression on Andro-cFVIII-iDCs along with the increased secretion of IL-10 and TGF-β induced by these cells. Unlike Foxp3+ T regs, the suppressive ability of Tr1 and Th3 T regs does not require direct contact with T effector cells. Instead, they release immunosuppressive cytokines that negatively regulate T effector cells. From our results, it can be suggested that the elaboration of immunosuppressive cytokines may have a more significant regulatory effect on FVIII-specific T cells

Studies conducted by Reipert et al [90] and Qian et al [91] used an anti-CD40 approach to tolerize hemophilic mice toward FVIII, but this tolerance was not long lasting. In contrast, while our studies with cFVIII-pulsed iDCs only reduced inhibitor formation by 25-40%, we were able to maintain a long-lasting reduction in immune reactivity towards FVIII.

In conclusion, this study has shown, for the first time, that FVIII immunogenicity can be reduced by tolerogenic presentation of the protein by infused iDCs. Depending upon the conditions under which the iDCs are cultured, the immunomodulatory mechanisms appear to involve the expansion of either Foxp3+ nTregs or IL-10 and TGF-β secreting T cell populations. The fact that FVIII inhibitor levels were only reduced and not eliminated by pulsed-iDC infusions indicates that this method of tolerance induction is inadequate to prevent at least some effector T cell activation following repeated intravenous FVIII administration. Although we can only speculate why this happens, a feasible explanation may be that there is an inadequate representation of FVIII peptides presented by the pulsed iDCs. Since FVIII is so large and has many immunogenic epitopes [92,93], we propose that some of these epitopes may not be presented sufficiently by the iDCs to achieve T cell tolerance. Future studies using this tolerance approach might achieve more success if the iDCs were pulsed with high concentrations of peptides representing the major immunodominant T cell epitopes instead of the full-length cFVIII protein.

Chapter 4

Recombinant and plasma-derived Factor VIII products induce distinct splenic cytokine micro-environments in hemophilia A mice

I performed all experiments. All animal techniques were performed by Erin Burnett. Scott Bradshaw helped me in analyzing the micro-array data.

Abstract:

The formation of Factor VIII (FVIII) antibodies after FVIII treatment in hemophilic patients is currently the major treatment-related complication faced by hemophilic patients. It has been reported that the use of plasma-derived FVIII (pdFVIII) in patients and animal models of hemophilia A will result in reduced anti-FVIII antibody formation. In an attempt to explain this phenomenon, an initial hypothesis relating to the uptake of FVIII by antigen presenting cells has been formulated. In contrast, here we are proposing that the cytokine micro-environment induced by pdFVIII has a critical influence on the regulation of anti-FVIII antibody titers in hemophilic mice. Our microarray analysis showed that pdFVIII infusion caused a different gene expression profile in dendritic cells than recombinant FVIII (rFVIII) administration. Both treatments caused the up-regulation of pro-inflammatory gene expression. However, the rFVIII and pdFVIII treatments caused the up-regulation of genes that induce Th1 and Th2 responses, respectively. Moreover, after administering rFVIII or pdFVIII concentrates to mice, we observed that each treatment induced a distinct T cell splenic cytokine micro-environment. Recombinant FVIII induced the release of Th1 cytokines and the Th2 cytokine IL10, while pdFVIII induced the release of Th2 cytokines and TGF-β. In this mouse model, we observed the formation of high titers of anti-human VWF antibodies in the pdFVIII-treated mice and we propose that in these animals, VWF competes with FVIII for antigenic presentation. We further investigated the potential of antigenic competition by treating mice with FVIII and increasing concentrations of another “irrelevant”

protein, recombinant human factor IX (FIX). Our studies have shown an inverse relationship between increasing concentrations of FIX and the production of anti-FVIII antibodies. In summary, these studies allude to new and additional mechanisms that may contribute to the reduction of anti-FVIII antibody development in hemophilia A mice treated with pdFVIII.

Introduction:

Hemophilia A is the most common severe inherited bleeding disorder. Approximately 25% of severe hemophilic patients undergoing FVIII replacement therapy develop antibodies that inhibit the activity of the infused FVIII (FVIII inhibitors). The formation of FVIII antibodies is currently the most significant treatment-related complication in the clinical care of hemophilic patients. The available treatments for hemophilia A patients who develop FVIII antibodies include the use of hemostatic bypassing agents or the induction of immune tolerance to FVIII using various FVIII dosing schedules. These approaches are all very costly and not always successful. The prevention of immunological responses toward FVIII during FVIII replacement therapy would represent a significant therapeutic advance.

The immune system in severe hemophilia A patients is activated after FVIII treatment, in part, because no circulating normal FVIII is present in these patients; thus, the infused FVIII may be viewed as a foreign protein. Infused FVIII will be internalized by professional antigen presenting cells such as dendritic cells (DCs) through mechanisms that include receptor-mediated endocytosis[14] or by antigen non-specific internalization pathways including pinocytosis[94]. Dendritic cells have the ability to process and present FVIII to $CD4^+$ T cells. Factor VIII-presenting dendritic cells (FVIII-DCs) interact with T cells in terms of signal 1 (MHC-II and TCR interaction), signal 2 (co-stimulatory molecules and their appropriate receptors on T cells) and signal 3 (cytokines that regulate the differentiation of $CD4^+$ T cells into either a Th1 or Th2 profile[95]). Th1 T cells

release pro-inflammatory cytokines such as IL2, IL12, and IFN-γ that trigger an inflammatory immune response and the activation of the T cell cytotoxic pathway[96]. In contrast, Th2 cells release anti-inflammatory cytokines such as IL4 and IL10 that inhibit Th1 cells and induce B cell activation[96].

Since DCs play a key role in directing the differentiation of $CD4^+$ T helper cells, we expect these cells to act as regulators for the formation of FVIII antibodies. We and others have shown that FVIII under in vitro sterile conditions does not induce the maturation of DCs [80]. Also, in T cell and FVIII-immature DC co-cultures, FVIII does not induce the release of pro-inflammatory cytokines [80]. However, repetitive infusions of FVIII into hemophilic mice result in the formation of FVIII antibodies in these animals. These findings suggest that the behavior of FVIII in vivo is different from its behavior in vitro. Thus, FVIII “in vivo” is expected to induce a “dangerous” inflammatory micro-environment that results in the maturation of DCs and the subsequent activation of the immune system.

In this chapter, we have studied the mechanisms associated with the differences in anti-FVIII antibody development in hemophilia A mice after rFVIII or pdFVIII treatment. This was achieved by investigating the effects of rFVIII and pdFVIII administration on the profile of immune gene expression of DCs, DC maturation, the T cell cytokine splenic micro-environment and anti-FVIII antibody titers in mice after repetitive FVIII treatments. These studies provide new information concerning the different splenic cytokine profiles induced by FVIII concentrates and the influence of these cytokines on the formation of anti-FVIII antibodies.

Materials and Methods:

Animals

Male and female Balb/c E16 hemophilia A mice, 6-10 weeks old were used in all experiments. Mouse genotype was assessed for the interruption of FVIII exon 16 by polymerase chain reaction (PCR) using genomic DNA isolated from tail clips as described by Connelly et al[57]. All mouse experiments were performed in accordance with the Canadian Council for Animal Care, and the Queen's University Animal Care Committee approved all animal protocols.

Factor VIII and von Willebrand Factor Concentrates

The following human FVIII and VWF concentrates were used in these studies. Recombinant human FVIII, Kogenate-FS® (Bayer Inc. Berkeley, CA), plasma-derived FVIII, Wilate® (Octapharma AG, Lachen, Switzerland), and a plasma-derived VWF concentrate (Biotest AG, Dreieich, Germany).

Design of The Immune Gene Micro-Array Chip

The chip contained 1095 immune relevant genes, 12 house keeping genes, 150 negative controls and 150 spike controls and positive controls. This chip was designed using Agilent Earray services and the gene probes were printed by the manufacturer (Agilent Technologies, Santa Clara CA, USA). Three replicates of each gene probe were printed on each array (8 arrays per slide).

RNA Isolation From FVIII In vivo Pulsed Dendritic Cells

Naïve hemophilia A mice of 6-10 weeks of age were infused intravenously with three different materials: 200 µL of HBSS, 2 IU (80 IU/kg) of human recombinant FVIII Kogenate FS® (rFVIII) or 2 IU (80 IU/kg) plasma-derived human FVIII Wilate® (pdFVIII). Twenty four hours later, the spleens were isolated. For each infusate, 6 mouse spleens were pooled to generate one experimental biological replicate. For each infusate, we had 4 experimental biological replicates (ie. 4 x 6 pooled splenocyte samples). The $CD11c^+$ dendritic cells were isolated from each splenocyte pool using magnetic beads coated with anti-CD11c (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's guidelines. The CD11c cellular pellets were resuspended in Trizol (Invitrogen, Carlsbad, CA, USA). RNA isolation and purification steps were performed according to the manufacturer's guidelines. RNA quantity was evaluated by Nanodrop while the RNA quality was assess by the Aglinet BioAnalyzer and there was no evidence of RNA degradation.

RNA Labeling and Hybridization

The isolated RNA was labeled with Cyanine 3-CTP and hybridized to the micro-array chip according to the manufacturer's guidelines (Agilent Technologies, Santa Clara CA, USA). Each biological replicate was hybridized on two separate arrays in order to generate two technical repeats. The arrays were then washed and scanned using an Agilent scanner according to the manufacturer's guidelines (Agilent Technologies, Santa Clara CA, USA).

Micro-Array Data Analysis

For micro-array hybridization analysis, the difference of the median intensity subtracted from the median background was divided by the standard deviation of the background. The data was normalized and the replicates were filtered as previously described by Stekel and Quackenbush[97,98]. The DC gene expression isolated from the HBSS-treated mice was compared to the DC gene expression isolated from either the rFVIII or pdFVIII-treated mice. Altered gene expression was considered significant if it had a fold induction of ≥2 or ≤ 0.5 in all of the four biological readings and their corresponding technical repeats.

Treatment of Hemophilia A mice with FVIII

Naïve hemophilic mice of 6 to 10 weeks of age received four weekly intravenous infusions of human recombinant FVIII Kogenate-FS® (rFVIII) or plasma-derived human FVIII Wilate® (pdFVIII). Each dose comprised of 2 IU of FVIII (200 ng: equivalent to 80 IU/kg) diluted with HBSS into a final volume of 200 µL. The negative control mice for this experiment received 4 intravenous injections of 200 µL of HBSS.

Blood Sampling from the Hemophilia A Mice

Mice were anesthetized with 0.2cc hypnorm/water/midazolam (1:2:1) and blood samples were obtained using uncoated microhematocrit capillary tubes (Fisher Scientific Pittsburgh, PA, USA) via the retro-orbital plexus. Blood was mixed with 1/10 volume of 3.2% sodium citrate. Plasma samples were isolated by

centrifugation of blood samples at 10,000 RPM for three minutes at 4°C, frozen on dry ice and stored at -70°C until tested.

Measurement of FVIII Antibodies in Mouse Blood Samples

Total anti-FVIII antibody titers were quantified by ELISA. In brief, microtiter plate wells were coated over night at 4°C with 100 μL of human recombinant FVIII (Kogenate-FS®) at a concentration of 10 IUs/mL . Wells were blocked using 10% BSA in PBS. Plasma samples were diluted in HBS-BSA buffer. The anti-FVIII antibody isotypes (IgG1, IgG2A, IgG2B and IgG3) were studied using the clonotype kit (Southern Biotech, Birmingham, Alabama USA). An anti-FVIII standard curve was generated using an anti-human FVIII antibody of known concentation (Abcam, Cambridge, MA, USA). The standard curve was used as a reference to quantify the FVIII antibodies in our samples.

Plasma samples were analyzed for FVIII inhibitory antibodies using a one-stage FVIII clotting assay. In brief, plasma samples were diluted in 2% BSA in HBS, mixed with human pool plasma in a 1:1 ratio and incubated at 37°C for 2 hours. Samples were then assayed for residual FVIII activity using APTT reagent (Organon Teknika; Durham, NC, USA) in an automated coagulometer (General Diagnostics Coag-A-Mate, Toronto, Canada) following the manufacturer's protocol. Inhibitor titers (Bethesda Units) were calculated as detailed in the standard Bethesda protocol[58].

Phenotypic Characterization of In-vivo Pulsed Dendritic Cells by Flow Cytometry.

Naïve hemophilia A mice of 6 to 10 weeks of age were treated with a single infusion of HBSS, 2 IU of rFVIII or 2 IU pdFVIII. Twenty four hours later, spleens were isolated and digested in collagenase D for 30 minutes. $CD11c^+$ DCs were isolated using magnetic beads coated with anti-CD11c antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's guidelines. All isolated DC populations were >90% PE-Cy5-CD11c+ by flow cytometric assessment. Co-stimulatory molecules CD80 and CD86 (BD Biosciences, San Diego CA, USA) were fluorescently labeled with PE and FITC in order to identify the maturation stage of the DCs. The cells were fixed with Cytofix/Cytoperm kit (BD Biosciences, San Diego CA, USA) and samples were analyzed by flow cytometry within 12 hours.

In-vivo Assessment of The Splenic T Cell Micro-Environment Induced by FVIII Infusions

Hemophilia A mice that were previously treated with either rFVIII or pdFVIII were used in this experiment. Mice were challenged with 2 IU of either rFVIII or pdFVIII 4 months after their last FVIII treatment. 24hrs later, CD4+ T cells were isolated from the spleens via the use of a CD4 negative selection isolation kit (Miltenyi Biotec Bergisch Gladbach, Germany) following the manufacturer's protocol. The CD4+ cells were stained with specific antibodies against CD4, CD25, Foxp3 and the intracellular cytokines IL2, IL4, IL5, IL10, IFN-γ, and TGF-β as described by

the manufacturer (BD Biosciences, San Diego CA, USA). The cells were fixed with the Cytofix/Cytoperm kit (BD Biosciences, San Diego CA, USA) and samples were analyzed by flow cytometry within 12 hours. The negative control mice for this experiment received 4 intravenous injections of 200 µL of HBSS. Mice were treated again with 200 µL of HBSS 4 months later.

In-vivo Studies of Antigenic Competition Between FVIII and FIX

Naïve hemophilia A mice of 6 to 10 weeks of age received four weekly intravenous treatments of either 2 IU human rFVIII or 2 IU rFVIII mixed with 3µg (0.6 IU), 10 µg (2 IU) or 20 µg (4 IU) of recombinant FIX BeneFix® (rFIX). Large amounts of FIX protein were used in this experiment to match the molar ratio of VWF found in pdFVIII i.e. in pdFVIII the molar ratio of FVIII:VWF is 1:100. All treatments were diluted with HBSS into a final volume of 200 µL. The negative control mice for this experiment received 4 intravenous treatments of 200 µL of 20 ug of FIX alone.

Statistical Analysis.

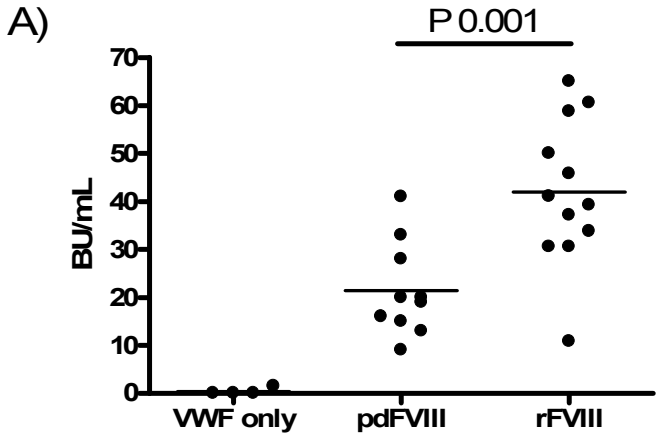
All data are presented as mean ± standard error of the mean (SEM). Statistical comparisons of experimental groups were evaluated with a Student T test; all P values are shown in the figures.

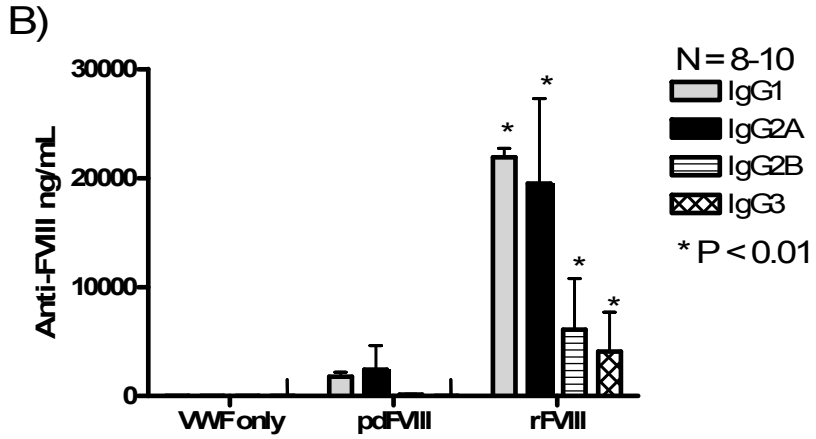
Results

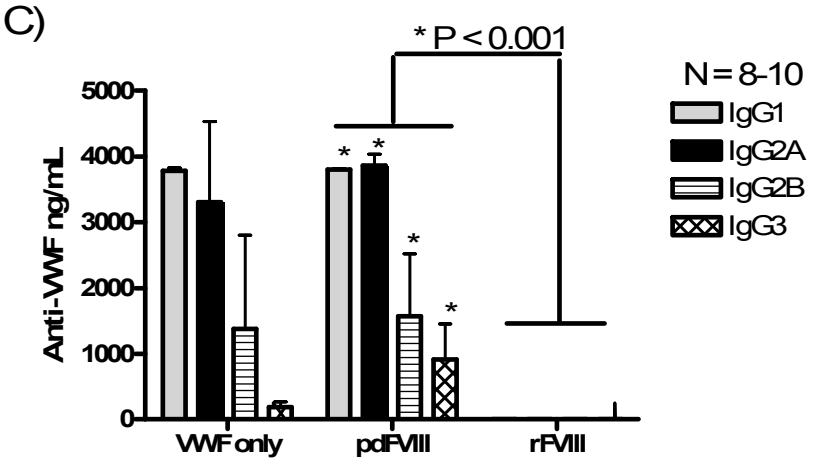
Treatment of Hemophilia A Mice with pdFVIII Results in Reduced anti-FVIII Generation

We were interested in learning more about the association between FVIII inhibitors and treatments with rFVIII or pdFVIII. This was achieved by treating hemophilia A mice with 4 weekly infusions of either 2 IU rFVIII or 2 IU pdFVIII. Our results show that the mice who received rFVIII had consistently higher anti-FVIII inhibitor titers compared to the pdFVIII-treated mice (Figure 4.1A). Anti-FVIII antibodies were not seen in the HBSS-infused mice. The anti-FVIII ELISA studies on the rFVIII-treated mice showed that the antibodies were predominantly of the IgG1 subclass (Th2 dependent) and to a lesser extent IgG2A and IgG2B (Th1 dependent) subclasses (Figure 4.1B). Moreover, the anti-FVIII isotyping studies on the pdFVIII-treated mice showed a marked reduction in the formation of anti-FVIII IgG1, IgG2A and IgG2B subclasses. The mice that were treated with pdFVIII developed high titers of anti-human VWF antibodies. These anti-VWF antibodies were predominantly IgG1 and IgG2A subclasses (Figure 4.1C). Thus, treating hemophilia A mice with pdFVIII results in low anti-FVIII and high anti-human VWF antibody titers. With this in mind, we investigated the cells of the innate and adaptive immune systems to identify the mechanism responsible for this observation.

Figure 4.1 Infusion of pdFVIII concentrate results in reduced anti-FVIII titers and the formation of VWF antibodies. Comparison of (A) FVIII inhibitors, (B) Anti-FVIII IgG subclasses and (C) Anti-VWF IgG subclasses in Balb/c mice after 4 infusions of either VWF alone, pdFVIII or rFVIII. Naïve hemophilic Balb/c mice were treated with 4 weekly infusions of 2 IU VWF or 2 IU pdFVIII or 2 IU rFVIII. Mice were sampled 1 week following the fourth treatment. The horizontal lines and error bars represent the mean and standard error of the mean for 8 to 10 mice, respectively.


A)
P 0.001
70
60
50
40
30
20
10
0
BU/mL
VWF only
pdFVIII
rFVIII


B)
30000
20000
10000
0
Anti-FVIII ng/mL
VWF only
pdFVIII
rFVIII
N = 8-10
IgG1
IgG2A
IgG2B
IgG3
* P < 0.01


C)
* P < 0.001
5000
4000
3000
2000
1000
0
Anti-VWF ng/mL
VWF only
pdFVIII
rFVIII
N = 8-10
IgG1
IgG2A
IgG2B
IgG3

Recombinant and Plasma-Derived FVIII Concentrates Induce Different Patterns of Immune Gene Expression in Dendritic Cells

Dendritic cells play a central role in directing the immune system towards either tolerance or immunity. Therefore, in order to investigate the influence of rFVIII and pdFVIII concentrates on DC immune gene expression, transcriptional profiling micro-array studies were performed. The transcript profile of DCs was investigated 24 hours after infusing hemophilic mice with either pdFVIII or rFVIII. The gene expression profile in DCs following administration of each concentrate (pdFVIII or rFVIII) was compared to that of DCs that were isolated from naïve hemophilic mice treated with HBSS. For each condition, we only considered the genes that had a 2-fold difference in gene expression in all of the four biological experiments and their corresponding technical replicates. Our results show that the pdFVIII infusion induced a different immune gene expression profile in DCs compared to the rFVIII treatment; these results are summarized in Table 4.1.

In vivo Administration of rFVIII and pdFVIII Concentrates Induces the Maturation of Dendritic Cells

In order to activate the immune system towards FVIII, the FVIII protein must be sampled, processed and presented to T cells by antigen presenting cells such as DCs. We studied the in vivo maturation of DCs due to FVIII treatment. Our results show that in vivo, rFVIII and pdFVIII concentrates both induce the maturation of DCs by increasing the expression of CD86 on DCs in comparison

to the HBSS control DCs (Figure 4.2). Interestingly, the pdFVIII-DCs had a higher CD86 expression than rFVIII-DCs. Dendritic cell CD86 expression was also

Table 1: Summary of the micro-array gene expression of DCs that were isolated 24 hours after (A) rFVIII or (B) pdFVIII infusion; all ratios are relative to HBSS-infused mice

A) Pathway and specific gene list of immune system genes that had a consistent 2-fold difference in gene expression after rFVIII treatment relative to the HBSS-treated mice

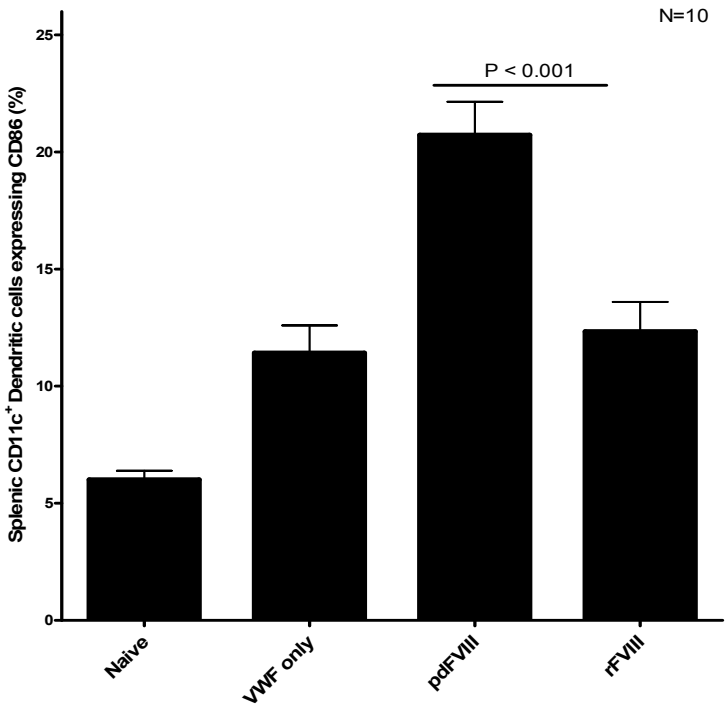
Functional Grouping : Gene Function	Gene Name	Gene Symbol	Intensity Ratio (rFVIII/HBSS)
Cell-Matrix Adhesion: Increases cell survival [99]	Connective Tissue Growth Factor	Ctgf	0.439013
Host Immune Defense to Bacteria: InducesTh1 responses [100]	Cathelicidin Antimicrobial Peptide	Camp	3.272401
Surface marker: Pro-inflammatory cytokines increasing phagocytoses [101]	S100 Calcium Binding Protein A8	S100a8	2.527378
Host Defense to Bacteria: Inhibits actions of TNF-α [102]	Proteoglycan 2	Prg2	0.364057
Oxidative Stress: prevents inflammation [103]	Glutathione Peroxidase 2	Gpx2	2.466754
Host Defense to Bacteria: Th1-polarized Ag-specific immune responses [104]	Lactotransferrin	Ltf	2.274128

B) Pathway and gene list of genes that had a 2-fold difference in the microarray gene expression after pdFVIII treatment relative to the HBSS-treated mice

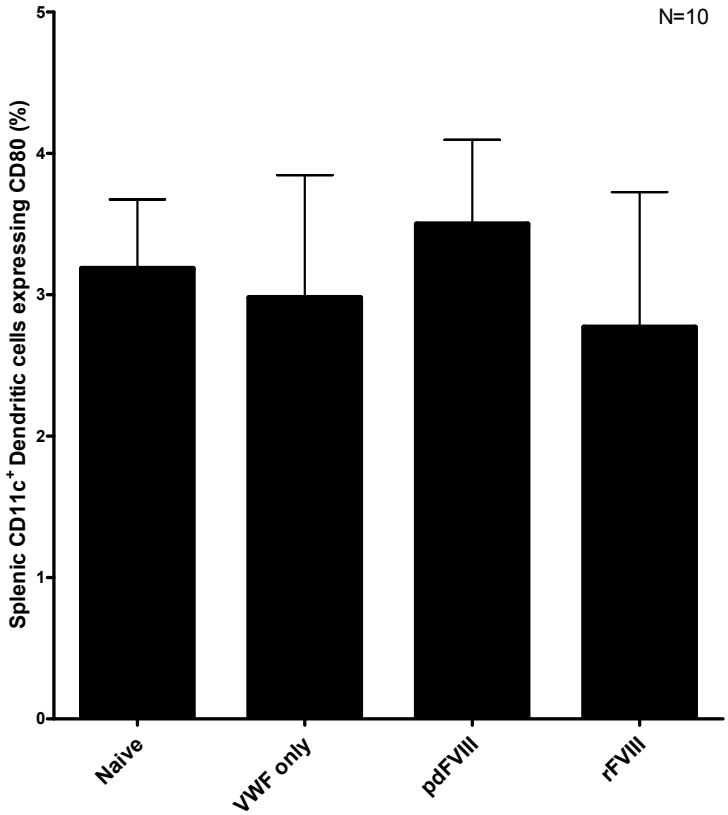
Functional Grouping : Gene Function	Gene Name	Gene Symbol	Intensity Ratio (pdFVIII/HBSS)
Cytokine-cytokine receptor interaction: Binds interleukin 8 receptor and recruits DCs and neutrophils [105]	Chemokine (C-X-C motif) Ligand 2	Cxcl2	3.107269
Cytokine-cytokine receptor interaction: Induce type 2 T helper cell polarization and recruits Natural Killer cells [106]	Chemokine (C-C motif) Ligand 2	Ccl2	2.091005
Host Defense to Bacteria: Inhibits actions of TNF-α [102]	Proteoglycan2	Prg2	0.426912
Involved in Septic Shock: Enhances inflammatory responses to microbial products [107]	Triggering Receptor Expressed on Myeloid Cells 1	Trem1	2.917323
Extracellular Matrix Protein: inhibits dendritic cell migration to lymph nodes [108]	Secreted Acidic Cysteine Rich Glycoprotein	Sparc	0.47198
Heat Shock protein (TLR Interacting Protein): Induce CD86 expression and release of TNF-α, IL-1β and IL-6 [109]	Heat Shock Protein 1A	Hspa1a	3.048223
Heat Shock protein: Induce the infiltration of NK cells and DCs [110]	HeatShock Protein 1	Hspb1	2.105893

Figure 4.2 The pdFVIII and rFVIII infusions cause the maturation of Dendritic Cells. Assessment of DC maturation after treating naïve hemophilic Balb/c mice with either HBSS, 2 IU VWF, 2 IU pdFVIII or 2IU rFVIII. Mice were sacrificed 2 hours after the treatment and CD11c+ DCs were purified from the spleens. The expression of (A) CD86 or (B) CD80 maturation markers was quantified on DCs via flow cytometry. The error bars represent the standard error of the mean for 10 mice.

A)


N=10
P < 0.001
Splenic CD11c+ Dendritic cells expressing CD86 (%)
25
20
15
10
5
0
Naive
VWF only
pdFVIII
rFVIII

B)


N=10
Splenic CD11c+ Dendritic cells expressing CD80 (%)
5
4
3
2
1
0
Naive
VWF only
pdFVIII
rFVIII

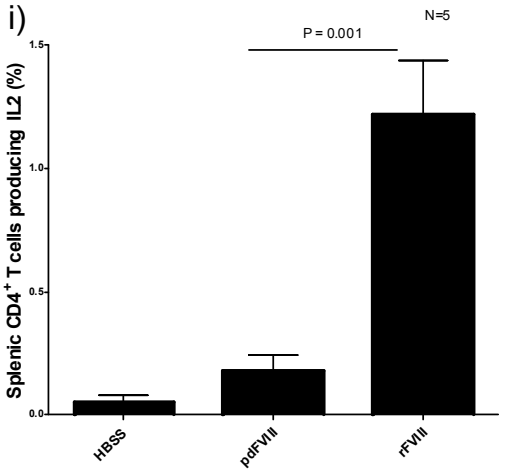
increased following infusion of the VWF concentrate. We propose that the presence of VWF and other co-purified proteins found in the pdFVIII concentrate are responsible for the increased CD86 expression documented following exposure to this product.

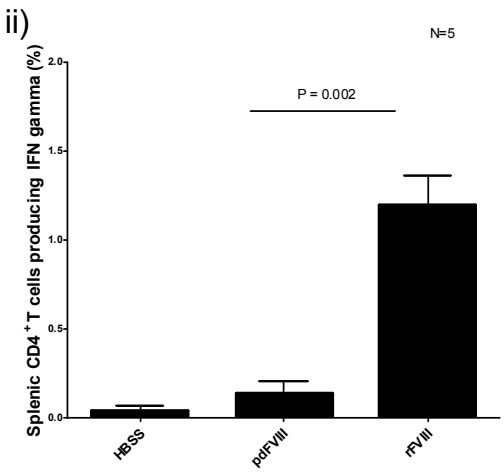
In vivo, Th1 and Th2 Adaptive Immune Responses Were Induced After the rFVIII and pdFVIII Infusions, Respectively.

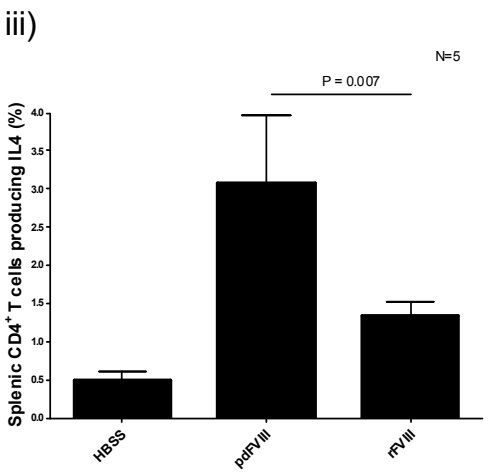
In order to learn more about the in vivo cytokine release induced by CD4+ T cells following infusion of rFVIII and pdFVIII concentrates, we assessed the intracellular cytokine production in CD4+ T cells isolated from rFVIII and pdFVIII-treated mice that were re-challenged with the respective FVIII concentrates after an interval. Our results show that the mice that were challenged with rFVIII had more T cells producing IL2, IL10 and IFN-γ and fewer T cells producing IL4, IL5, and TGF-β (Figure 4.3 A). A contrasting cytokine profile was documented in the mice that were challenged with pdFVIII, in which we observed more T cells producing IL4, IL5, and TGF-β and fewer T cells producing IL2, IL10 and IFN-γ (Figure 4.3 A). We also evaluated the CD4+CD25+Foxp3+ regulatory T cell population in the spleen after rFVIII or pdFVIII challenges. Our results show that the mice that received pdFVIII treatment had more splenic $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory cells (12.5 vs 6%) than the mice that were treated with rFVIII (Figure 4.3 B).

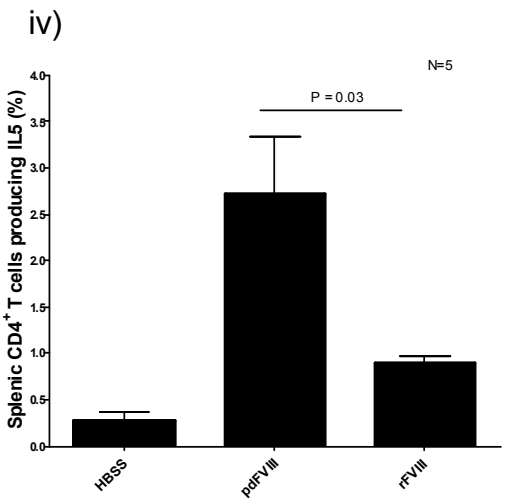
Figure 4.3 pdFVIII concentrate induces the development of T regulatory cells and a Th2 cytokine profile while rFVIII induces a Th1 cytokine profile. Detection of (A) intracellular Th1 and Th2 cytokines produced by CD4+ T cells and (B) CD4+CD25+Foxp3+ T regulatory cells isolated from HBSS, rFVIII or pdFVIII-treated mice. Mice were treated with 4 infusions of HBSS , 2 IU pdFVIII or 2 IU rFVIII; 4 months later mice were challenged with HBSS, 2 IU rFVIII or 2 IU pdFVIII. 24 hours later, CD4+ T cells were purified from the spleens. The Th1 cytokines IL2, IFN (3A- i and ii), Th2 cytokines IL4, IL5, IL10 and TGF-β (3B-iii-vi) and CD4+CD25+Foxp3+ expression was quantified by flow cytometry. The error bars represent the standard error of the mean for 5 mice.

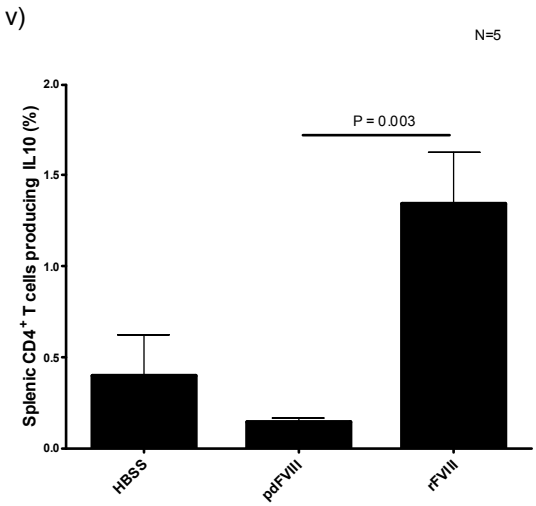
A)

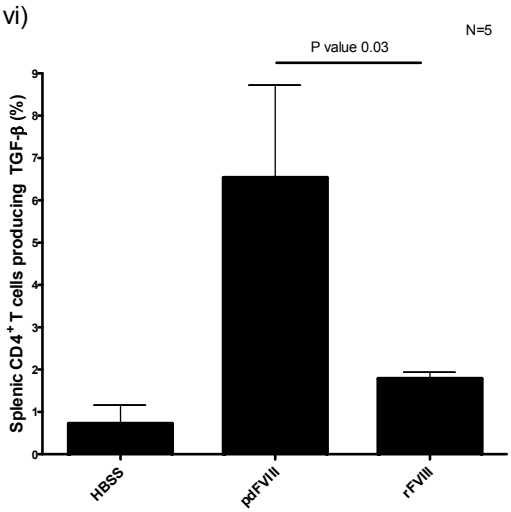

i)
N=5
P = 0.001
Splenic CD4+ T cells producing IL2 (%)
HBSS
pdFVIII
rFVIII


ii)
N=5
P = 0.002
Splenic CD4+ T cells producing IFN gamma (%)
HBSS
pdFVIII
rFVIII

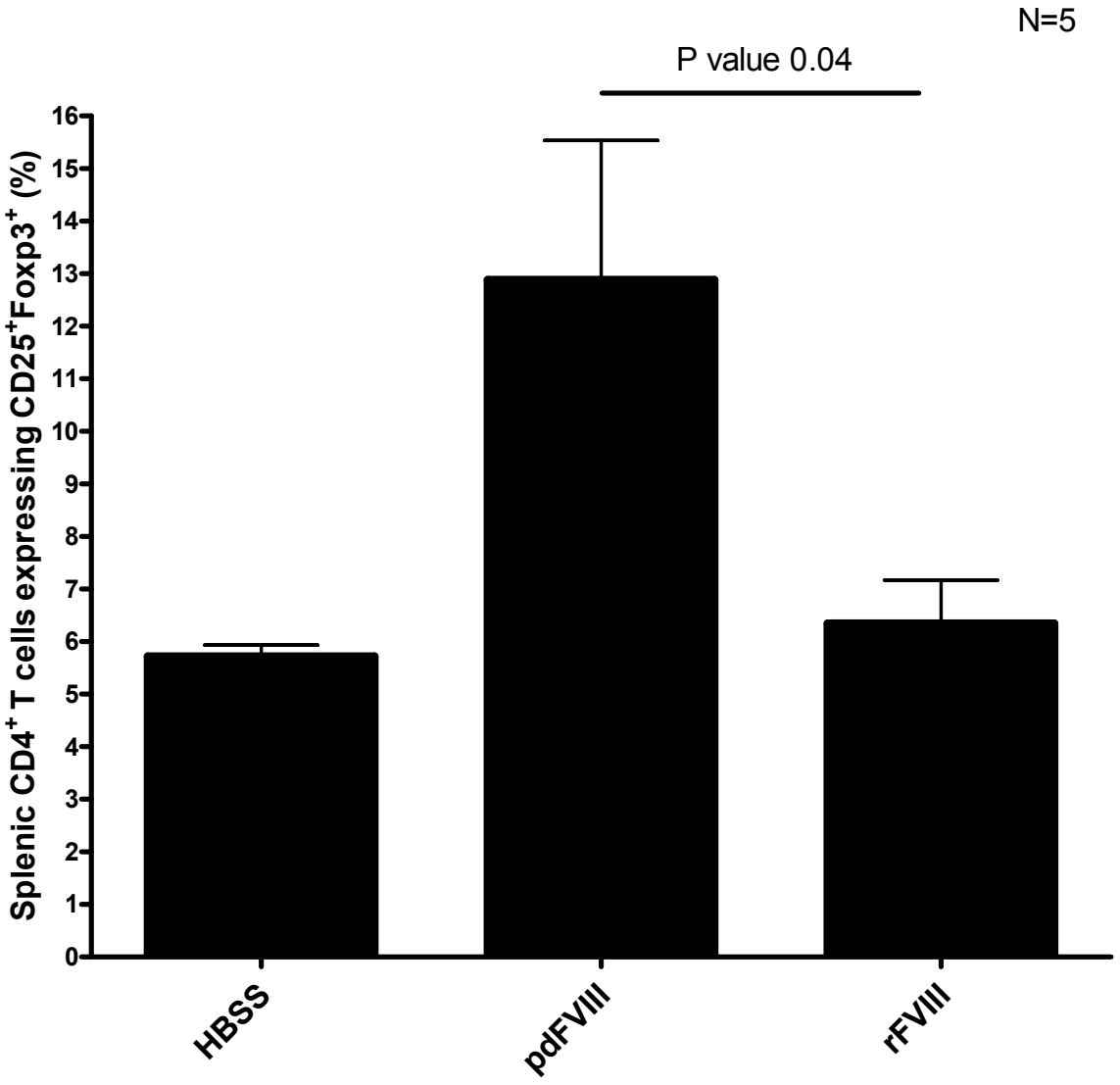

iii)
N=5
P = 0.007
Splenic CD4+ T cells producing IL4 (%)
HBSS
pdFVIII
rFVIII


iv)
N=5
P = 0.03
Splenic CD4+ T cells producing IL5 (%)
HBSS
pdFVIII
rFVIII


v)
N=5
P = 0.003
Splenic CD4+ T cells producing IL10 (%)
HBSS
pdFVIII
rFVIII


vi)
N=5
P value 0.03
Splenic CD4+ T cells producing TGF-β (%)
HBSS
pdFVIII
rFVIII

B)

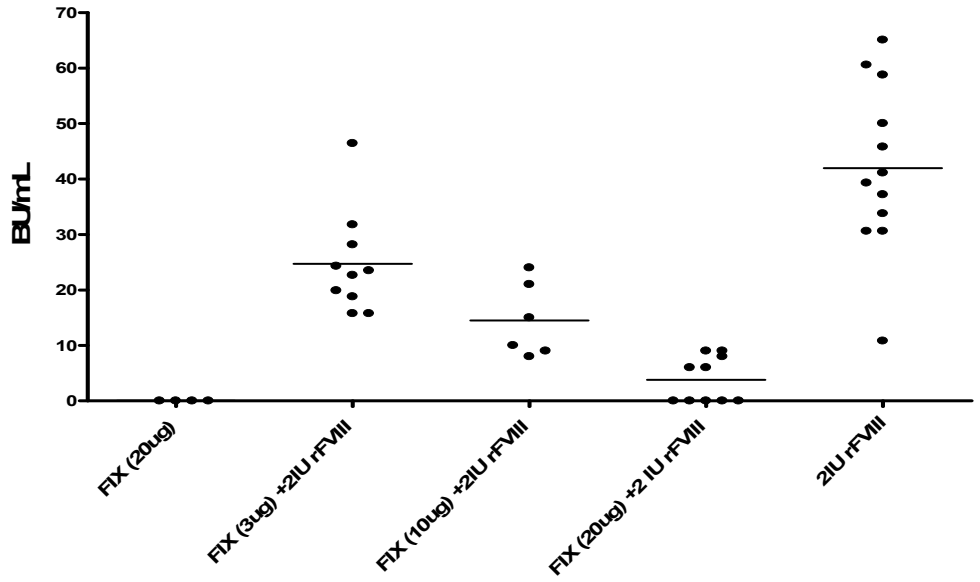

N=5
P value 0.04
Splenic CD4+ T cells expressing CD25+Foxp3+ (%)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
HBSS
pdFVIII
rFVIII

Treatment of Hemophilia A Mice With rFVIII Mixed With rFIX Results in Reduced Levels of FVIII Antibodies

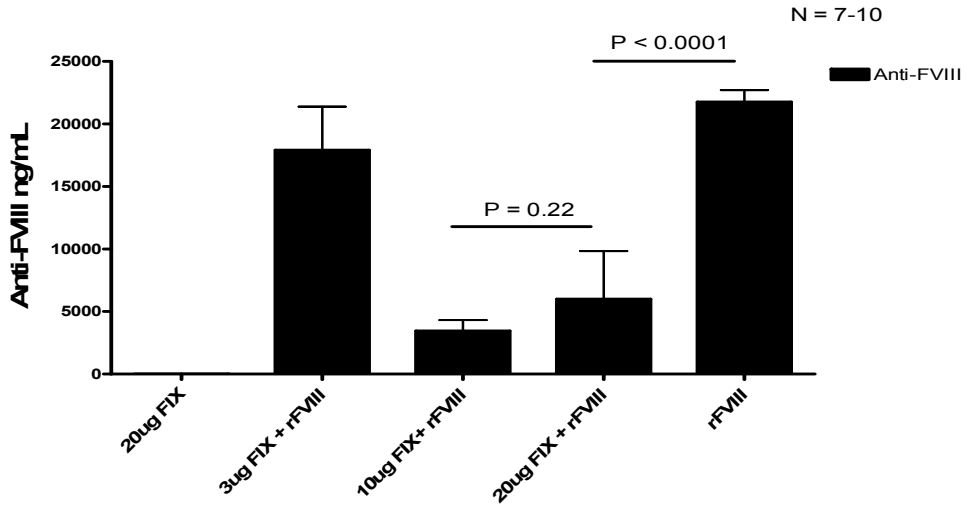
Reipert and colleagues previously suggested that VWF found in pdFVIII can compete with FVIII for immunogenic recognition[111]. Therefore, in order to assess the possibility of antigenic competition between FVIII and other proteins, we performed the following experiment. Hemophilia A mice were treated with four weekly infusions of 2 IU rFVIII alone, 2 IU rFVIII + 10 µg rFIX or 2 IU rFVIII + 20 µg rFIX. Our results show that the mice who received increasing concentrations of rFIX mixed with rFVIII had reduced FVIII inhibitor titers compared to the mice that were treated with rFVIII alone (Figure 4.4 A). Particularly noteworthy, 5 of the mice that were treated with 2 IU rFVIII + 20 ug rFIX did not develop FVIII inhibitors. Our experiments also show that the rFVIII/rFIX treated mice had lower total anti-FVIII antibody titers as measured by ELISA than the rFVIII-treated mice (Figure 4.4B). We also evaluated the presence of anti-FIX antibodies in the mice after rFVIII+rFIX treatment. Our results show that the mice that were treated with rFIX developed low levels of anti-FIX antibodies (Figure 4.4 C). Therefore, treating hemophilic mice with rFVIII + FIX results in reduced anti-FVIII titers which might be due to antigenic competition between FIX and FVIII for immunogenic presentation by APCs and subsequent activation of effector T cells.

Figure 4.4 The presence of high FIX protein concentrations during FVIII treatment results in reduced anti-FVIII antibodies. Comparison of (A) FVIII inhibitors, (B) Anti-FVIII antibodies and (C) Anti-FIX antibodies in hemophilic mice after 4 treatments of either 3ug FIX+2IU rFVIII, 20 ug FIX, 10 ug FIX+2 IU rFVIII, 20 ug FIX+2 IU rFVIII or 2 IU rFVIII. Mice were sampled 1 week following the fourth treatment. The horizontal and error bars represent the mean and standard error of the mean for 8 to 10 mice, respectively.

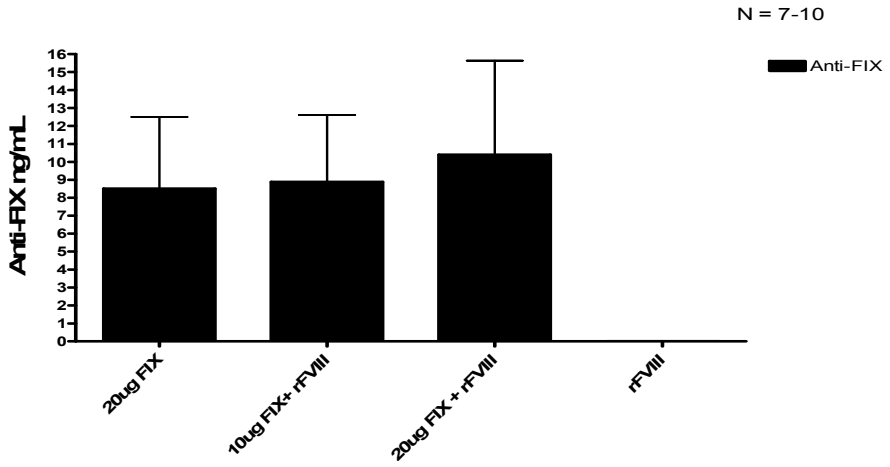
A)


BU/mL
70
60
50
40
30
20
10
0
FIX (20ug)
FIX (3ug) +2IU rFVIII
FIX (10ug) +2IU rFVIII
FIX (20ug) +2 IU rFVIII
2IU rFVIII

B)


N = 7-10
P < 0.0001
Anti-FVIII
Anti-FVIII ng/mL
25000
20000
15000
10000
5000
0
P = 0.22
20ug FIX
3ug FIX + rFVIII
10ug FIX+ rFVIII
20ug FIX + rFVIII
rFVIII

C)


N = 7-10
Anti-FIX
Anti-FIX ng/mL
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
20ug FIX
10ug FIX+ rFVIII
20ug FIX + rFVIII
rFVIII

Discussion

We have shown that treating hemophilia A mice with rFVIII results in higher levels of anti-FVIII antibodies compared to pdFVIII-infused mice. We have subsequently investigated the key players in the initiation of the adaptive immune response, DCs and T cells. Our results suggest that the differences in immune responsiveness between the rFVIII and pdFVIII-treated mice may be related to the different splenic cytokine milieu induced by each type of concentrate. The cytokine context of antigen presentation is an important determinant of immunogenicity and recent studies have documented an association between FVIII antibody generation and polymorphisms in the promoter regions of the IL10 and TNF-α genes[22,23]. Moreover, Sasgary et al. reported an association between rFVIII treatment and IFN-γ production by T cells isolated from hemophilic mice [68].

Dendritic cells regulate the immune response by expressing cell surface proteins and by releasing cytokines and chemokines that influence the interaction with T cells. The immune gene expression profile of DCs isolated from pdFVIII or rFVIII-treated mice was distinct. The rFVIII and pdFVIII treatments resulted in a consistent >2-fold change of expression of 6 and 7 genes involved in the immune response, respectively. After analyzing the differentially expressed genes following rFVIII treatment, we observed the enhanced expression of genes that induce inflammation, promote Th1 responses, enhance phagocytosis and prevent oxidative metabolic stress. The pdFVIII treatment induced the expression of genes that recruit inflammatory cells, promote inflammation and stimulate the

production of TNF-α, IL-1β and IL-6 inflammatory cytokines. These studies indicate that the rFVIII and pdFVIII treatments induce the expression of two different groups of immune system genes in DCs. The Ltf and CAMP genes that were up regulated following rFVIII administration are involved in inducing Th1 cytokines. In contrast, the Ccl2 gene which is involved in inducing Th2 T cell cytokine polarization was up regulated after the pdFVIII treatment. Since DCs regulate the differentiation of T cells via signal 3, we expect that the expression of these genes has an influence on the development of Th1 and Th2 T cells after rFVIII and pdFVIII treatment, respectively.

The flow cytometry studies on the DCs isolated from the pdFVIII-treated mice showed an increased expression of the costimulatory molecule CD86. This was expected since the DCs obtained from the pdFVIII mice over-expressed the Heat Shock Protein A1 which is known to increase CD86 expression. We have also shown that DCs will over express CD86 following the infusion of VWF alone. Therefore, we expect that the higher CD86 expression on DCs obtained from pdFVIII-treated mice is due to several factors, including the presence of VWF in pdFVIII and the up regulation of genes such as Hspa1a after pdFVIII infusion. Interestingly, the expression of CD80 was not over expressed after either rFVIII or pdFVIII treatment. This was expected because the DCs were isolated from the mice 24 hours after the first FVIII treatment and Hausl et al. showed that CD86 but not CD80 is involved in the primary anti-FVIII immune response in hemophilic mice[112]. Consequently, we have shown, for the first time, that the rFVIII and

pdFVIII treatments induce different immune gene expression in DCs. This difference may well underlie a subsequent differential adaptive immunological reactivity to FVIII.

Our intracellular cytokine studies have shown major differences in the secondary immune response following rFVIII and pdFVIII infusions. We have shown that rFVIII and pdFVIII treatments induced the generation of a predominantly Th1 and Th2 secondary immune response, respectively. Reding et al. have suggested that Th1 cytokines have an essential role in maintaining the anti-FVIII immune response (secondary immune response) and Sasgary et al. reported that FVIII-specific T cells are dependent on the production of IL2, IL10 and IFN-γ[68]. We observed high levels of these cytokines produced by T cells isolated from the rFVIII-treated mice and reduced levels expressed by T cells obtained from pdFVIII-treated mice. Our findings support the results obtained by Hu et al. and Sasgary et al. and indicate that rFVIII induces the production of IL2, IL10 and IFN-γ by CD4+ T cells[68,69,113]. We further investigated the importance of IFN-γ on the formation of FVIII inhibitors by pre-treating hemophilic mice with neutralizing anti-IFN-γ antibodies before and after the four weekly 2 IU rFVIII treatments. The anti-IFN-γ treated mice showed a 30% reduction in FVIII inhibitor formation in comparison to the isotype control treated mice (data shown in the supplementary figures). This data provides additional evidence of the importance of IFN-γ on the formation of FVIII antibodies. In contrast to the results of rFVIII infusion, following pdFVIII administration, IL4, IL5, and TGF-β were expressed by CD4+ T cells.

Notably, TGF-β has been reported to suppress immune reactivity in hemophilic mice resulting in a reduced anti-FVIII titer[114,115,116]

We also observed higher levels of CD4+CD25+Foxp3+ T regulatory cells in the spleens of mice that were treated with pdFVIII compared to rFVIII. We propose that this has occurred due to a number of factors, including the release of the immunosuppressive cytokine TGF-β by the CD4+ T cells obtained from pdFVIII-treated mice. The increased numbers of these T regulatory cells may play a role in reducing immune reactivity towards FVIII but additional experiments must be conducted to confirm this hypothesis. The involvement of T regulatory cells in reducing the formation of anti-FVIII antibodies in pdFVIII-treated mice has previously been reported by Kallas et al[18].

A reduction in FVIII inhibitor development following pdFVIII infusion into hemophilia A mice has been observed previously. However, the mechanisms responsible for this phenomenon remain unresolved. Reipert et al. have suggested that antigenic competition between FVIII and other proteins in the pdFVIII concentrates, including VWF, may account for a reduction in presentation of FVIII by DCs[111]. Our results show that pdFVIII induces DC maturation, the release of Th2 cytokines and formation of high titer anti-human VWF antibodies. Thus, in the hemophilia A mouse model, human VWF appears to compete very effectively for immunological recognition.

We have further confirmed the possibility of antigenic competition between FVIII and another unrelated protein, factor IX. Our results agree with previous studies on antigenic competition[117,118]. These studies suggest that the presence of high

concentrations of other infused proteins during the FVIII treatment (in these studies, an approximately 100, 300 or 600 fold molar excess of FIX molecules) will result in reduced titers of anti-FVIII antibodies. By increasing the concentrations of FIX, we expect that we are increasing the competition between FIX and FVIII for antigenic sites on APC and antigenic recognition. The presence of high FIX concentrations during FVIII treatment might therefore result in fewer APCs presenting FVIII and thus a reduced anti-FVIII antibody titer. Significantly, we did not observe high anti-FIX antibodies in these mice. We propose that this is likely due to at least two factors: the expression of normal murine FIX protein in hemophilia A mice and the inherent reduced immunogenicity of FIX due to it's homology with other vitamin K-dependent plasma proteins.

As a result of these studies, we propose that the following mechanisms contribute to the observed reduction of FVIII inhibitor formation in the pdFVIII-treated mice. First, proteins found in pdFVIII concentrates will be internalized by DCs and will compete for antigenic presentation. Secondly, we have shown that pdFVIII concentrates elicit a different profile of immune gene expression in DCs compared to rFVIII. This difference in gene expression will influence the maturation state and cytokine production of DCs. In the hemophilia A mice, more DCs will present VWF to T cells resulting in the activation of more VWF-specific T cells. With the subsequent generation of a Th2 cytokine response, and the activation of VWF-specific B cells, a potent anti-human VWF antibody response develops. Thus, through mechanisms involving antigenic competition and

differential immune gene expression the anti-FVIII immune response will be substantially reduced.

In hemophilic patients who will be tolerant to human VWF and lack VWF-specific T cells there will be a different outcome. Nevertheless, we still expect that the VWF from pdFVIII products will compete with FVIII for antigenic presentation by DCs and that a different immune gene expression profile will be generated in these cells. We propose that these mechanisms will contribute to a reduction in FVIII immune reactivity.

In summary, we have shown that treating hemophilia A mice with pdFVIII results in the reduction of FVIII antibody formation. We propose that this differential effect occurs as a result of several factors including competition for antigen presentation, a different immune gene expression profile, and the production of Th2 instead of Th1 cytokines.

Chapter 5

General Discussion and Perspectives

General Discussion

The formation of FVIII antibodies in patients with hemophilia A occurs because the infused FVIII is viewed as a foreign protein triggering an immune response. There are a series of immunological interactions that are needed to activate the immune response. First, FVIII must be sampled by APCs. Second, $CD4^+$ T cells must be activated followed by the activation and differentiation of B cells into plasma cells. Finally, the plasma cells will produce and release anti-FVIII antibodies. Normally, healthy individuals do not develop FVIII antibodies due to self tolerance; whereby, most FVIII-specific T cells are deleted by a negative selection process in the thymus (central tolerance). However, previous studies have suggested that approximately 10-15% of healthy individuals possess anti-FVIII antibodies, suggesting that the central tolerance process is not completely exhaustive [119].

Central tolerance occurs until puberty while peripheral tolerance occurs throughout the life span of the individual. Induction of immune tolerance (ITI) towards FVIII is currently used to overcome the activation of the immune system resulting from FVIII immunogenicity in hemophilia A patients. Although this method of tolerance induction is widely used, it involves repeated daily intravenous infusions of FVIII, often for many months and can be very costly. In addition, in approximately 20% of cases the tolerance induction is unsuccessful. Therefore, other means of tolerance induction are being investigated. We have proposed the use of immature dendritic cells (iDCs) to induce tolerance to FVIII. Immature DCs were used because they are the first cells involved in activating

the immune system and they can regulate the immune system towards either immunity or tolerance [120]. iDCs lack the maturation markers that are needed to activate naïve T cells into effector cells [121]. Instead, iDCs induce the development of T regulatory cells that will suppress the immune system and result in the induction of peripheral tolerance [122]. By infusing FVIII-iDCs into hemophilia A mice, we were able to induce the development of two distinct subsets of T regulatory cells, the Tr1/Th3 and naturally occurring Foxp3 T cells. Peripheral tolerance was induced in our hemophilic mice after treating the mice with FVIII-iDCs followed by FVIII treatments. While this novel approach was associated with a reduction of neutralizing anti-FVIII antibodies of 25-40 %, complete tolerance towards FVIII in hemophilic mice was not achieved. Therefore, before pursuing further immunomodulatory studies, we attempted to elaborate some of the mechanisms that regulate the immune response in hemophilic mice to FVIII.

Our initial studies demonstrated that the immunologic response to FVIII in vitro is different to its behavior in vivo. After culturing DCs and hemophilic T cells with FVIII, we did not observe DC maturation or T cell activation. This indicates that FVIII in vitro does not behave as an immunogen and does not induce inflammatory “danger” signals. In contrast, intravenously infusing hemophilic mice with FVIII results in the activation of the immune system and formation of anti-FVIII antibodies and inhibitors. At the present time, we are lacking very important basic knowledge regarding the in vivo behavior of FVIII. Therefore, we investigated the interactions of FVIII in vivo with a variety of cells involved in innate and adaptive immune responses. We showed that FVIII in vivo results in

the release of inflammatory cytokines from cells derived from the spleen, this in turn, activates FVIII-specific CD4+ T cells and CD19+ B cells. Interestingly, the cytokine profile of the FVIII-specific T cells changes during the primary and secondary stages of the immune response. The cytokine profile shifts from a predominantly Th2 (primary immune response) into a predominantly Th1 (secondary immune response) profile. We observed that the Th1-prone C57BL/6 mouse model developed higher anti-FVIII antibody titers than the Th2-prone Balb/c mice. Our experiments have shown that the anti-FVIII antibodies are associated with the expression of higher IFN-γ levels. Therefore, we believe that there is a network of interactions between cells of the innate and adaptive immune systems that results in the immunogenic presentation of FVIII. This interaction is mainly regulated by cytokines and results in the formation of high anti-FVIII titers. Thus, interfering with the cytokine profile induced by FVIII might affect the development of FVIII antibodies. In this research program, we have shown that the cytokine profile towards FVIII is influenced by the genetic background of the hemophilic animals. It has been reported that the immunogenotype has an influence on the formation of FVIII inhibitors in hemophilic patients [24]. Our studies confirmed this finding in hemophilic mice and we have demonstrated that the Th1-prone C57BL/6 mice released more of IFN-γ and have higher anti-FVIII antibody titers. These data strongly suggest that IFN-γ has an important role in the splenic cytokine micro-environment and the subsequent level of FVIII inhibitor formation.

We were able to modulate the immune response towards FVIII in hemophilic mice by treating the animals with plasma-derived FVIII products. The introduction of human FVIII and VWF into the murine model resulted in the activation of the immune system towards both proteins. Therefore, different cytokines were released when mice were treated with pdFVIII in comparison to rFVIII. This resulted in a reduction of FVIII antibody development in the pdFVIII-treated mice. This finding further demonstrates the importance of the cytokine microenvironment on the formation of anti-FVIII antibodies.

Immune Tolerance Induction

Activation of the immune response and formation of anti-FVIII antibodies in hemophiliacs is currently the major clinical obstacle faced by hemophilia A patients. The most widely used strategy for treating inhibitor patients is the process of immune tolerance induction (ITI)[28]. ITI refers to the repetitive infusion of FVIII for a prolonged period of time, until tolerance to FVIII is achieved. ITI is considered to be successful in hemophilic patients with inhibitors when the patient has: an end result inhibitor titer ≤ 0.6 BU/mL, a plasma recovery of infused FVIII greater than 66% and a FVIII half-life of 6 hours or more[123]. After successful ITI, patients are placed on FVIII prophylactic treatment in order to maintain the FVIII immune tolerance [124]. However, ITI is only successful in approximately 87% of patients, and when FVIII inhibitors are still present after 33 months of the treatment course, ITI is stopped[125,126].

The first protocol for ITI was described in 1977 and since this time, several new treatment regimens have been introduced[28]. The most commonly used protocols

are the Bonn and Van Creveld protocols [29,30]. The Bonn protocol (high dose FVIII ITI) is divided into two phases. Phase one involves the infusion of 100 IU/kg FVIII twice daily and 100 IU/kg FEIBA twice daily. Phase two involves a gradual reduction in FVIII treatment over 3 months after achieving a normal FVIII half-life. The rate of success for this protocol is 87%[29]. The Van Creveld regimen (low dose FVIII ITI) also includes two phases, a neutralizing and a tolerizing phase. In the neutralizing phase, patients are treated with 25–50 IU/kg FVIII twice daily for 1–2 weeks. In the tolerizing phase, patients are treated with 25 IU/Kg FVIII every second day. The rate of success for this protocol is also 87%[30]. New ITI protocols that include the use of immune-modulatory products have also been introduced. There has been some focus on the use of anti-CD20, a B cell-deleting antibody (Rituximab™) in ITI. The use of Rituximab in ITI is shown to be beneficial, but the side effects of anti-CD20, especially the long-term depletion of B cells, are still unknown [127].

Although the ITI protocol seems straightforward, there are many complications that can be faced by patients while undergoing this treatment. Central venous catheter infections in young children, during the repetitive infusions have been reported [128]. These infections can prolong the time course of ITI and in some cases can result in ITI failure, most likely due to “inflammatory” conditions that preclude tolerance. Therefore, arteriovenous fistulae have been tested as an alternative to catheters, and there is preliminary data showing promising results[129]. Also, there is an association between the presence of anti-FVIII IgG4 subclass antibodies in hemophilic patients and prolonged ITI treatment[130]. These

data suggest that the immunological state of patients has an influence on the success rate of ITI. Another factor that is considered while pursuing an ITI regimen is the type of the FVIII product used[131]. Either plasma-derived or recombinant FVIII products can successfully induce immune tolerance [132]. The success rate of ITI using each product varies between different studies. However, the importance of VWF in pdFVIII concentrates on the success rate of ITI has not been investigated.

The exact mechanisms of ITI are not understood. There is a previous study by Hausl et al. demonstrating that high doses of FVIII will inhibit the differentiation of FVIII memory B cells into anti-FVIII antibody secreting plasma cells[133]. Interestingly, the authors show that this inhibition of memory B cells is irreversible and is independent of FVIII-specific T cells.

We attempted to induce immune tolerance through the tolerogenic presentation of FVIII by iDCs. We were able to reduce the immune response by 25-40% in hemophilic mice. However, we were not successful in achieving complete tolerance towards FVIII. Therefore, protocols for using FVIII-iDCs as potential strategies for ITI would need to be modified.

Treatment of Hemophilic Patients Using Recombinant FVIII or Plasma-Derived FVIII Concentrates

There are currently many studies investigating the associated risk of FVIII inhibitor development in patients treated with either plasma derived (pd) or recombinant (r) FVIII. The prospective German study demonstrated that patients treated with pdFVIII experienced a reduced incidence of FVIII inhibitor formation

in comparison to the rFVIII-treated patients[134]. However, the authors did not consider important additional factors that influence the risk of inhibitors such as the type of FVIII mutation, age of first exposure to FVIII and ethnicity. A group in France also studied the formation of FVIII inhibitors in patients who were treated with pdFVIII or rFVIII[19]. Their studies demonstrated that there is a higher risk of FVIII inhibitor development in patients treated with rFVIII in comparison pdFVIII products. In the French study, the finding that the risk of FVIII inhibitor development was higher with rFVIII was independent of FVIII genotype, ethnicity, a family history of FVIII inhibitor development and the age at first FVIII infusion. Finally, the CANAL study, which is considered to be the most credible study because it involved a large patient population and considered most of the factors that are associated with inhibitor formation[135], demonstrated no significant difference in the incidence of FVIII inhibitor formation between patients treated with the two types of FVIII concentrate.

How pdFVIII products might result in reduced FVIII inhibitor formation is being investigated. It is known that VWF binds the C2 domain of FVIII. Therefore, a research group investigated the binding affinity of anti-C2 antibodies to FVIII in patients treated with either rFVIII or pdFVIII. The result of this study showed that anti-C2 antibodies are less inhibitory in patients who are treated with pdFVIII[136]. The authors concluded that VWF will compete with the anti-C2 antibodies for the C2 domain binding sites[137]. Therefore, VWF shields the C2 domain epitopes of FVIII and prevents the anti-C2 antibodies from binding FVIII. Moreover, VWF has been shown to protect FVIII from endocytosis by DCs and thus, reduces FVIII

presentation to the immune system[14]. In our studies we proposed a mechanism that helps to clarify why the rFVIII-treated mice develop more FVIII inhibitors than pdFVIII-treated mice.

All of the clinical studies that compared the immunogenicity of rFVIII to pdFVIII in hemophilic patients are valid and can be used to assess the potential safety of future treatments. However, the organization of these studies was not randomized and the patient study populations were small. Therefore, many research groups around the world are now collaborating on a large international clinical study that will address the question of whether rFVIII products possess a higher risk of FVIII inhibitor development than pdFVIII in hemophilic patients. Previously untreated patients will be treated in a randomized fashion with pdFVIII and rFVIII. Patients will then be followed longitudinally with periodic measurement of FVIII antibodies and inhibitors.

Modulation of The Immune Response During FVIII Treatment

One of the most interesting observations in hemophilic patients under going FVIII treatment is that only 25-30% of these patients develop inhibitors against FVIII. There are reports that suggest the importance of immuno-modulating cytokines on the formation of FVIII inhibitors[22,23]. The formation of anti-FVIII antibodies is controlled by a complex interplay between cells of the immune system. Many researchers are investigating the use of products that can be co–administered with FVIII treatments in order to reduce the formation of anti-FVIII titers. There are many different ways in which the immune response can be modulated. Some research groups are using immuno-suppressive agents that target cells of the

innate immune system, while other groups are studying products that target cells of the adaptive immune system.

Salooja et al demonstrated that the immune system can be modulated if anti-CD4 antibody is co-administrated with the FVIII treatment[138]. The authors reported a significant reduction in the formation of FVIII inhibitors. Also, in our lab we have modulated the immune response towards FVIII by treating mice with anti-CD3 antibody before they are exposed to FVIII treatments. This strategy resulted in robust antigen-specific immune tolerance to FVIII. Machado et al targeted B cells using the chimeric anti-CD20 monoclonal antibody Rituximab while treating patients with FVIII inhibitors[139]. This treatment resulted in the depletion of FVIII inhibitors in these patients. These studies indicate that by targeting T or B cells of hemophilic animals or patients, a reduction in the FVIII inhibitory titers can be achieved. Nevertheless, the mechanisms responsible for these treatments are not fully understood and further studies need to be conducted.

In rare cases, healthy individuals can develop FVIII inhibitors; so called “acquired hemophilia A”. In most cases of acquired hemophilia A, patients are treated with a combination of immune suppressive agents such as corticosteroids and cyclophosphamide. Pejsa et al reported a rapid disappearance of FVIII inhibitors in acquired hemophilia A patients undergoing treatment with corticosteroids and cyclophosphamide[140]. These agents mainly interfere with the synthetic functions of inflammatory cells resulting in reduced release of inflammatory cytokines or through the cytotoxic removal of cells of the immune system.

Immunomodulation of hemophilic mice was studied by Reipert et al and Rosi et al [59 ,90]. The authors pre-treated the mice with anti-CD40 antibody before they received FVIII treatments. Their studies showed that hemophilic mice that received the anti-CD40 treatment had a reduction in their anti-FVIII antibody titer in comparison to the mice treated with FVIII alone. These findings provide additional evidence of the importance of the cellular interactions between DCs, T cells and B cells. Whereby, the CD40 co-stimulatory molecule that is found on DCs and B cells is critical for the activation of T cells and the subsequent development of FVIII antibodies.

Furthermore, Lei et al modulated the immune response towards FVIII through the presentation of A2 and C2 domains of FVIII on Ig backbones on activated B cells[44]. The authors demonstrated that this tolerogenic presentation of FVIII domains resulted in the expansion of the naturally occurring Foxp3 T regulatory cells. The authors also mentioned that this approach can be used to reduce the anti-FVIII titer in FVIII immunized mice.

We were able to modulate the immune system of hemophilic mice by co-administering rFVIII and the anti-inflammatory agent Andrographolide or anti-IFN-γ. We were able to reduce the FVIII inhibitory titer by 50% and 30% for the Andrographolide and IFN-γ treated mice, respectively (Data shown in Appendix AII). We presume that this is the result of the inhibition of inflammatory cytokine release and the neutralization of IFN-γ's pro-inflammatory activities.

Overall, these studies demonstrate the importance of cytokines, immune cells and immune cellular interactions on the formation of FVIII antibodies. Therefore,

future treatments using anti-inflammatory products, and T and B cell depleting agents should be considered in the management of hemophilic patients with FVIII antibodies.

Genetic Differences Between Hemophilic Animal Models

The murine FVIII protein is 77% homologous to the human FVIII protein, and the availability of hemophilia A mouse models has contributed significantly to the current understanding of the immunogenicity of FVIII. Additionally, hemophilia A mice consistently develop high FVIII inhibitory titers when repeatedly infused intravenously with human FVIII. Therefore, hemophilic mouse models can be used in pre-clinical studies to assess the efficacy of FVIII treatment.

In hemophilic patients, there are genetic factors such as HLA haplotypes and genetic polymorphisms that are associated with the development of FVIII antibodies[60]. This is also true for the animal models. We have investigated the immunogenicity of FVIII in different strains of hemophilic mice and different results were obtained in each mouse strain. There are several immuno-genetic differences between the C57BL/6 and Balb/c hemophilia A mice. The C57BL/6 mice express the MHC-II $H2^B$ haplotype while the Balb/c mice express the MHC-II $H2^D$ haplotype. The MHC-II molecules are needed for the presentation of peptides to CD4+ T cells and different haplotypes present different peptides to T cells. Moreover, the C57BL/6 mice are prone to develop a predominantly Th1 polarised immune response, whereby they tend to be resistant to intracellular infections due to their enhanced ability to produce IFN-γ[141]. However, the Balb/c mice are more likely to develop a predominantly Th2 type of response and they

tend to be resistant to bacterial infections due to their ability to produce IL4[142]. The causes of these immunological responses might be due to the differences in the cells of the innate immune system in each mouse strain[143]. The cells of innate immunity are responsible for directing the differentiation of CD4+ T cells into either Th1 or Th2 type cells.

This is also true for hemophilic patients. Polymorphisms within the IL10 and TNF-α genes were shown to be associated with inhibitor formation [22,23]. Also, White et al reported that specific HLA haplotypes have increased risk of FVIII inhibitor formation[20] and Hay et al showed an association between the HLA DRB1*1501 haplotype and FVIII inhibitors [21]. These findings support the results observed in the murine hemophilic model where a significant influence was apparent for the immunogenotype. This information further justifies the use of hemophilic mouse models to predicate the immunological outcomes of FVIII treatment in hemophilic patients.

All of these genetic differences between mouse strains can possibly have an influence on the formation of FVIII inhibitors. These genetic variables within the mouse models should be taken into account when designing and interpreting the results of pre-clinical FVIII treatment studies and gene therapy projects.

Modification of Clotting Factor Concentrates to Reduce The Immunogenicity of FVIII

A number of pharmaceutical companies and research laboratories are pursuing the goal of developing coagulation proteins with reduced immunogenicity. As one example, Baxter Bioscience have developed FVIII and VWF proteins with

reduced immunogenicity due to their chemical modification with the polymer, polyethylene glycol. While the specific coagulant activity of these modified proteins is reduced by the “pegylation” process, preclinical studies have shown that the immunogenicity of FVIII by can be reduced compared to the unmodified protein[144].

Furthermore, another group has reduced the immunogenicity of FVIII by preparing phosphatidylserine (PS)- liposomes containing FVIII[145,146]. These PS liposomes do not affect the functional activity of FVIII; in contrast, they increase the stability of FVIII. The authors reported that the hemophilic mice that were treated with (PS) - liposomes containing FVIII had reduced FVIII antibody titers than the mice that were treated with FVIII alone. This is expected to happen since the exposure of PS will reduce inflammation, adaptive immunity and antigenic protein presentation [147].

In the past, hemophilia A patients with inhibitors have been treated with porcine FVIII in order to bypass the human anti-FVIII antibodies. However, after exposure to porcine FVIII, anti-porcine FVIII antibodies develop in some of these patients. Barrow and colleagues reduced the immunogenicity of porcine FVIII by engineering seven human/porcine FVIII protein hybrids, the highly immunogenic sites of human FVIII “A2, *ap*, A3, and C2” were substituted with the less immunogenic porcine FVIII segments. The authors reported that the substitution of the human A2, A3, C2, and ap regions with the corresponding porcine sequences resulted in a significant reduction of porcine FVIII antigenicity relative to native porcine FVIII.

In our research group, Dr. Rawle mutated the C2 domain of FVIII in order to reduce its immunogenicity. The following two mutations were introduced into the C2 domain: Q2311T and V2314A. These two mutations reduced the C2 domain specific FVIII antibodies. However, we have no knowledge of the effects of these mutations on the activity of FVIII.

Currently, there are many companies/research groups who are interested in further improving the quality of life of hemophilic patients by developing new modified coagulation products. Their ultimate aim is to introduce new products with reduced immunogenicity, longer half-life and reduced cost.

Conclusions

FVIII in vivo behaves as a strong immunogen. This results in the release of pro-inflammatory factors and cytokines causing the activation of the immune system. The information obtained from this thesis along with the current knowledge on FVIII treatments can be used to introduce new treatments for hemophilic patients with antibodies and inhibitors. For example, patients can be treated with FVIII along with anti-inflammatory agents such as anti-IFN-γ treatments; which might result in minimal activation of the immune system and improved life qualities of hemophilic patients.

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Appendix I

General Methods

A1.1 Isolation of Splenocytes For In vitro Cytokine Studies

Purpose: To isolate splenocytes for in vitro antigen (rFVIII or pdFVIII or VWF) stimulation

Materials

1) Cell Culture Media (1000ml)

i. RPMI-1640 media with L-glutamine, without sodium bicarbonate (GIBCO)
ii. 2.0g/L sodium bicarbonate ($NaHCO_3$) (Sigma)
iii. 10% Fetal Bovine Serum (FBS-heat inactivated)
iv. 0.5% Penicillin/Streptomycin (GIBCO)
v. 0.1mM non-essential amino acids (GIBCO)
vi. 50μM 2-mercaptoethanol (Sigma)

2) Equipment

i. 3mL syringes (B-D, Luer-Lok)
ii. 25 G5/8 PrecisionGlide Needles (B-D)
iii. 70μm Nylon Cell Strainer (Falcon)
iv. Conical Tubes: 15 and 50mL (Starstedt)
v. 100mm Petri dishes-tissue culture (Starstedt)
vi. Sterilized surgical tools (scissors, forceps)
vii. Various sizes of pipettes (Rainin)

3) Read blood cell lyses: 0.16M NH_4Cl and 0.17M Trip, pH 7.2

4) Animals: male and female E16-Balb/c and C57BL/6 mice aged 6-10 weeks of age

Method

1. Anesthetize mouse with Hypnorm: Midazolam: Water (1:1:2)
2. Sacrifice mouse by cervical dislocation (forceps across cervical vertebrae and pull tail)
3. Repeatedly disinfect mouse with spray bottle of ethanol
4. Pull back skin of mouse to expose spleen
5. Use scissors and forceps to cut around the spleen
6. Remove the spleen using tweezers
7. Place the spleen in a 6 cm petri-dish with Collagenase D solution (5 mL/spleen).
8. Inject spleen with 1000 μL of Collagenase D solution, then cut the tissue into smaller pieces using scissors.
9. Incubate the spleen pieces in Collagenase D solution for 30 minutes at 37 °C.

10. Pass the whole material, i.e. remaining fragments and Collagenase D-released cells, through a 70 µm cell strainer using a plunger.
11. Collect all cells in a 50 mL tube and wash the cells by adding HBSS to a final volume of 40 mL
12. Spin the cells at 350g for 10 minutes at 4 °C
13. Wash the cell pellet in 20 mL HBSS
14. Repeat step 12
15. Re-suspend the cell pellet in 1 mL HBSS
16 Lsye the red blood cells with tris-buffered ammonium chloride by adding 2 mL of the mixture: 90% 0.16M NH_4Cl and 10% of 0.17M Tris. (incubate for 3 minutes at room temperature)
17. Quench the cellular mixture by adding 20 mL HBSS
18. Repeat step 12
19. Wash the cell pellet in 10 mL RPMI media
20. Count cells using hemocytometer
21. Adjust the cellular concentration to 1 million cells per 150µL using RPMI media
22. Add the stimulating antigen 10 IU/mL of rFVIII or pdFVIII or VWF to the splenocytes
23. Add 150µL of cells + condition to each well in U- bottomed sterile 96- well plates
24. Plate each condition in quadruplicate
25. Culture the cells for 120 hours at 37°C
26. Remove all media from each well and spin cells at 350g for 10 minutes at 4 °C
27. Analyze the cell supernatant for the prenscne of IL2, IL4, IL5, IL6, IL10, IFN-γ and TGF-β by ELISA following the manufacturer's protocol

A1.2 Isolation of Bone Marrow

Purpose: To isolate mouse bone marrow for dendritic cell purification

Materials

1) Cell Culture Media (1000ml)

i. RPMI-1640 media with L-glutamine, without sodium bicarbonate (GIBCO)
ii. 2.0g/L sodium bicarbonate ($NaHCO_3$) (Sigma)
iii. 10% Fetal Bovine Serum (FBS-heat inactivated)
iv. 0.5% Penicillin/Streptomycin (GIBCO)
v. 0.1mM non-essential amino acids (GIBCO)
vi. 50μM 2-mercaptoethanol (Sigma)

2) Equipment

i. 3mL syringes (B-D, Luer-Lok)
ii. 25 G5/8 PrecisionGlide Needles (BD)
iii. 70μM Nylon Cell Strainer (Falcon)
iv. Conical Tubes: 15 and 50mL (Starstedt)
v. 100mm Petri dishes-tissue culture (Starstedt)
vi. Sterilized surgical tools (scissors, forceps)
vii. Various sizes of pipettes (Rainin)

3) Read blood cell lyses: 0.16M NH4Cl and 0.17M Trip, pH 7.2

4) Animals: male and female E16-Balb/c and C57BL/6 mice aged 6-10 weeks of age

Method

1. Anesthetize mouse with Hypnorm: Midazolam: Water (1:1:2)
2. Sacrifice mouse by cervical dislocation (forceps across cervical vertebrae and pull tail)
3. Repeatedly disinfect mouse with spray bottle of ethanol
4. Pull back skin of mouse and dissect muscle and fat to expose femur
5. Use scissors and forceps to cut bone below knee joint, slice muscle along the femur and cut the top of the bone at hip joint above femoral head
6. Remove the other femur and place bones in 100mm Petri dish in ethanol
7. Wash femurs with HBSS
8. Transfer femurs to 100mm Petri dish in media
9. After femurs have soaked in media, remove the remaining muscle and tissue with scissors and Kim-wipes.
10. Cut both ends of bones as close to the ends as possible.
11. Flush marrow into strainer on conical tube using 3mL of RPMI media with a

25 G5/8 needle, alternating 3 mL through top and 3 mL through bottom.

12. Flush until bone is transparent.
13. Filter marrow suspension through a 70μM nylon strainer to remove any bone particles and debris
14. Centrifuge at 350 g for 10 minutes at 4 °C
15. Remove supernatant and resuspend cells in 30 mL media
16. Count on hemocytometre
17. At day 0, seed BM mixed cell population at a concentration of $1x10^6$ cells/mL
18 At day 0, add cytokines
    a. GM-CSF (1000U/mL)
    b. IL-4 (1000U/mL)
19. At day 2, remove 80% of the media and replenish with fresh media
20. On day 4, repeat step 19
21. On day 5, harvest the loosely attached cells
22. Continue with one or more of the following protocols
    a. CD11c isolation via magnetic labeling following the manufacturer's protocol
    b. Flow Cytometry following the manufacturer's protocol
    c. T cell-DC co-culture studies

A1.3 Isolation of CD4 T cells From Mouse Spleen

Purpose: To isolate CD4 T cells for intracellular cytokine staining studies and T cell-DCs co-cultures,

Materials

1) Cell Culture Media (1000ml)

i. RPMI-1640 media with L-glutamine, without sodium bicarbonate (GIBCO)
ii. 2.0g/L sodium bicarbonate ($NaHCO_3$) (Sigma)
iii. 10% Fetal Bovine Serum (FBS-heat inactivated)
iv. 0.5% Penicillin/Streptomycin (GIBCO)
v. 0.1mM non-essential amino acids (GIBCO)
vi. 50μM 2-mercaptoethanol (Sigma)

2) Equipment

i. 3mL syringes (B-D, Luer-Lok)
ii. 25 G5/8 PrecisionGlide Needles (B-D)
iii. 70μM Nylon Cell Strainer (Falcon)
iv. Conical Tubes: 15 and 50mL (Starstedt)
v. 100mm Petri dishes-tissue culture (Starstedt)
vi. Sterilized surgical tools (scissors, forceps)
vii. Various sizes of pipettes (Rainin)

3) Read blood cell lyses: 0.16M NH4Cl and 0.17M Trip, pH 7.2

4) Animals: male and female E16-Balb/c and C57BL/6 mice aged 6-10 weeks of age

Method

1. Anesthetize mouse with Hypnorm: Midazolam: Water (1:1:2)
2. Sacrifice mouse by cervical dislocation (forceps across cervical vertebrae and pull tail)
3. Repeatedly disinfect mouse with spray bottle of ethanol
4. Pull back skin of mouse to expose spleen
5. Use scissors and forceps to cut around the spleen
6. Remove the spleen using tweezers
7. Place the spleen in a 6 cm Petri-dish with Collagenase D solution (5 mL/spleen).
8. Inject spleen with 1000 μL of Collagenase D solution, then cut the tissue into smaller pieces using scissors.
9. Incubate the spleen pieces in Collagenase D solution for 30 minutes at 37 °C.

10. Pass the whole material, i.e. remaining fragments and collagenase D released cells, throught a 70 70 µm cell strainer using a plunger.
11. Collect all cells in a 50 mL tube and wash the cells by adding HBSS to a final volume of 40 mL
12. Spin the cells at 350g for 10 minutes at 4 °C
13. Wash the cell pellet in 20 mL HBSS
14. Repeat step 12
15. Re-suspend the cell pellet in 1 mL HBSS
16. Lsye the red blood cells with tris-buffered ammonium chloride by adding 2 mL of the mixture: 90% 0.16M NH_4Cl and 10% of 0.17M Tris. (incubate for 3 minutes at room temperature)
17. Quench the cellular mixture by adding 20 mL HBSS
18. Repeat step 12
19. Wash the cell pellet in 10 mL HBSS
20. Count cells using hemocytometer
21. Proceed to CD4 T cell indirect magnetic labeling following the manufacturer's protocol.

A1.4 T cell and Dendritic Cells Co-Cultures

Purpose: To study T cells proliferation and cytokine release in T cell-DCs co-cultures

Materials

1) Cell Culture Media (1000ml)

i. RPMI-1640 media with L-glutamine, without sodium bicarbonate (GIBCO)
ii. 2.0g/L sodium bicarbonate ($NaHCO_3$) (Sigma)
iii. 10% Fetal Bovine Serum (FBS-heat inactivated)
iv. 0.5% Penicillin/Streptomycin (GIBCO)
v. 0.1mM non-essential amino acids (GIBCO)
vi. 50μM 2-mercaptoethanol (Sigma)

2) Cells: purified FVIII pulsed DCs and T cells as explained in 1.2 and 1.3
3) U bottom sterile 96-well plate

Methods

1. Add 10,000 FVIII-DCs to 100,000 T cells to each well in U-bottomed 96 well plates in a volume of 150μL RPMI media. Plate wells in quatroplicates
2. After 72 hours after the addition of antigen, collect the cellular cultures for
    i. Analyses of cytokines in the media

A1.5 Anti-FVIII, Anti-FIX and Anti-VWF ELISA

Purpose: to quantify the titer of Anti-FVIII, Anti-FIX and Anti-VWF antibodies in mouse plasma

Materials

1. rFVIII or rFIX or VWF (capture Ag)
2. Anti-FVIII or Anti-FIX or Anti-VWF standard curve (Abcam)
3. Coating buffer (50 mM Carbonate buffer) 1.59g Na_2CO_3, 2.93g $NaHCO_3$, fill beaker up to 1L dH2O, pH 9.6
4. Washing buffer (0.1% Tween 20-PBS)
5. Blocking buffer (PBS-BSA) dissolve 5g of BSA in 250 PBS pH=7.4 aliquot and store at -20
6. Secondary antibody: for total antibody or antibody isotype titre, isotype specific goat anti-mouse Ig-HRP (Southern Biotech)
7. Sample Diluent HBS-BSA-EDTA-T20 : 5.95g HEPES, 1.46NaCl, 0.93g Na_2EDTA, 2.5g BSA, 250uL Tween-20 in 250 in H2O, pH=7.2 (Use NaOH) aliquot and store at -20
8. OPD buffer (0.1M Citric acid-phosphate buffer): 0.0347M citric acid (3.34g citric acid), 0.0667M Na_2HPO_4 (4.73g Na_2HPO4), fill up to 500 mL dH2O, pH 5.0
9. Colour Reagent: 15 mL of OPD buffer (described above), 2 x 5 mg tablets of OPD, dissolve in OPD buffer, immediately prior to loading on plate, add 6.2 µL 30% H_2O_2
10 Stop solution (1.0 M H_2SO_4)
11 VERSAmax microplate reader

Method

1. Coat a 96-well microtiter plate with 100 µL of capture Ag 2µg/mL per well suspended in a 0.1M carbonate buffer, and incubate at 4°C overnight.
2. Wash plate 3 times with 300 µL of 0.1% Tween 20 in PBS.
3. Block plate with 200 µL of Blocking buffer for 2 hrs at room temperature.
4. Prepare the standard curve by performing 1:2 dilution starting from 0.5µg/mL of FVIII or 0.25µg/mL of FIX or 0.125µg/ml of VWF
5. During the blocking incubation, prepare plasma samples. Dilute plasma samples with sample diluent. Use normal mouse plasma as your control
6. Wash plate 3 times with 300 µL of 0.1% Tween 20 in PBS.
7. Load 100 µL of samples in duplicate to the plate.
8. Incubate plasma samples on plate for 2h at room temperature.
9. Wash plate 5 times with 300 µL of 0.1% Tween 20 in PBS.
10. Add 100 µL of the secondary antibody. For determining total antibody titre, dilute polyclonal goat anti-mouse Ig-HRP 1:500 in sample diluent. For

determining antibody isotype titre, dilute isotype-specific goat anti-mouse Ig-HRP 1:500 in blocking buffer. Incubate at room temperature for 1 hour.

11. Wash plate 7 times with 300 μL of 0.1% Tween 20 in PBS.
12. Add 100 μL of OPD substrate reagent to each well. Incubate for 20 minutes in the dark.
13. Add 50 μL of 1M H_2SO_4.
14. Read the optical density at 490 nm on a microplate reader within 5min after adding H_2SO_4.
15. Subtract the blank values from all sample values.

A1.6 One Stage Factor FVIII Assay (APTT)

Purose: to detect functional FVIII in plasma , and it is used indirectly to quantify the levels of functional Anti-FVIII inhibitors

Materials

1. Activated Agent: Biomerieuz automated APTT reagent
2. 25mM $CaCl_2$
3. HBS-BSA: HEPES buffered saline with 0.1% BSA pH 7.4 (50 mM Hepes, 100mM NaCl, 0.1% BSA)
4. COAG-A-MATE MAX machine
5. Human reference standard pooled plasma
6. FV/FVIII deficient canine plasma
7. Bovine FV

Method

1. Turn on machine
2. Set up the machine with all reagents: APTT reagent, 25mM $CaCl_2$ and HBS-BSA
3. Add bovine FV at 1/50 dilution to the FV/FVIII deficient plasma
4. Make a calibration curve by performing 1:2 dilution of the reference plasma in HBS-BSA
5. Dilute the mouse plasma in HBS-BSA (1:5, 1:10 and 1:20) then add 1:1 ratio of human reference plasma pool
6. Prepare a control sample that has HBS-BSA and human reference at 1:1 ratio
7. Incubate the diluted samples for 2 hours at 37 ºC
8. Enter sample information into the machine program
9. Start the machine to measure the clot time
10. Plot the results on analyzing software while taking into account all performed dilutions
11. Determine the Bethesda units level in each sample by relating the clotting time of each sample to the clotting time of control sample
12. Measure the bethesda values only on samples that have between 25%-75% FVIII activity

Appendix II

Supplementary Experiments

Supplementary experiments

AII.1 The Modulation of The Immune System Toward FVIII (Approach A):

Aim: To modulate the immune response by treating hemophilic mice with Anti -IFN-γ before and after rFVIII treatments

Materials and Methods:

Male and female Balb/c E16 hemophilia A mice, 6-10 weeks old were used in all experiments. Mouse genotype was assessed for the deletion of FVIII exon 16 by polymerase chain reaction (PCR) using genomic DNA isolated from tail clips as described by Connelly et al.[57] All mouse experiments were performed in accordance with the Canadian Council for Animal Care and the Queen's University Animal Care Committee approved all animal protocols.

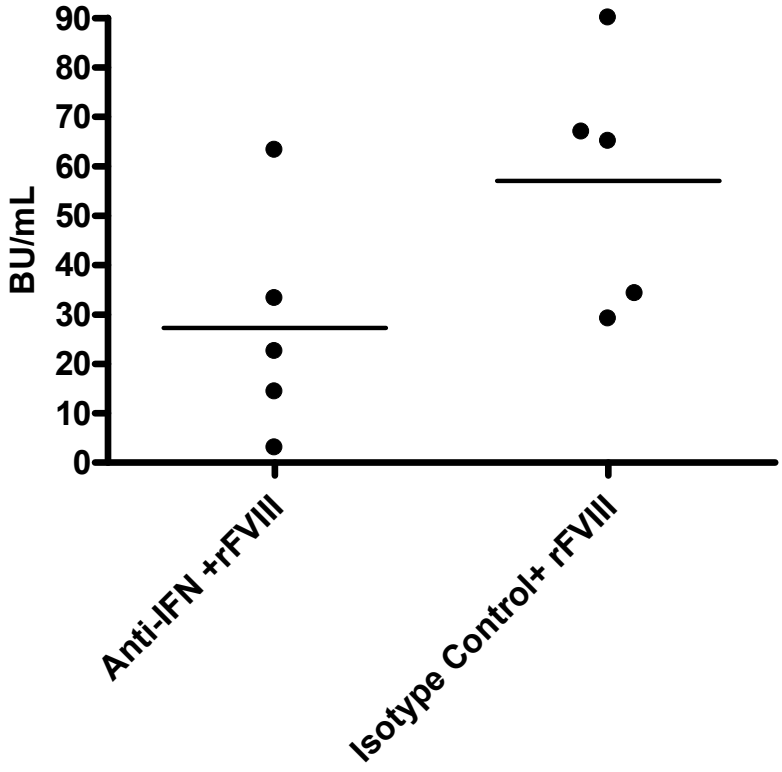
Treatment of Mice With Recombinant Human FVIII

Naïve hemophilic mice received four weekly intravenous infusions of 2 IU human recombinant rFVIII Kogenate® (rFVIII). Twenty four hours before and after each rFVIII infusion, the mice were treated with 300 μg of either anti-IFN-γ or isotype control antibody.

Results and Discussion

IFN-γ is a pro-inflammatory cytokine and important in maintaining T and B cell survival and promoting antibody class switching. The presence of IFN-γ indicates the involvement of Th1 T cells. As shown in figure AII.1, the inhibitor level in the mice that were treated with anti-IFN-γ before/after each rFVIII infusion was lower than the inhibitor titer of the isotype control mice. These experiments demonstrate, for the first time, the importance of IFN-γ in vivo on the formation of FVIII inhibitors.

Figure AII.1 Treating hemophilic mice with neutralizing anti-IFN-γ antibody before and after rFVIII treatment results in reduced FVIII inhibitor formation. Naïve hemophilic mice of 6-10 weeks of age were treated with four weekly infusions of 2 IU rFVIII. These mice were pre-treated with either neutralizing anti-IFN-γ or isotype control antibody 24 hours before and after each rFVIII treatment. Mice were sampled 1 week following the fourth treatment. The horizontal lines represent the mean of inhibitor titer for 5 mice.


BU/mL
90
80
70
60
50
40
30
20
10
0
Anti-IFN +rFVIII
Isotype Control+ rFVIII

We have previously shown that rFVIII induces the production of IFN-γ by CD4+ T cells. We expect that the IFN-γ is needed for the survival of FVIII-specific T and B cells. Therefore, by neutralizing the biological effects of IFN-γ, the FVIII inhibitor titer will be reduced. These studies confirm the results obtained by Sasgary et al. and demonstrate the importance of Th1 cytokines on the formation of FVIII inhibitors [68]. Therefore, future FVIII treatments can include a combination of therapeutic FVIII infusion and Th1 cytokine inhibitors such as anti-IFN-γ to minimize the risk of an anti-FVIII immune response.

AII.2 The Modulation of The Immune System Towards FVIII

(Approach B)

Aim: To modulate the immune response by treating hemophilic mice with FVIII and the anti-inflammatory agent Andrographolide

Animal Model:

Male and female Balb/c E16 hemophilia A mice, 6-10 weeks old were used in all experiments. Mouse genotype was assessed for the deletion of FVIII exon 16 by polymerase chain reaction (PCR) using genomic DNA isolated from tail clips as described by Connelly et al. All mouse experiments were performed in accordance with the Canadian Council for Animal Care and the Queen's University Animal Care Committee approved all animal protocols.

Treatment of Mice With Recombinant Human FVIII

Naïve hemophilia A mice received four weekly intravenous infusions of 2IU human recombinant FVIII Kogenate® (rFVIII). One hour before each rFVIII

treatment, the mice were intravenously infused with either 100 µL of 10 µM of the anti-inflammatory agent Andrographolide (Andro) or 100 µL of HBSS.

Results and Discussion

Andrographolide is an anti-inflammatory agent. It blocks the activity of the NF-κB transcription factor resulting in reduced expression of pro-inflammatory genes.

As shown in figure AII.2 , the inhibitor titer of the mice that were treated with Andro before FVIII infusion was 50% lower than the control mice. This data shows a direct association between the inflammatory immune response and FVIII inhibitor formation. In order to further understand the mechanisms responsible for these findings, additional studies including splenocyte cultures and intracellular T cell cytokine staining must be conducted.

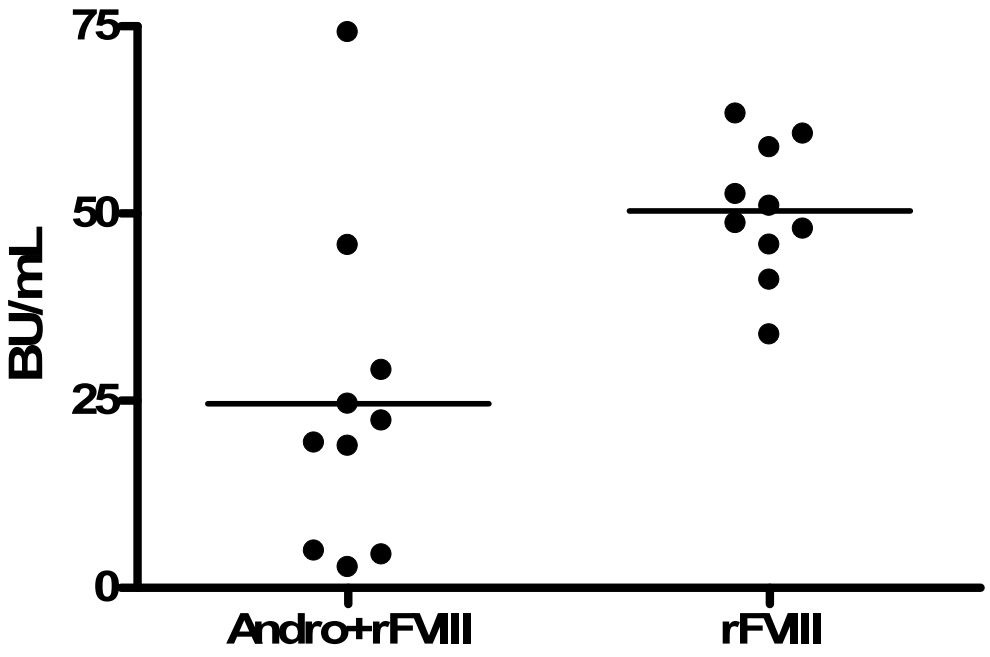
AII.3 Investigating The Immunogenicity of Human Plasma-Derived FVIII and Recombinant FVIII in C57BL/6 VWF/FVIII Double Knock Out Mice.

Aim: To study the immunogenicity of FVIII and VWF in VWF/FVIII double KO mice following the infusion of pdFVIII and rFVIII

Materials and Methods:

Male and female C57BL/6 VWF/FVIII KO, 6-10 weeks old mice were used in all experiments. Mouse genotype was assessed for the deletion of FVIII exon 16 by polymerase chain reaction (PCR) using genomic DNA isolated from tail clips as described by Connelly et al. The absence of VWF was assessed by Southern blot as previously described by Denis et al [148]. All mouse experiments were performed in accordance with the Canadian Council for Animal Care and the Queen's University Animal Care Committee approved all animal protocols.

Figure AII 2. Treating hemophilic mice with Andro before rFVIII treatment results in reduced FVIII inhibitor formation. Naïve hemophilic mice of 6-10 weeks of age were treated with four weekly infusions of 2 IU rFVIII. These mice were pre-treated with either Andro in HBSS or HBSS only 1 hour before and after each rFVIII treatment. Mice were sampled 1 week following the fourth treatment. The horizontal lines represent the mean of inhibitor titer for 10 mice.


75
50
25
0
BU/mL
Andro+rFVIII
rFVIII

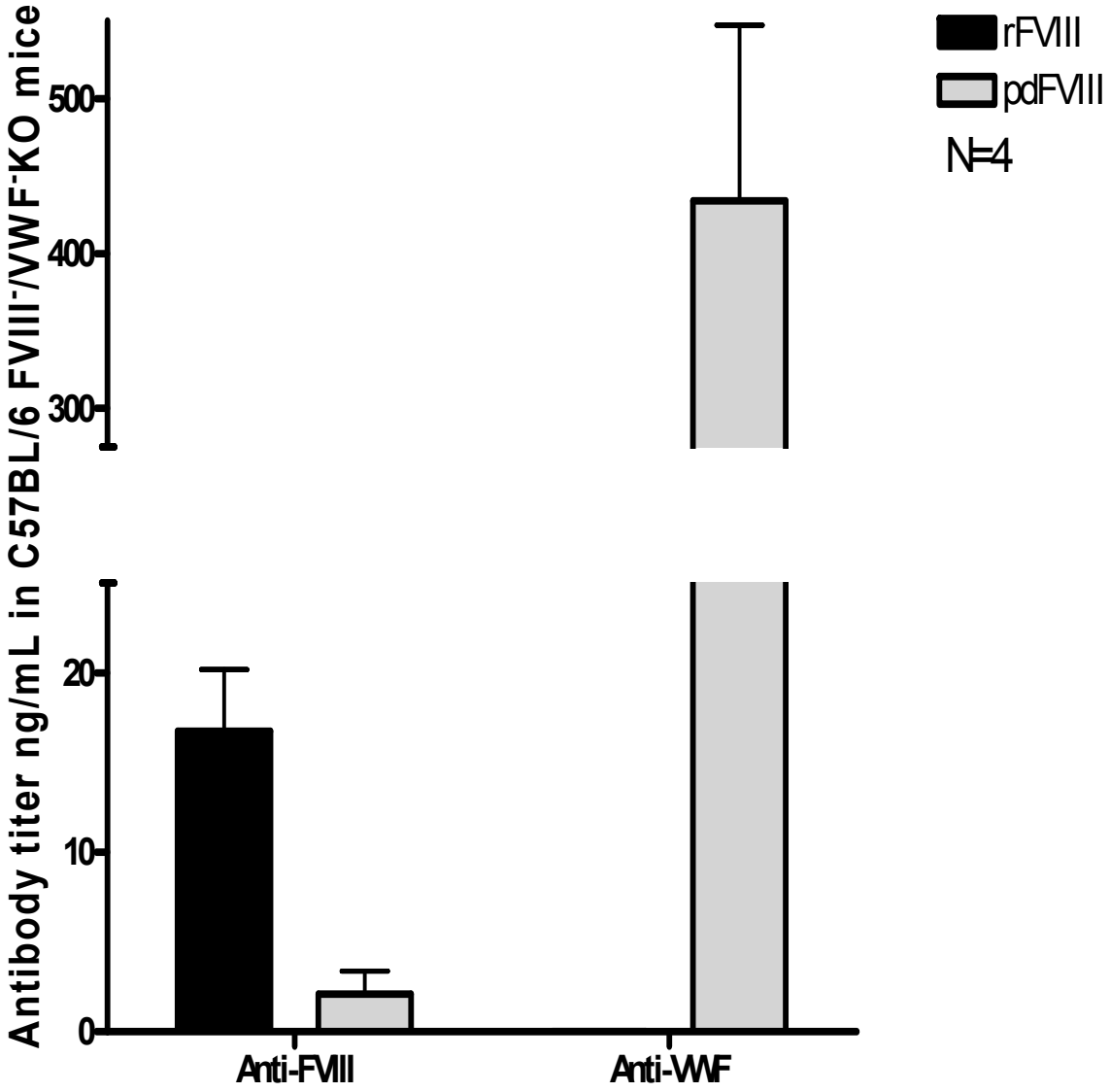
Treatment of Mice With Human Recombinant FVIII or Plasma-Derived FVIII

Naïve C57BL/6 VWF/FVIII KO mice received four weekly intravenous infusions of human recombinant FVIII Kogenate® (rFVIII) or plasma-derived human FVIII Wilate® (pdFVIII). Each dose comprised of 2 IU of FVIII (200 ng: equivalent to 80 IU/kg) diluted with HBSS into a final volume of 200µL. Total anti-FVIII and anti-VWF antibody titers were quantified by ELISA.

Results and Discussion

The immunogenicity of VWF and FVIII was assessed in FVIII/VWF double knock out (DKO) mice. This unique mouse model allowed us to better investigate the immunogenicity of VWF and FVIII in mice who are not naturally tolerized to either of these proteins. Our results show that infusing pdFVIII into C57BL/6 VWF/FVIII DKO animals results in the formation of high titers of anti-VWF antibodies and reduced levels of anti-FVIII antibodies in comparison to the mice treated with rFVIII alone figure AII. 3. These results suggest that the VWF found in pdFVIII has competed with FVIII for immunological recognition and has significantly reduced the formation of anti-FVIII antibodies. We expect that the immune response towards VWF has suppressed the immune response towards FVIII as a result of competition for antigenic presentation. Reipert defined antigenic competition as the inhibition of the immune response to one antigen by the

Figure AII 3. Treating VWF/FVIII double knockout mice with pdFVIII results in reduced FVIII antibody formation and the development of high Anti-VWF titers. Naïve VWF/FVIII double KO mice 6-10 weeks of age were treated with four weekly infusions of either 2 IU rFVIII or pdFVIII. Mice were sampled 1 week following the fourth treatment. The horizontal lines represent the mean of antibody titer for 4 mice.


rFVIII
pdFVIII
N=4
Antibody titer ng/mL in C57BL/6 FVIII-/VWF-KO mice
500
400
300
20
10
0
Anti-FVIII
Anti-VWF

administration of another antigen. This phenomenon is well described in the literature.

AII.4 Investigating the Internalization of VWF and FVIII by Dendritic Cells

Aim: To study the in vitro internalization of FVIII and VWF by dendritic cells

Materials and Methods:

Hematopoietic cells were isolated as described by Inaba et al. The culture regimen was designed so that the cells were fed on days 0, 2 and 4. On day 5, loosely-adherent cells were aspirated and washed four times with HBSS (GIBCO BRL, Rockville, MD). DCs were isolated using magnetic beads coated with anti-CD11c antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's guidelines. Seven hundred and fifty thousand DCs were cultured in serum-free RPMI 1640 (Invitogen Burlington, Ontario, Canada) with either 1 IU rFVIII, 1 IU VWF, 1 IU pdFVIII and 10 µg rFVIII+ 0.1 µg VWF for 2 hours at 37 ºC. Then, cells were isolated, washed, fixed and made permeable using Cytofix/Cytoperm kit (BD Biosciences, San Diego CA, USA). The dendritic cells were then stained for intracellular VWF and FVIII via FITC-anti-VWF (Affinity Biologicals, Ancaster, Canada) and PE-Biotin (Miltenyi Biotec, Bergisch Gladbach, Germany) + Biotin-Anti-FVIII (Affinity Biologicals, Ancaster, Canada) respectively. Cells were then analyzed by flow cytometry within 12 hours.

Results and Discussion

We were able to investigate the competition between FVIII and VWF for dendritic cell internalization. This was achieved by staining the DCs cultured with rFVIII, pdFVIII or VWF with FITC-VWF and PE-FVIII antibodies. For the DCs cultured with 1 IU pdFVIII (10 ug VWF and 0.1 µg FVIII), we observed more FITC-VWF^+ $CD11c^+$ cells than PE-$FVIII^+CD11c^+$ cells, figure AII.4. However, when the DCs were cultured with 10 µg FVIII and 0.1 µg VWF, we observed more PE-$FVIII^+CD11c^+$ than FITC-VWF^+ $CD11c^+$ cells. Our results show that the protein with the higher concentration will be internalized preferentially by DCs. These studies indicate that there is competition between FVIII and VWF for internalization by dendritic cells.

AII.5 Validation of Microarray Studies

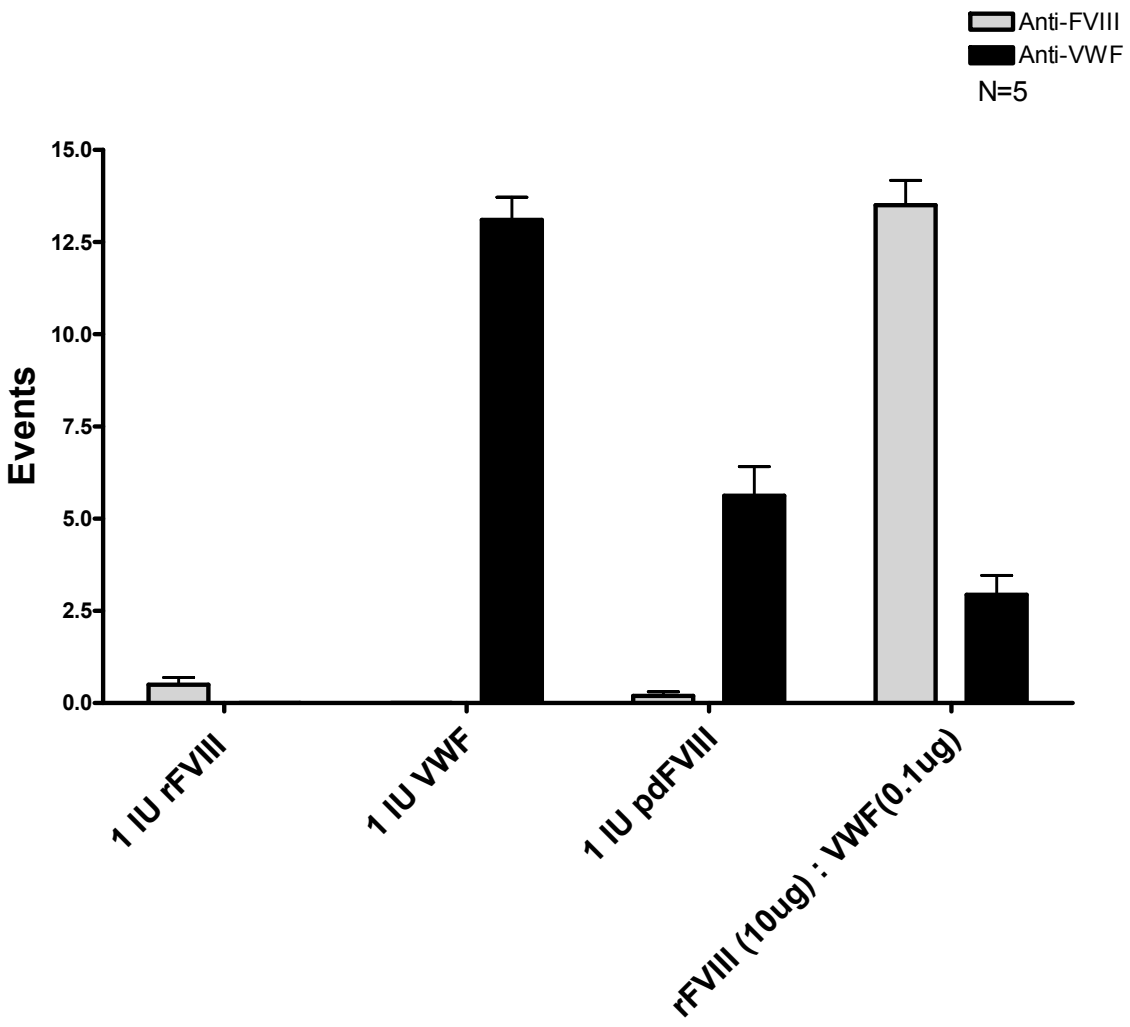
Aim: to validate the gene expression in DCs via real time PCR after treating mice with rFVIII or pdFVIII

Materials and Methods

RNA isolation from FVIII in vivo pulsed dendritic cells

Naïve hemophilia A mice of 6-10 weeks of age were infused intravenously with three different materials: 200 µL of HBSS, 2 IU (80 IU/kg) of human recombinant FVIII Kogenate FS® (rFVIII) or 2 IU (80 IU/kg) plasma-derived human FVIII Wilate® (pdFVIII). Twenty four hours later, the spleens were isolated. For each

Figure AII 4. More VWF is internalized than FVIII by dendritic cells cultured with pdFVIII. Investigation of the in vitro uptake of FVIII and VWF by CD11c DCs. Bone marrow derived CD11c+ DCs obtained from naïve hemophilia A mice were cultured with VWF, rFVIII or pdFVIII for 2 hours at 37 ºC. The intracellular proteins were quantified by flow cytometry after permeabilizing and staining the DCs with anti-FVIII-PE or anti-VWF-FITC. The error bars represent the standard error of the mean for 5 separate experiments.


Anti-FVIII
Anti-VWF
N=5
Events
15.0
12.5
10.0
7.5
5.0
2.5
0.0
1 IU rFVIII
1 IU VWF
1 IU pdFVIII
rFVIII (10ug) : VWF(0.1ug)

infusate, 6 mouse spleens were pooled to generate one experimental biological replicate. For each infusate, we had 4 experimental biological replicates (i.e. 4 x 6 pooled splenocyte samples). The $CD11c^+$ dendritic cells were isolated from each splenocyte pool using magnetic beads coated with anti-CD11c (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's guidelines. The CD11c cellular pellets were resuspended in Trizol (Invitrogen, Carlsbad, CA, USA). RNA isolation and purification steps were performed according to the manufacturer's guidelines. RNA quantity was evaluated by Nanodrop while the RNA quality was assessed by the Aglinet BioAnalyzer and there was no evidence of RNA degradation.

Quantitative real-time PCR

Expression of the differentially-induced genes as determined by micro-array evaluation was validated by quantitative real time PCR (qRT -PCR). The RNA was isolated as described earlier. The qRT -PCR experiments were conducted by SuperArray Bioscience Corporation (Frederick, Maryland, USA). For each condition, each gene was run in quadruplicate. Each sample was normalized to the (ACTB) house keeping gene. The HBSS samples were used as the control samples for analysis. The fold change was calculated based on the $2^{-\Delta\Delta Ct}$ method[149].

Results and conclusion

All of the interesting gene expressions that were obtained by the microarray (table 4.1) studies were validated via qRT–PRC as shown in Figure AII.5. Table AII.1 summaries the genes were common in both treatment groups.

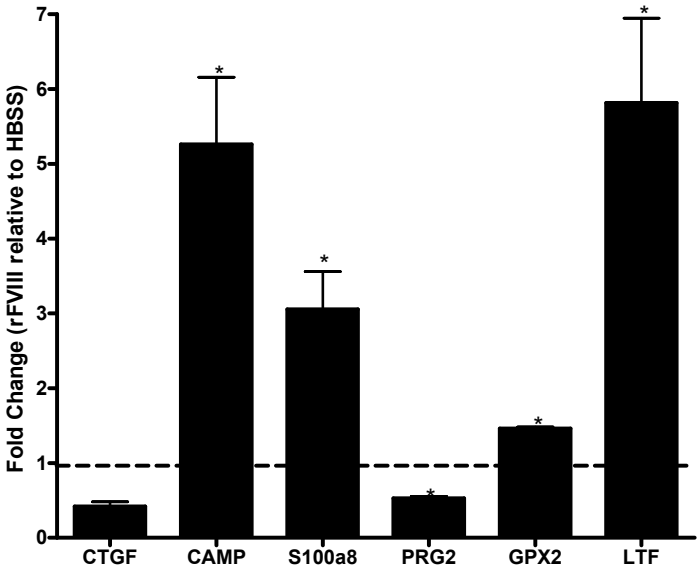
Table AII.1. Common genes that were differently expressed due to rFVIII or pdFVIII treatment in dendritic cells

Gene ID	Gene symbol	rFVIII/HBSS	pdFVIII/HBSS
20440	St6gal1	0.61	0.66
14219	Ctgf	0.44	0.56
12822	Col18a1	0.63	0.66
16176	Il1b	1.53	1.61
20201	S100a8	2.53	1.99
20296	Ccl2	0.57	2.09
19074	Prg2	0.36	0.43
17474	Clec4d	1.99	1.74
58217	Trem1	1.66	2.92
20692	Sparc	0.58	0.47
14776	Gpx2	2.47	1.70
14824	Grn	1.50	1.80
12826	Col4a1	0.50	0.58
268697	Ccnb1	0.65	0.65
193740	Hspa1a	1.60	3.01

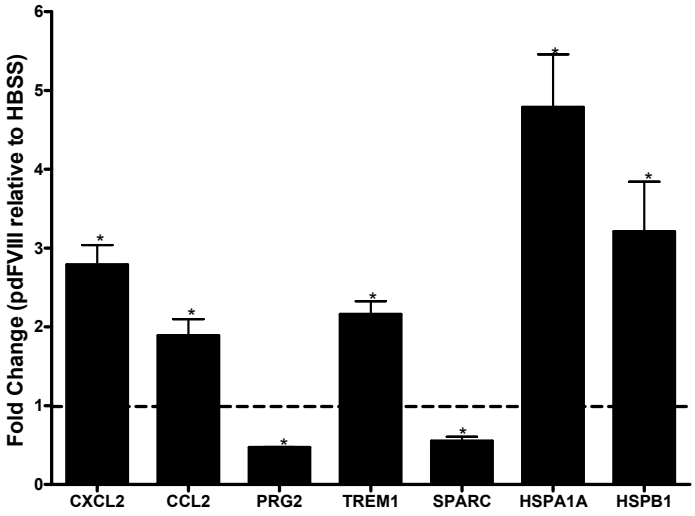
Figure AII.5 The pdFVIII and rFVIII treatments induce the expression of different genes in dendritic cells. Gene expression in DCs 24 hours following the (a) rFVIII or (b) pdFVIII treatments was analyzed via qRT-PCR. The HBSS treated mice were used as a control. The fold change for each gene was calculated as explained in the materials and methods section. Fold inductions were considered statistically significant if the P value was <0.05 in at least 3 of the 4 biological repeats. The dashed line represents the gene expression of the control samples. The error bars represent the standard error of the mean.

N= 3-4

A)


Fold Change (rFVIII relative to HBSS)
0
1
2
3
4
5
6
7
CTGF
CAMP
S100a8
PRG2
GPX2
LTF

B)


Fold Change (pdFVIII relative to HBSS)
0
1
2
3
4
5
6
CXCL2
CCL2
PRG2
TREM1
SPARC
HSPA1A
HSPB1