DISSERTATION

CHARACTERIZATION AND ANALYSIS OF FVIII - SPECIFIC T CELLS IN HEMOPHILIC MICE TREATED WITH HUMAN FACTOR VIII - DEVELOPMENT OF NEW STRATEGIES FOR TOLERANCE INDUCTION

Ausgeführt zum Zwecke der Erlangung des akademischen Grades
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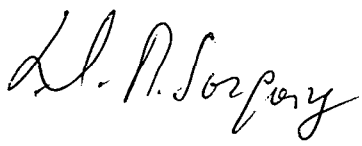

Wien, im Oktober 2003

Für meine beiden Schätze:
Victoria und Adrian

Der ist der Selbsterkenntnis und Selbstverwirklichung am nächsten, der mit seinem Schicksal zufrieden und einig ist. Denn die Zufriedenheit ist die Fröhlichkeit des Menschen auch in der Bitterkeit des täglichen Lebens.

Zun-Nun

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ZUSAMMENFASSUNG

Die Behandlung von Hämophilie A Patienten mit Substitutionsprodukten (Faktor VIII Produkten) kann zur Ausbildung einer Immunantwort führen, wobei Alloantikörper (Inhibitoren) gebildet werden. Diese inhibitorischen Antikörper sind gegen spezifische Epitope auf dem Faktor VIII Molekül gerichtet. Als Folge wird die Aktivität des betroffenen Koagulationsfaktors neutralisiert und die Koagulationsfaktor-Ersatztherapie ist für Hämophilie Patienten teilweise oder vollständig wirkungslos. Das Auftreten solcher inhibitorischen Antikörper stellt gegenwärtig die Hauptkomplikation bei der Behandlung von Hämophilie Patienten dar. Die Mechanismen, die die Immunantwort gegenüber Faktor VIII bestimmen, sind weitgehend unbekannt.

Diese Arbeit wurde durchgeführt, um die zelluläre Immunantwort gegen ein extrazelluläres Protein-Antigen (Faktor VIII) auf der Ebene der T-Lymphozyten zu studieren. Es wurden Studien zur Kinetik und die Charakterisierung der T-Helfer Zellen ($CD4^+$ Zellen), die nach der Präsentation von Antigen aktiviert werden und die Produktion der Antikörperbildenden B Zellen anregen, durchgeführt. Die Bedeutung der Interaktion von CD40/CD40L (Ligand) für die Entstehung der anti-Faktor VIII Immunantwort wurde ebenso untersucht. Die gewonnenen Erkenntnisse sollen helfen, neue Strategien zur Behandlung von Hämophilie A Patienten zu entwickeln und die Mechanismen, die bei der Ausbildung der Immunantwort gegen Faktor VIII eine Rolle spielen, besser zu verstehen.

Für die Untersuchungen wurde ein murines knockout Modell verwendet, das durch Ausschalten des Exon 17 im Faktor VIII Gen, mit Hämophilie A Patienten vergleichbare Immunantworten und Symptome ausbildet. Die angewandten Protokolle zur Behandlung der hämophilen Mäuse entsprachen den Behandlungsprotokollen für Hämophilie A Patienten mit Substitutionstherapie oder den Protokollen zur Toleranzinduktion in hämophilen Patienten. Hämophile Mäuse wurden in wöchentlichen Abständen mit humanen Faktor VIII (Recombinate, Baxter BioScience) behandelt. Die Analyse Faktor VIII-spezifischer T Zellen in den Milzen wurde in wöchentlichen Abständen mittels Multiparameter-Durchflusszytometrie auf Basis von Einzelzellen analysiert. Die

Detektion intrazellulärer Zytokine in den $CD4^+$ Zellen erfolgte nach in vitro Restimulierung mit humanem rekombinantem Faktor VIII. Die Entstehung der anti-Faktor VIII Antikörper wurde durch ELISA Systeme (Bestimmung der Gesamt- anti-Faktor VIII Antikörper) und funktioneller Tests (Bethesda-Test zur Bestimmung der neutralisierenden Antikörper) analysiert.

Zwischen dem Auftreten von Faktor VIII-spezifischen $CD4^+$ Zellen in der Milz und der Detektion von anti-Faktor VIII Antikörpern im Plasma konnte eine zeitliche Korrelation festgestellt werden. Es wurde gefunden, dass Faktor VIII-spezifische $CD4^+$ Zellen IFN-γ (Interferon-γ) in hohem Ausmaß produzieren. Daraus lässt sich schließen, dass Th1 Zellen (T-Helfer Zellen vom Typ 1) eine bedeutende Funktion auf die Regulation der anti-Faktor VIII Immunantwort ausüben. Am zweithäufigsten wurden IL-10 (Interleukin 10) produzierende $CD4^+$ Zellen gefunden. IL-10 produzierende Zellen wurden nach 2 Dosen FVIII gefunden und zeigten eine Zunahme der Häufigkeit nach der 4. Dosis. Durch Co-Expressions-Analysen intrazellulärer Zytokine konnte gezeigt werden, dass Faktor VIII-spezifische $CD4^+$ Zellen IL-10 und IFN-γ auch in einer Zelle gemeinsam produzieren können. IL-2 (Interleukin 2) produzierende T Zellen wurden nach Verabreichung von 2 Dosen Faktor VIII gefunden. IL-4 (Interleukin 4) produzierende Zellen wurden in einigen wenigen Experimenten mit geringer Häufigkeit gefunden, meistens konnte IL-4 aber nicht detektiert werden. Die Analyse intrazellulärer Zytokine in Faktor VIII-spezifischen T Zellen zeigte, dass mindestens 4 Arten von Zytokin-produzierenden Zellen nach Faktor VIII Verabreichung vorhanden sind: IL-2, IFN-γ, und IL-10 produzierende Zellen und Zellen, die IFN-γ und IL-10 co-expremieren. Welche Rolle IL-10 produzierende Faktor VIII-spezifische T Zellen bei der Regulation der Immunantwort ausüben, ist zur Zeit Gegenstand weiterer Arbeiten.

Die Ergebnisse dieser Arbeit zeigen, dass die Entstehung von anti-Faktor VIII Antikörpern T-Zellen abhängig ist und durch die Induktion von T-Helferzellen des Typs 1 als auch des Typs 2 bestimmt wird. Die komplexen Interaktionen der verschiedenen Subpopulationen Faktor VIII-spezifischer T-Helfer Zellen führen entweder zur Stimulierung oder Niederregulierung der anti-Faktor VIII Immunantwort. Es konnte auch gezeigt werden, dass das Induzieren neutralisierender anti-Faktor VIII Antikörper durch Hemmung der CD40/CD40L Interaktion mit einem anti-CD40L Antikörper vollständig verhindert werden

kann. Allerdings konnte mit diesem Ansatz konnte keine dauernde Immuntoleranz gegen Faktor VIII erreicht werden.

1. ABSTRACT

The development of neutralizing anti-FVIII antibodies in patients with hemophilia A is currently the most serious complication of factor VIII replacement therapy. Despite progress in recent years the mechanisms regulating the immune response to factor VIII and the determinants of immunogenicity of FVIII products remain unclear. Previously a murine knockout model of hemophilia A generated by a targeted disruption in exon 17 of the factor VIII gene was used to study the immune response to human factor VIII. Using this animal model it was demonstrated that hemophilic mice have characteristics of antibody responses to human factor VIII similar to those in patients with hemophilia A. Intravenous injection of therapeutic doses of human FVIII give rise to high titers of anti-FVIII antibodies directed against both functional and non-functional parts of the FVIII molecule. This animal model is, therefore, suitable to search for new strategies to induce immune tolerance against factor VIII. This animal model was used in this Ph.D. thesis to study the kinetics and the features of FVIII-specific T cells that regulate the induction of anti-FVIII antibodies. The importance of CD40/CD40L (ligand) interactions for the initiation of the anti-FVIII immune response was also under investigation. The used treatment protocols mimicked either the replacement therapy of patients with factor VIII or the induction of tolerance against factor VIII in patients. Attention was in particular focused on the induction of neutralizing anti-FVIII antibodies and the Th1/Th2 polarization of FVIII-specific T cells. For this purpose, sensitive assays were developed that were suitable to analyze factor VIII-specific T cells on a single cell level. These assays were used to follow the kinetic of factor FVIII-specific T cells after treatment of hemophilic mice with human factor FVIII or after treatment with an anti-CD40L antibody (MR1) that blocked the CD40/CD40L interaction.

The results of this work demonstrate that the induction of anti-FVIII antibodies is T cell dependent and is characterized by the induction of both Th1 and Th2 cells. This T cell response involves a complex interaction between different subsets of FVIII-specific helper T cells that might either stimulate or down-modulate the anti-FVIII-immune response. Furthermore it is shown that the induction of neutralizing anti-FVIII antibodies in hemophilic mice after treatment with human FVIII could be completely prevented by a blockade of CD40/CD40L

interactions using a monoclonal anti-CD40L antibody (MR1). However, the initial blockade of CD40/CD40L interactions was not sufficient to induce a lasting immune tolerance against FVIII.

TABLE OF CONTENTS

2. INTRODUCTION

2.1 HEMOPHILIA

The inherited deficiency of blood coagulation factors leads to lifelong bleeding disorders commonly called hemophilias. The factors most frequently found deficient in hemophilias are factors VIII (FVIII) and IX (FIX), whose genes are located on the X chromosome and, when mutated, cause the X-linked recessive traits hemophilia A and B. Females are carriers of this trait.

The reported incidence of hemophilia A is 1 in 10,000 men and is caused by incomplete function or lack of circulating coagulation factor VIII. Hemophilia B, also called Christmas disease is less common (1 in 60,000 birth). Hemophilia causes bleeding episodes, either spontaneously or as a result of trauma. Typically, bleeding occurs into joints or into muscles. Recurrent hemarthroses can lead to severe arthritis. Hemophiliacs are also susceptible to intracranial bleeding, causing strokes.

2.1.1 HEMOPHILA A

The clinical classification of hemophilia A is based on the residual activity of factor VIII in the circulation. A factor activity of less than 1% is designated as severe hemophilia. Levels between 1 and 5 % are defined as moderate severity, and when the levels are above 5% the disease is of the mild form.

Form of hemophilia	**severe**	**moderate**	**mild**
Factor VIII activity	<1%	1 to 5 %	> 5 %
Characteristic mode of bleeding	Frequent spontaneous hemorrhages	Bleeding after slight injuries	Bleeding after severe injuries and during surgery
Frequency of bleeding	Once or twice a week	About once a month	Rare (no spontaneous bleeding)

Tab.1: clinical classification of hemophilia A

People without hemophilia A have between 50-150% of the normal level of factor VIII (normal concentration of FVIII in blood plasma is 0,1 μg/ml). Severe hemophilia is actually the most common form; about 40% of all hemophilia patients have the severe form. The main symptoms of severe forms of hemophilia A are spontaneous bleedings mainly into joints or muscles. They usually occur at the end of the first year of life for the first time. The deficiency of the coagulation factor is treated with infusions of FVIII concentrates. However, in many patients, particularly those with severe hemophilia A, therapeutically administered exogenous factor VIII comes to be recognized as a foreign protein, and thus stimulates the production of anti-FVIII antibodies. These antibodies are frequently directed against functionally important regions of the protein and, therefore, inhibit the functional activity of factor VIII. Inhibitors to factor VIII have been recognized as a complication of hemophilia since the introduction of replacement therapy about 40 years ago (1). Today there are no effective strategies to prevent the antibody response in patients, and efforts to establish immune tolerance are not always successful once antibodies have developed. Understanding the mechanisms underlying hemophilia A requires detailed knowledge of structure and function of FVIII.

Specific antibodies against factor VIII have also been detected in patients without hemophilia. Some of these anti-factor VIII antibodies in non-hemophiliacs are similar to those in classic hemophilia but many of them recognize different sites on the factor VIII molecule. Many factor VIII inhibitors appear to arise in patients with diseases associated with altered immune function or other conditions such as the postpartum period, certain types of malignancy, systemic lupus erythematosus and chronic lymphocytic leukemia. Presumably the anti-factor VIII inhibitors in non-hemophiliacs are autoantibodies. Frequently, these autoantibodies disappear with time, sometimes without specific therapy. However, management of these patients is as difficult as management of hemophilic patients with inhibitors.

2.2 STRUCTURE AND FUNCTION OF FVIII IN HEMOSTASIS

FVIII is a plasma glycoprotein with procoagulant activity. The molecule is a large multidomain protein (about 330 kDa) produced mainly by the liver as a single-stranded polypeptide chain of 2332 amino acids. Factor VIII shows a repeating domain structure A1-A2-B-A3-C1-C2 (2) that is in close homology to coagulation factor V (also A1-A2-B-A3-C1-C2). It is highly sensitive to proteolytic processing before and after secretion. Only a small fraction of circulating FVIII is in the single-chain form. The predominant form in the circulation consists of two polypeptide chains. These chains are formed when the FVIII precursor is proteolytically cleaved to generate the heavy chain of variable length (consisting of the A1 and A2 domains together with variable lengths of B domain due to partially proteolysis of B domain) and the light chain consisting of the A3, C1 and C2 domains. The heavy and light chains that comprise the heterodimer form of FVIII are non-covalently linked via a divalent metal bridge. Between the A domains there are a high number of negatively charged residues that are also known as acidic rich regions (*a1, a2, a3*) (Fig.1).

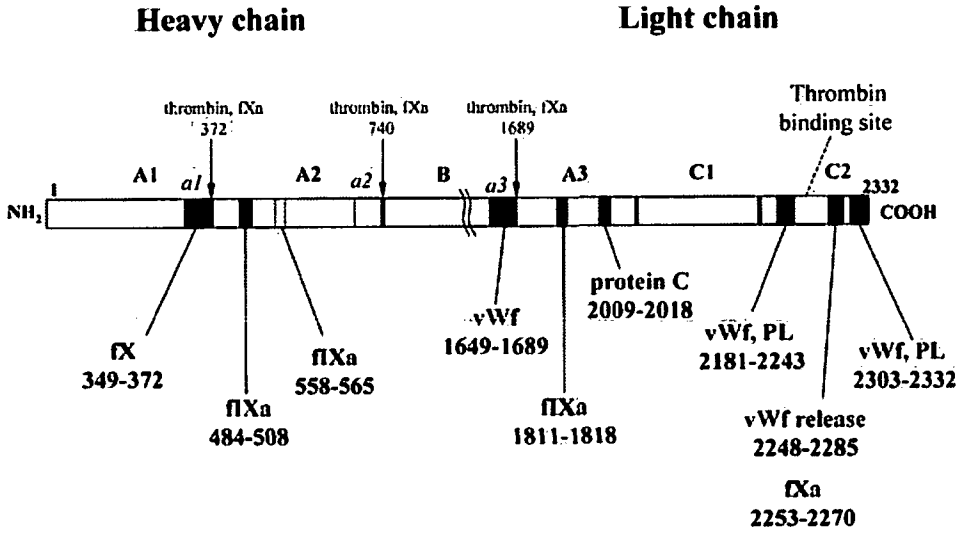

Fig. 1: major functional binding sites and epitopes of inhibitory antibodies within the FVIII molecule. Binding sites for major FVIII ligands (VWF, FX, FIXa, PL, FXa and aPC) are shown. Ligand binding sites that are also targets for inhibitory antibodies are shown in blue. (3)

VWF	von Willebrand factor	PL	phospholipid
FX	coagulation factor X	FXa	coagulation factor Xa
FIXa	coagulation factor IXa	aPC	activated protein C

After release into the circulation factor FVIII binds noncovalently to von Willebrand factor with high affinity. Each monomer of the multimeric VWF protein is able to bind one factor VIII molecule. In vivo, however factor VIII heterodimers form a complex with the VWF in a molar ratio of 1:50. The multimeric adhesive protein VWF acts as a carrier protein complex for FVIII during its secretion and circulation in the plasma. Complex formation with VWF is required for maintaining the normal FVIII level in plasma and prevents the premature formation of the Xase complex (a complex of activated FVIII and activated FIX). VWF binds to the light chain of FVIII and thereby inhibits the binding sites of important players of the coagulation cascade that initiate clotting. The interaction of FVIII with VWF is of great importance in vivo, as it has been shown that lack of VWF reveals a secondary deficiency of factor VIII suggesting a lack in protecting FVIII from proteases and a faster clearance of factor VIII from the circulation (4).

Biochemical evidence and molecular cloning data have previously indicated that blood coagulation involving tissue factor, prothrombin and fibrinogen is present in all vertebrates and might have evolved over 430 million years ago. In mammalian blood coagulation five proteases, factor VII (FVII), factor IX (FIX), factor X (FX), protein C (PC) and prothrombin act with five cofactors (tissue factor [TF]; factor V [FV]; factor VIII [FVIII]; thrombomodulin and protein S) to control the generation of fibrin (5).

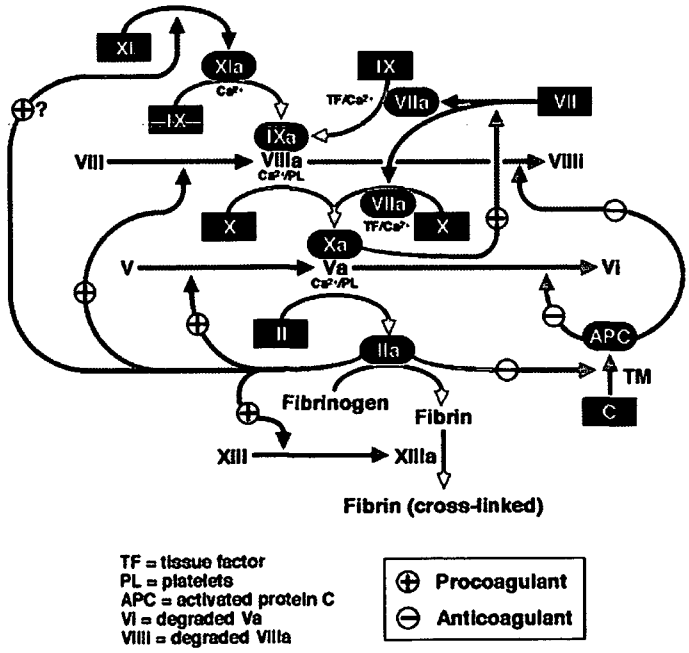

Fig.2: Amplification and localization of coagulation reactions (6)

There are two major activation pathways in blood coagulation. The extrinsic pathway in which the physiological activation of the coagulation cascade is mediated almost exclusively via the interaction of tissue factor with FVII and the intrinsic pathway that is activated via the contact system. The two pathways are are highly interconnected. For example, the tissue factor/VIIa complex activates not only factor X but also factor IX of the intrinsic pathway. The catalytic nature of coagulation reactions greatly amplifies the initial stimulus. Each VIIa/tissue factor complex can produce many Xa molecules, which subsequently produce an even greater amount of thrombin. Amplification also results from positive feedback reactions like the activation of V, VIII, and possibly XI by thrombin, as well as activation of VII by Xa (6).

Factor VIII is an essential component of the intrinsic pathway of blood coagulation cascade, where it serves as an accelerating cofactor for activated factor IX (FIXa), a serine protease. Factor VIII is activated by thrombin through cleavage of its heavy and light chain at precisely defined sites (positions Arg 372, 740 and 1689) or by FXa-mediated proteolysis.

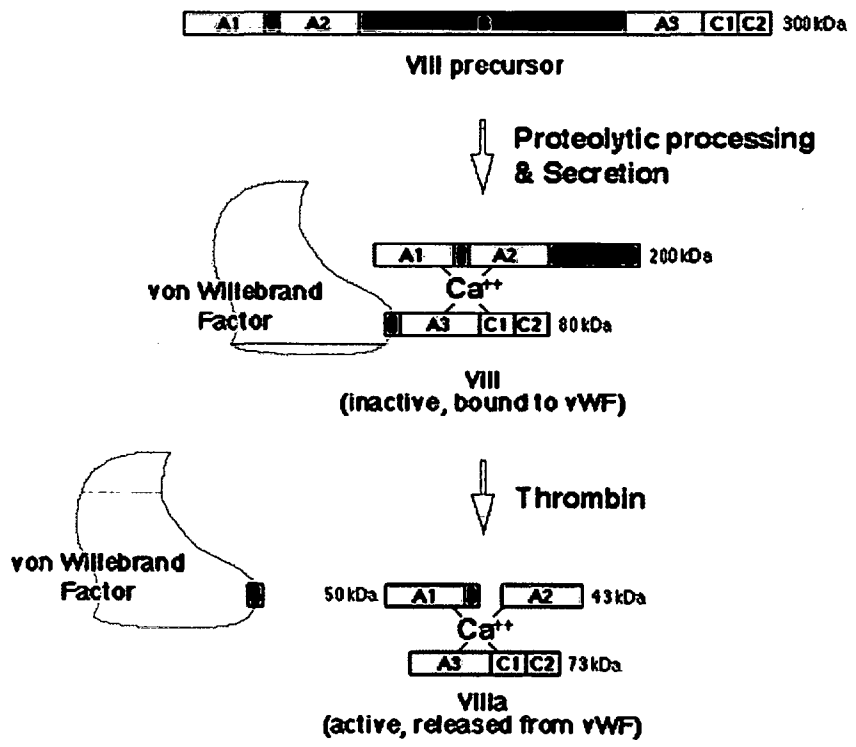

Fig. 3: activation processes during the life cycle of factor VIII (6)

Activation of factor VIII by thrombin leads to release of the B domain that is described not to participate in the co-enzymatic action of factor VIIIa in the coagulation process (7) but is suggested to have an important role in the regulation and quality control of factor VIII biosynthesis and activation by thrombin (8). The release of the B domain reduces the size of the molecule and separates VWF. During this locally controlled mechanism, factor VIII remains bound to the phospholipid surface on platelets via its C2 domain and form a macromolecular complex with membrane-bound factors IXa and X. Conversion of factor X into its active form (FXa) is catalyzed by FIXa. Subsequently FXa participates in conversion of prothrombin into thrombin, the key enzyme of the coagulation cascade. Further proteolytic cleavage by activated protein C (aPC), thrombin, FIXa or FXa may specifically inactivate factor VIIIa and are important down-modulating mechanisms for factor VIII activity after coagulation. Thus, the normal physiologic function of coagulation factor VIII requires its interactions with physiologically important ligands, including vWF, PL, FIXa, FX, thrombin and FXa.

Mechanisms responsible for factor VIII turnover have been recently established (9). A low-density-lipoprotein receptor-related protein (LRP), which is a hepatic clearance receptor with a broad ligand specifity, mediates the catabolism of factor VIII together with cell surface heparan sufate proteoglycans (HSPGs) that facilitates the clearance of factor VIII from VWF complex. The regions of factor VIII involved in binding of the FVIII-VWF complex to LRP and HSPGs were found to be localized in the A2 domain. The catabolic pathway of FVIII involves the initial binding of the FVIII-VWF complex to HSPGs, which serves to concentrate the complex on the cell surface and to present it to LRP. As the half-life of factor VIII is relatively short (about 12 hours) the finding of specific receptors involved in the turnover of factor VIII might help to improve hemophilia A therapy by the design of factor VIII molecules with prolonged lifetime in circulation.

2.3. MOLECULARE DEFECTS IN HEMOPHILIA

Technical innovations to probe for gene defects have allowed the definition of the molecular defect in patients with clinically significant bleeding due to haemophilia A. A database of hemophilia A mutations and phenotype data can be found on the Internet at http:// europium.csc.mrc.ac.uk. There are two prevalent mutations in severe hemophilia A. In general, null mutations (deletions, stop codons, insertions, splicing abnormalities) predicting no or truncated protein production are associated with severe factor VIII deficiencies and clinical phenotypes. About 40 % of the cases of severe hemophilia A are caused by an inversion within intron 22 of the factor VIII gene. Missense mutations lead to severe deficiencies too. In these cases, it is thought that impaired folding and/or altered conformation of the mutant factor VIII lead to intra-and extracellular instability, which in turn causes severe factor VIII deficiencies in plasma (10). Analysis of patients`DNA has permitted identification of the great majority of the gene lesions that cause hemophilia A and has allowed secondary control of the disease through carrier detection and antenatal diagnosis.

2.4 FVIII INHIBITORS - Development and characterization

Inhibitory antibodies to factor VIII may arise in hemophilic and in nonhemophilic patients in response to factor VIII infusions. They are diagnosed by the sudden lack of responsiveness to FVIII replacement and develop usually in the early childhood; they arise less commonly after the age of 5 years (11). The Bethesda assay is the accepted standard to detect and quantify anti-factor VIII antibodies in plasma of patients with congenital or acquired hemophilia (12). This assay is based on testing the ability of patient's plasma to inactivate factor VIII in normal plasma. As there is evidence that antibody formation to factor VIII in hemophilia A patients is more frequent than it is indicated by Bethesda assays more sensitive assays e.g. immunoprecipitation assays or enzyme-linked

immunosorbent assays are used additionally for detecting anti-factor VIII antibodies that cause reduced factor VIII recovery (13).

Factor VIII contains three epitopes that are usual targets of clinically significant inhibitory antibodies. Most inhibitors are directed against epitopes located in the A2 or C2 domains of factor VIII and to a lesser extent to an epitope within the A3-C1 region. Inhibitor binding to these domains prevents factor VIII from binding to factor IXa (A2, A3), factor Xa (C2), and phospholipids (C2). Binding to the acidic region between A1 and A2 domains interferes with factor X binding. The release of activated factor VIII from von Willebrand factor can be slowed by inhibitor binding to a minor C2 epitope and is shown to prevent factor VIII association with phospholipid. Inhibitory antibodies in hemophiliacs are usually directed to at least two or three epitopes, whereas autoantibodies that can appear in non-hemophiliacs are more prone to be specific for a single domain (14).

Mutation analysis revealed a correlation between the type of mutation and inhibitor formation and has also been applied to predict the development of antibodies inhibiting factor VIII. It is suggested that, among patients with severe hemophilia, those with mutations, which interfere with VIII biosynthesis - intron 22 inversions, large deletions, and nonsense mutation leading to stop codons, have a significantly higher risk (about 30 %) to develop inhibitors than those with missense mutations (4 %) or small deletions (7%) (15).

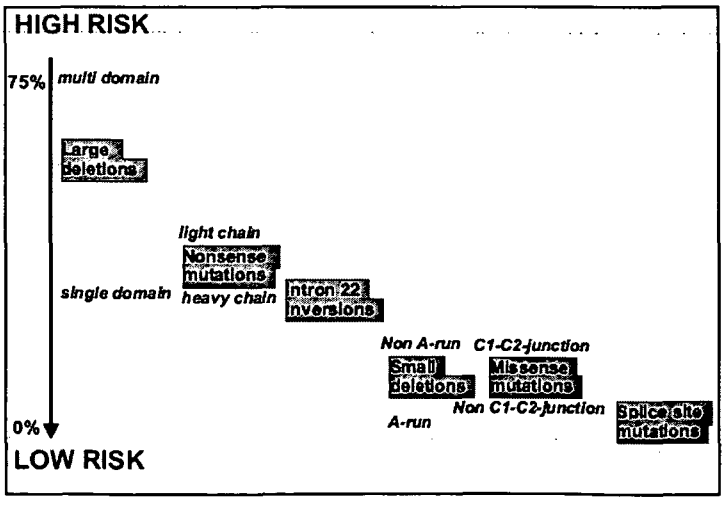

Fig.3: risk of inhibitor development (16)

For patients with the mild or moderate forms of hemophilia A it was recently shown that carrying certain mutations in the C1 domain of factor VIII give rise to a higher risk of inhibitor occurrence (17).

In addition, other genetic factors such as HLA haplotype are believed to influence the anti-factor VIII immune response. Furthermore, variables such as family history of inhibitors, race, and type of clotting factor concentrate; therapy regimen, age at first exposure and other medication etc have to be considered in influencing inhibitor development.

2.5 TREATMENT OF HEMOPHILIA A

Replacement therapy with factor VIII concentrates started 1970 and has revolutionized hemophilia care. An increased availability of plasma concentrates of coagulation factors and home replacement therapy led to the early control of hemorrhages. Prophylactic treatment was successfully implemented in many countries to prevent the majority of bleeding episodes and further reduce the impact of arthropathy. The infection of about 70% of patients with severe hemophilia with human immunodeficiency virus (HIV) in the early 1980`s has led to the production of safer plasma concentrates of coagulation factor VIII. After cloning the human factor VIII gene between 1982 and 1984 by Gitschier and colleagues (18) the use of DNA technology revealed recombinant factor VIII products that were introduced in 1990. In 2003 U.S. Food and Drug Administration (FDA) approved the first recombinate factor VIII product that is produced without any added human or animal plasma proteins (ADVATE, Baxter BioScience). This product virtually eliminates the risk of transmission of human or animal pathogens and provides supreme pathogen safety.

Mild forms of hemophilia A can usually be treated with desmopressin, a synthetic analogue of natural vasopressin. This treatment works by liberating factor VIII from its storage compartments and can significantly boost circulating levels of factor VIII.

Currently, in developed countries patients with hemophilia A are treated either with plasma-derived factor VIII concentrates or with recombinant products. A

higher safety regarding virus transmission for plasma-derived factor VIII products was achieved by the implementation of at least two virus-inactivation procedures. A recently introduced step further is based on pre-screening plasma for viral load. Manufacturers test now for human immunodeficiency, hepatitis C and hepatitis B viruses (HIV, HCV and HBV) using nucleic acid amplification assays and some test also for hepatitis A virus (HAV) and B19 parvovirus.

To choose a product for replacement therapy one has to consider that on the one hand plasma-derived factor VIII products have become safer; on the other hand recombinant products are more expensive, they cost 2 to 3 times more than plasma-derived factors. Despite all that progress in the safety of products for treatment of hemophilia A the problem of inhibitor development against factor VIII is still present.

2.6 TREATMENT OF HEMOPHILIA A PATIENTS WITH INHIBITORS

As mentioned before, patients with hemophilia A are treated by administration of recombinant or plasma-derived factor VIII concentrates. This substitution therapy induces the production of factor VIII inhibitor antibodies in about 30 % of patients with severe hemophilia A (19) and leads to increased morbidity in hemophiliacs. The severity of the disease seems to be the most important risk factor for the development of inhibitors (3).

The development of inhibitory antibodies (inhibitors) has emerged to be currently the most severe complication of hemophilia A. Factor VIII inhibitors rapidly inactivate residual autogenous factor VIII or exogenous factor VIII when given as substitute to hemophilia A patients. Depending on the type of gene mutation, the factor VIII in these concentrates might be recognized by the patient's immune system as a foreign protein or an altered self-protein. The efficiency of treatment is often dramatically decreased and alternative treatment strategies have to be used to control acute bleeding. Therefore the management of patients with inhibitors has to consider two elements: the treatment of bleeding episodes and the abolishment of inhibitors.

The treatment of patients with inhibitors is strongly individualized as the inhibitor concentration, the patients response to factor VIII challenge, anamnestic factors and type of bleeding has to be considered. Patients with inhibitors to factor VIII can be roughly divided into 2 categories: 1. low responders that have maximal inhibitor titers of 2-3 Bethesda units (BU) / ml even when challenged with factor VIII. About 20 % of the inhibitor population are low responders. 2. high responders defined with inhibitor level ≥ 10 BU/ml in response to factor VIII infusions. 50 – 60% of inhibitor patients are high responders. Patients with inhibitor titers between 3 to 10 BU/ml fall between these two groups. The distinction has important therapeutic implications because low-responding patients can still be treated effectively with factor VIII although frequently a significant higher dosage will be required than in treatment of hemophiliacs without inhibitors. High responder patients must be treated with other hemostatic agents than factor VIII (20,22).

2.6.1 PORCINE FACTOR VIII

The most common use of porcine factor VIII has been to manage severe bleeding in patients with intermediate- or high-titer inhibitors to factor VIII (21). It is suggested that inhibitors generally react less strongly to porcine factor VIII than to human factor VIII and porcine factor VIII is therefore less rapidly neutralized. Although cases of transfusion reactions and anamnestic rises in inhibitor titers against both porcine and human factor VIII are reported the overall risk of reactions is found to be low and appears to be largely dose-related (22).

2.6.2 BYPASSING AGENTS

The principle underlying the treatment with factor VIII bypassing agents is the correction of the defect in intrinsic coagulation. Prothrombin complex

concentrates (containing FII, FVII, FIX, FX), activated prothrombin complexes or recombinant factor VIIa (rVIIa) preparations are used for this purpose. Recombinant FVIIa is thought to ensure hemostasis by binding either directly or in complex with tissue factor to phospholipids on platelet surface. Alternatively it is thought that the therapeutic effect is due to increasing the ratio of factor VIIa to factor VII (23). Owing to its short half-life administrations have to be at short intervals, typically every 2-3 h.

Although bypassing agents are widely and efficiently used to treat hemorrhages in hemophiliacs they are sometimes associated with thrombotic complications and myocardial infarction. These complications are however rare and are considered to be caused by an increase in concentration of native or activated coagulation factors in the recipient thus influencing the balance of hemostasis.

2.6.3 SUPPRESSION OR REMOVAL OF INHIBITORS

Early attempts to induce immune tolerance involved the use of immunosuppressive drugs, in particular steroids, cyclophosphamide and azathioprine. These drugs act non-specifically on immune cells and cause a general suppression of the patient's immune system. The use of immunosuppressive drugs alone for the elimination of inhibitors was not successful. Currently, they are sometimes used in conjunction with immune tolerance therapy, but only in rare cases.

Plasmapheresis

If factor VIII is to be used in patients whose inhibitor titer is higher than 10 Bethesda units/ml, it is usually necessary to remove some of the inhibitors using either plasmapheresis alone or plasmapheresis together with immunoadsorption. Additionally, plasmapheresis may be helpful in emergencies in patients with relatively low inhibitor levels. It has also been used in conjunction with various chemotherapeutic agents for temporary removal and

suppression of inhibitor formation. The exchange of one volume of circulation plasma reduces the inhibitor titer by about half. To reduce 80-90 % of the inhibitors the exchange of 3 volumes of circulating plasma is required (24).

Extracorporeal adsorption of inhibitory antibodies

A temporary reduction of inhibitors can be achieved by extracorporal adsorption of inhibitory antibodies to staphylococcal protein A. Anti-factor VIII antibodies are immunoglobulin G antibodies, predominantly of subclass 4, and bind to the Fc part of the staphylococcal protein. The treatment, which is part of the Malmö protocol for tolerance induction, is very efficient and enables conventional replacement therapy with factor VIII for 5 to 9 days. However, in patients with inhibitor titers >30-50 Bethesda units the removal of inhibitors by immunoadsorption procedures is often not sufficient to permit the use of replacement therapy. Therefore, it has to be repeated several times. Immunoadsorption requires good venous access and must be carried out in specialized centers (24).

2.6.4 IMMUNE TOLERANCE INDUCTION (ITI)

Immune tolerance to factor VIII can be achieved in patients with inhibitors through the regular administration of factor VIII over prolonged periods of time. The mechanism by which immune tolerance is induced is still unclear. It is suggested that regular administration of factor VIII might interrupt the normal immune response, maybe by inducing T or B cell anergy through altered antigen presentation. Tolerance induction by treating high responder hemophiliacs with high doses of factor VIII was first demonstrated by Brackmann in 1974 whose treatment regimen is known as the "Bonn protocol" This regimen uses 150 IU FVIII/ kg BW twice a day. Today several treatment schedules are proposed, most of them are modification of the original Bonn protocol (25-27); others employing considerably lower doses (25-50 IU FVIII/kg BW per day) have also shown to be successful (28). However, high-titer inhibitors appear to be less

likely to respond to the low-dose protocols than to the high doses protocol. Protocols for ITI differ in many aspects, such as the daily dosages of factor VIII, the number of factor VIII administration per day or the use of immunosuppressive drugs. Another important issue is the definition of success in terms of tolerance attainment. To evaluate and compare different treatment schedules a stringent definition of outcome based on the assessment of the factor VIII kinetics (FVIII half-life), a negative outcome of the Bethesda assay and a normal recovery has to be used. Depending on the regimen used, ITI has been shown to be successful in eliminating factor VIII inhibitors in 50 – 90 % of the patients. Currently, inhibitor eradication with ITI remains the best way for treating hemophilia patients with high titer antibodies. It is the only efficient method that is currently available for inhibitor elimination that enables subsequent treatment with factor VIII.

The advantages of the induction of immune tolerance are that the patients can be treated with conventional doses of factor VIII. The induced tolerance is specific for factor VIII since immune responsiveness to other antigens and other cellular immune responses are not affected. The main disadvantages of the ITI regimens are unpredictable and incomplete efficacy, the extensive costs and the long duration of therapy (29).

In summary, the treatment of inhibitor patients is a continuing challenge as there is no consensus yet obtained regarding how to treat acute bleedings and how to induce immune tolerance in the most efficient way and how to treat patients who have failed ITI.

2.7 FUTURE ASPECTS OF TREATMENT OF INHIBITOR PATIENTS

In future, further genetic characterization, including the identification of host genetic markers could help to accurately predict which patients are at risk for developing anti-factor VIII antibodies and then it would be possible that patients at risk are subjected to tolerance regimens early in life. The exposure to factor factor VIII in utero via fetal gene therapy would also be a possibility to prevent early disease manifestation and is aimed to induce natural tolerance. Evidence

for this approach is given by the experiments of Waddington et al who administered intravascular prenatal an adenoviral vector carrying the human factor IX. In adult mice treated with repeated human factor IX protein injection, after prenatal vector injection, a persistent level of human factor IX was found and in most animals no antibodies to human factor IX were detected (30).

The production of less immunogenic forms of factor VIII preparations lacking the most common epitopes for inhibitors is another way to prevent the complication of inhibitor development. Defined B cell epitopes on factor VIII provide the basis for the modification of antigenic sites of factor VIII. Constructs of modified factor VIII with normal activity but with reduced antigenicity are thought to be helpful. However, future studies in animal models will show if factor VIII`s full biological activity is maintained by reducing the immunogenicity. It has also been suggested to prepare synthetic peptides that mimic the factor VIII epitopes recognized by the inhibitor and and might be capable to neutralize the inhibitory antibodies (31). Another approach to prevent development of inhibitory antibodies is the production of recombinant human-porcine hybrid factor VIII constructs. These molecules have variable porcine substitutions of sectors of the human A2 and C2 domains, the domains were inhibitors preferentially bind to the factor VIII molecule. Lollar et al. (32) has already shown that a chimeric molecule with the porcine A2 domain, which is recognized by a number of human anti-factor VIII antibodies, substituted for the human may not be recognized by the inhibitor.

The preparation of antibodies against idiotypic determinants of the factor VIII inhibitors is also suggested as these antibodies can neutralize anti-factor VIII antibodies and could be used for active immunization. Polypeptides that mimic idiotypes of anti-factor VIII antibodies could be used to generate anti-idiotypes capable of neutralizing anti-FVIII antibodies.

The overall goal for future therapies of hemophilia patients however is the inhibitor prevention. Therefore, understanding the immunological mechanisms which either promote or fail to induce tolerance are crucial for developing new strategies to prevent inhibitor formation in future.

2.8 FVIII AND THE IMMUNE RESPONSE

The immune system with its range of cellular and humoral components is fundamentally directed towards recognizing, neutralizing and eliminating foreign antigen populations of every possible kind. Confrontation with antigens induces reactive adaptive responses in the immune system that have the purpose of eliminating potentially harmful antigens such as pathogens.

The cellular immune response to a soluble protein antigen such as factor VIII occurs through a cellular cascade of interactions between different cells of the immune system. The initial event in this cascade is the contact between the FVIII molecule and the antigen–presenting cell (APC). Factor VIII is taken up by the APC and is afterwards processed to peptide sequences in endosomes. Factor VIII peptides (T cell epitopes) interact with class II MHC molecules. The FVIII-peptide-MHC-complex is transported to the surface of the APC and presented to the T cell receptor on $CD4^+$ cells. This APC-T cell interaction that includes not only peptide-MHC-complex interaction with the T cell receptor but also costimulatory interactions, stimulates the production of cytokines and the upregulation of a number of surface molecules, including CD25, CD69, CD30, CD28 and CD40 ligand. Cytokine production by T cells and the interaction with paired molecules (C30 ligand, CD80/CD86 and CD40) on the surface of B cells results in the proliferation of B cells and a further differentiation into antibody producing cells. As the immune reaction to factor VIII follows a cascade there are a number of potential strategies to intervene with the immune responses and to induce tolerance.

2.8.1 STATE OF TOLERANCE

As the immune response to an antigen is dependent on the collaboration of specific T and B cells, tolerance to an antigen can result from either unresponsiveness at the T cell level, the B cell level or at both levels simultaneously. Tolerance can be induced by three basic mechanisms: clonal deletion, anergy and ignorance. In the case of antigen specific clonal deletion

the removal of immune responsive cells through programmed cell death or apoptosis is mediated by hyperstiumaltion with antigen. A failure of adequate co-stimulatory interactions may result in the development of anergy. The state of ignorance occurs if immune responsive T and B cells have no access to the antigen because the antigens are expressed in so called immunological privileged sites. Finally, there are mechanisms of tolerance that involve T-cell-T-cell-interactions, also known as immune suppression. The importance of regulatory T cells for immune suppression has attracted a lot of interest in recent years.

2.8.2 MANIPULATION OF THE IMMUNE RESPONSE

The large complexity of interactions between APC, T cells and B cells in generating an immune response offers many possibilities for tolerance induction. T cells are actually the main target for tolerance induction protocols as almost every stage in the T cell activation pathway represents a potential target for disruption that may lead to tolerance induction. Self-reactive T cells that express high affinity T cell receptors are removed in the thymus during T cell development by a process known as negative selection that leads to central tolerance. Any remaining self-reactive T cells are inactivated once they leave the thymus through a peripheral tolerance mechanism. Central and peripheral tolerances neutralize self-reactive T cells by several different processes such as apoptosis, anergy or suppression mediated by a T cell subset with regulatory properties.

Manipulation of APC – Tcell interaction

For optimal T cell activation at least two signals are required. Signal one is the interaction of the antigen specific T cell receptor (TCR) with a peptide bound to the MHC complex. By blocking the TCR with antibodies to the T cell receptor or by altering peptide presentation via mutant tolerizing peptides, the interaction

between T cell receptor and MHC-peptide complex can be inhibited. This would reveal an incomplete T cell activation and could render the T cell anergic.

Manipulating the costimulatory signal

Another approach that has recently received a lot of attention is the inhibition of the second signal for optimal T cell activation that is delivered though the interaction of co-stimulatory molecules, present on the T cell and on the APC. T cells that encounter antigen without the appropriate signals from APCs and become non-reactive or tolerant. Over the last couple of years a number of co-stimulatory interactions have been identified that may promote or down-regulate the proliferation on T cells. One is the interaction of CD28, a surface molecule that is constitutively expressed on T cells, and its ligands CD80 and CD86 present on activated professional APCs. This interaction favors proliferation of T cells and exerts their biological function. Excessive proliferation of T cells is prevented by CTLA-4 (cytotoxic T lymphocyte antigen), which is upregulated after T cell activation. CTLA-4 competes with CD28 for binding to CD80/CD86 on APCs and acts antagonistically. Ligation of CTLA-4 to CD80/CD86 down regulates T cell activation and prevents proliferation of T cells. Recently, the modulating role of CTLA-4 on inhibitor formation in factor VIII knock out mouse was investigated by constructing a fusion protein consisting of soluble CTLA-4 and part of the heavy chain of IgG1 (CTLA4-Ig) (33). It was shown that co-administration of CTLA4-Ig with factor VIII could completely block inhibitor formation and that the co-stimulatory molecule CD86 is likely to have an essential role in humoral immune response as hemophilic mice lacking CD86 did not develop an immune response.

Costimulatory interactions are also involved in the interaction of antigen - specific B and T cells. B cells can only be fully activated with the help of T cells. B cells that are not adequately stimulated will be deleted or anergized. An important co-stimulatory signal for productive interaction between B and T cells is mediated by CD40/CD40 ligand binding. The interaction of CD40 on B cells with CD40 ligand on activated T cells provides a strong activation stimulus for B cells and results in the stimulation of differentiation, somatic mutations and

antibody class switching. The blockade of the CD40/CD40L interaction for suppression of inhibitor formation has been evaluated in several studies. The modulating effect of anti-CD40 ligand treatment on high titer inhibitors in hemophilic mice had been shown by Qian et al (34). Others and we have shown that treatment with anti-CD40 ligand antibody co-administered with factor VIII can indeed prevent anti-factor VIII antibody formation in hemophilic mice. However, our results indicate that long-term tolerance cannot be achieved (35, 36).

Active suppression of T cell responses

The regulation of the function of immune responsive cells is mediated by active suppression via specialized T cells. Certain T cells appear to be capable to specifically suppress the immune response of other lymphocytes to antigen. T cells with these inhibitory properties are referred to as suppressor or regulatory T cells (37). These cells are believed to play a role in peripheral tolerance to self-antigens and in inhibiting or limiting responses to foreign antigens. They have been described to be important players in down regulating antigen responses. Regulatory T cells (T_R) are a subset of $CD4^+$cells, most of which express the activation marker CD25 and are present in healthy individuals at about 5-10% of the total CD4 population. These $CD4^+$ T_R have been demonstrated to prevent a number of immune-mediated diseases, including autoimmune disorders, transplant rejection and inflammatory bowel disease (38, 39) and might be crucial for parasite persistence and long lasting immunity (40) as well as for induction of alloantigenspecific tolerance by costimulatory pathway blockade (41). These natural occurring $CD4^+$ $CD25^+$ T_R, together with other immunoregulatory cells, have an important physiologic function in balancing self-recognition and reactivity with self-tolerance. Several subsets of T_R with distinct phenotypes and distinct mechanisms of action have now been identified. This include type 1 T_R (Tr1), which secrete high level of interleukin 10 (IL-10), type 3 T cells (Th3), which primarily secrete transforming growth factor–ß (TGF-ß), and $CD4^+$ $CD25^+$ cells, which inhibit immune response via direct cell-cell contact. Future studies on the factors controlling the development and

activation of regulatory T cells should make it possible to shift the balance either toward regulatory cell activity (e.g. for treating autoimmune diseases) or away from regulatory cell activity to enhance tumor rejection and vaccination.

Modulating the cytokine environment

Another strategy for modulating immune responses is to manipulate cytokine expression as the pattern of cytokines expressed by T cells is critical in determining the maintenance and expression of diseases. This immune modulation involves manipulation of the cytokine environment in which T cell activation takes place or the antigen presentation as these factors have been observed to influence the differentiation and cytokine secretion of activated T cells (42, 43). CD4 T cells can be subdivided into two major subsets, the Th1 subset, which secrete Interferon–γ and drive inflammatory immune response, and the Th2 subset, which secrete interleukin-4, 5 and 13 and are associated with a protective role against Th1 mediated diseases such as murine diabetes or experimental induced encephalitis (EAE) (44, 45). The cytokine environment is important for driving the $CD4^+$ T cell response towards a predominantly Th1 response or Th2 response. *In vitro* experiments indicate that skewing the immune response is possible by e.g. blocking the Th1 dominated immune response with anti-IL-12 mAb and anti-IFN-γ mAb. The altered cytokine environment reveals the release of cytokines associated with the Th2 subsets. A major role for IL-10 and TGF-ß has been shown in several animal models in influenencing the polarization of T cells and it is suggested that these cytokines are also important for the T-cell mediated regulation of immune responses (46, 47).

Manipulating antigen presentation via Dendritic cells

More recently it has become apparent that dendritic cells (DC) not only are cells that present antigen to corresponding T cells. Instead, DC are engaged in cross

talks with T and B cells through surface interactions and soluble mediators. DCs are essential elements in the initiation of immune responses: They are able to discriminate between self and non-self and may guide the immune system to either potent effector activity to pathogens or to silence against self-reactive lymphocytes. Depending on their maturation stage and on environmental signals they are thought to induce tolerance (immature or semi-mature DC) or immunity (mature DC). The decisive immunogenic signal towards immunity seems to be the release of proinflammatory cytokines from DCs (IL-12, IL-6 IL-1ß and TNF-α. Therefore, activation of DCs with an appropriate stimulus such as TNF-α or filamentous heamagglutinin (FHA) to keep DC immature or semi-mature or inhibition of DC maturation by specific blocking of maturation stimuli is an attractive opportunity to induce a state of immune tolerance (48-51). . However, mature DC have also been implicated in the induction of both anergic and regulatory T cells (52, 53) and have been shown to be able of antigen-specific inhibition of effector T cell function in vivo in humans (54).

2.8.3 METHODS FOR THE CHARACTERIZATION OF ANTIGEN- SPECIFIC T CELLS

The quantitative and qualitative measurement of antigen-specific T cells is important for monitoring of immune status during a disease and for getting a picture of the ongoing immune response. Various methods have been developed to identify antigen-specific T cell responses. Traditional assays have analyzed bulk populations of T cells for proliferation by ^{3}H-thymidin incorporation or for cytotoxity by ^{51} Cr release assay. These methods tend to be long and labor-intensive. Furthermore the results cannot be compared quantitatively.

Single–cell based assays of antigen-specific T cells have come into use recently, including MHC-peptide tetramer staining (55), Elispot (enzym-linked immunospot) assays (56), cytokine secreting assays and intracellular cytokine staining assays (57). The advantage of these assays is that they provide truly quantitative results. Antigen specific T-cells are enumerated without long in vitro

restimulation that would allow time for proliferation and /or apoptosis. Additionally, Elispot assays and intracellular cytokine staining measures a functional readout (cytokine production) and can determine the complete T cell responses to a particular antigen or pathogen. Tetramer staining instead measures antigen specifity without regard to function and can only identify T cells with single peptide /MHC specificities.

A major advantage of intracellular cytokine staining and cytokine secretion assays in comparison to T cell Elispots is the ability to analyze multiple parameters per cell. It is possible to analyze expression of phenotypic markers on the cells of interest in addition to a functional readout. This more on information will give greater assurance that the events being identified as cytokine positive cells are indeed the cells of interest.

Intracellular cytokine staining, cytokine secretion assays and Elispot assay have been made possible by the coming up of high-affinity anti-cytokine antibodies and optimized protocols for their use. These techniques allow the detection of functional populations of memory T cells that respond to specific soluble antigens in short term restimulation assay. I established these assays in our lab where they are used routinely for several different projects.

2.8.4 ANIMAL MODEL FOR HEMOPHILIA A

As mentioned above, the immune response to a protein antigen like factor VIII is very complex and involves the interaction and migration of different types of immune cells within immunological relevant compartments of the immune system. No in vitro system will ever match these requirements and due to ethical reasons access to cells from human lymphoid tissues is limited. Thus, *in vivo* animal models are required to understand the mechanisms of antibody formation and to develop new therapeutic approaches for inducing immune tolerance to factor VIII in humans. Animal models offer also the opportunity to test a variety of treatment protocols that could be of clinical relevance. Important advances in human therapy have been made using appropriate animal models.

For hemophilia A Bi et al (58) described two murine models. These models have a targeted gene disruption in exon 16 (E-16) or exon 17 (E-17), which results in translation of a truncated factor VIII molecule. Consequently the murine plasma shows a complete deficiency of factor VIII. Phenotypically, hemophilic mice are characterized by severe hemophilia A that can be corrected by the administration of human factor VIII (59). Due to the sequence homology between murine and human factor VIII the use of human factor VIII leads to the interaction with the murine proteins of the coagulation system and to clot formation (60). After intravenously treatment of hemophilic E17 mice with human factor VIII they develop anti-factor VIII antibodies with properties similar to those in hemophilic patients with factor VIII inhibitors. The anti-factor VIII antibodies raised in mouse and humans recognize similar major epitopes in FVIII and it is suggested that blockade of its functional activity require identical mechanisms.

The murine model for hemophilia A has proved to be suitable for studying a number of scientific questions. It has been used to investigate mechanisms responsible for the regulation of immune responses against factor VIII and for the search for new approaches to induce immune tolerance (35,36,58,61-64). However, it should be noted that the use of relevant animal models for human disease is important. Hemophilic E-17 mice reflect the situation in patients in some aspects but not in all. Because of the heterogeneity of the disease different animal models will have to be used to cover all aspects of the immune response against factor VIII in patients.

2.9. HEMOPHILIA AND GENETHERAPY

As replacement therapy is costly and needs a lot of effort, researchers have turned to investigate the possibility to use gene therapy as means of maintaining adequate levels of the required protein in patients.

Gene transfer approaches for hemophilia are attractive because the gene for clotting factor can be expressed in any tissue as long as it gains access to the circulation. Even a modest increase in protein levels (1-5% of normal level) is

expected to significantly improve clinical symptoms. Furthermore, there is detailed understanding of the factor VIII and factor IX genes, the structure, function, and biology of the proteins. Furthermore, extensive experiences with protein therapy to evaluate therapeutic endpoints and the availability of canine and mouse hemophilia models that phenotypically mimic the human disease have already been acquired (65-67). It is thought that hemophilia is likely to be the first widespread severe genetic condition to be cured by gene therapy.

So far, many different approaches with viral or non-viral vectors in different gene delivery systems ex vivo and in vivo have been tested in hemophilia animal models and some human trials of factor VIII or factor IX gene therapy have been initiated or concluded over the past 3 years. The results of these trials indicate that gene transfer in hemophilia with the vectors and doses used is generally well tolerated and no inhibitors have been observed. As only patients without development of inhibitors participate in these trials, the outcome of gene therapy in inhibitor patients is unknown. Furthermore, two of the clinical trials were put on hold because sequences of the vector were found in semen samples. In some studies minimum elevations of factor VIII or IX were observed which resulted in less product need for the patient. Nevertheless, plasma levels of factor VIII or factor IX that had been obtained so far have been insufficient to free patients from replacement therapy. It turned out that the major problem of all these gene therapy approaches has been the fact that no persistent gene expression levels were achieved that indicates that much more effort has to be put into vector developing.

However, if one considers the growing costs of clotting factor replacement therapy and the fact that 80 % of the world's hemophiliacs do not receive any treatment at all, affordable gene therapy for every patient would be of great value for patients suffering from hemophilia.

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3. THESIS OBJECTIVES

Current therapeutic strategies for the treatment of patients with inhibitors to FVIII are far from satisfactory. At present immune tolerance induction is the only way to eliminate inhibitors but this therapy is cost intensive and the outcome is not predictable. Sometimes drugs that non-specifically target proliferating cells are used but they are associated with undesirable side effects.

Regarding this and the fact, that immune response against factor VIII is T cell dependent, my thesis was aimed to investigate the kinetics and the features of factor VIII-specific T cells that regulate the induction of anti-factor VIII antibodies. The identification and functional characterization of different factor VIII-specific T cell subsets will help to understand the regulation of the induction and maintenance of factor VIII inhibitors.

Quantitative and qualitative measurements of antigen-specific T cells are important for monitoring the ongoing immune response. By establishing suitable assays like intracellular cytokine staining of T cells and the Elispot assay for detection of cytokine secreting cells I was able to detect functional populations of memory T cells that respond to factor VIII in a short term restimulation assay. Previous functional assays for cytokine measurements by other groups were mainly done by ELISA techniques in supernatants of secreting cells rather than on a single cell level. Using more sophisticate techniques like the intracellular staining of cytokines I was able to show a much more complex picture of the cytokine response against human factor VIII in the hemophilic animal model used. The obtained results should help to understand the mechanisms underlying anti-FVIII immune response and to develop new strategies for specific immunotherapy of hemophiliacs.

4. RESULTS

4.1 MURINE MODELS FOR THE STUDY OF FACTOR VIII INHIBITORS

Key words
Hemophilia A, factor VIII inhibitors, animal model

4.1.1 INTRODUCTION

Regulation of the immune response to a complex protein antigen like factor VIII (FVIII) requires interaction between different types of immune cells and migration of these cells between several compartments of the immune system. Access to such cells and the compartments they traverse is limited in humans for obvious ethical reasons. Hence, in vivo animal models are needed to understand the mechanisms of antibody formation and to develop new therapeutic approaches for inducing immune tolerance to FVIII in humans. As imperfect as any animal model is, important advances in human therapy have resulted from appropriate use of such models. Our present knowledge of how neutralizing antibodies develop against FVIII is based predominantly on clinical findings in hemophilic patients. The presence of FVIII inhibitors makes patients refractory to replacement therapy and is, therefore, a serious complication of modern treatment. The risk of patients with severe hemophilia A developing anti-FVIII antibodies is clearly associated with the type of FVIII gene mutation they have. Patients affected with nonsense mutations, large deletions and intrachromosomal recombinations (inversions) in the FVIII gene show the highest inhibitor incidence (1-3, HAMSTeRS: The hemophilia A mutation, structure, test, resource site: http://europium.csc.mrc.ac.uk). These mutations are predicted to cause a complete deficit of any endogenous FVIII production. Therefore, human FVIII is probably a protein foreign to the immune system in these patients. Other mutations such as missense mutations and small deletions are associated with a much lower incidence of inhibitor formation (1-3). In these cases, non-functional FVIII antigen may circulate in the blood (4)

and render the immune system tolerant to the mutant FVIII protein. Native FVIII, present in FVIII products could, therefore, be recognized as an altered self-protein by the immune system in these patients. Apart from the type of FVIII gene mutation, other genetic factors such as the HLA haplotype seem to be important for the development of FVIII inhibitors. To investigate how important the HLA haplotype is in an animal model, animals with different genetic backgrounds, e.g. different inbred strains of mice, are needed.

The application route and dose of an antigen determines the subsequent immune response. In patients, FVIII is given intravenously. Therefore, the spleen is probably the major location for the development of anti-FVIII immune responses. An animal model should reflect this and develop detectable anti-FVIII immune responses after intravenous injection of FVIII doses equivalent to the doses used for patients.

Factor VIII is a protein antigen with some extra features that distinguish it from other protein antigens. In its activated form, activated FVIII (FVIIIa), is an essential cofactor in blood coagulation. In the presence of negatively charged phospholipids, FVIIIa directly interacts with the serine protease factor IXa and forms a complex that converts factor X into activated factor X (factor Xa). Factor Xa, in the presence of activated factor V (factor Va), phospholipids and calcium ions, is then able to convert prothrombin to its active enzymatic form, thrombin. Apart from their function in blood coagulation, both factor Xa and thrombin have been shown in vitro to activate one or more of the protease-activated receptors (PAR receptors) expressed on endothelial cells (5-7). This activation induces proinflammatory stimuli. Therefore, each time FVIII is given it is possible that it induces proinflammatory stimuli that directly influence the regulation of the immune response to FVIII. An animal model in which a severe bleeding diathesis is associated with a lack of FVIII would be optimal for immunological studies.

Given the diversity of the disease and the heterogeneity of the genetic background of the patients with hemophilia A, who develop anti-FVIII antibodies, no single animal model could cover all aspects of the immune response against FVIII. Therefore, different models are needed to research the various facets of the response. Murine models in which the mechanism and

genetic of the immune response is extremely well characterized have potential advantages and meet many of the above requirements.

4.1.2 FACTOR VIII KNOCK-OUT MICE

In 1995, Bi et al. described two murine models of hemophilia A in which a targeted gene disruption in exon 16 (E-16) or exon 17 (E-16) of the FVIII gene resulted in a complete deficiency of FVIII (8). These mice express a typical phenotype of hemophilia A (9, 10) which can be corrected by human FVIII (11). Qian et al. showed that E-16 and E-17 knockout mice develop anti-FVIII antibodies after intravenous injection of human FVIII in therapeutic doses and that this immune response is dependent on the induction of FVIII-specific T-lymphocytes (12). Human FVIII is certainly more foreign to hemophilic mice than to humans with hemophilia and can be expected to induce a stronger immune response in mice than murine FVIII would do. The lack of a convenient source of murine FVIII limits its use but human FVIII can be used instead because it interacts with the murine proteins of the coagulation system (13) due to its sequence homology with the murine FVIII (14). As mentioned above, this interaction in an environment of a severe bleeding syndrome might be important for regulating the immune response. Therefore, human FVIII can be considered a suitable model antigen in the search for new strategies to induce immune tolerance.

The E-17 model in a series of different studies was used. In these experiments, all E-17 mice developed detectable anti-FVIII antibodies after two doses of human FVIII (200 ng recombinant FVIII, free from albumin) that increased in titer after subsequent doses (15, 16). Titers of total anti-FVIII antibodies analyzed by ELISA correlated with titers of neutralizing anti-FVIII antibodies measured by Bethesda assays (16, Fig. 1). Anti-FVIII antibody secreting cells (ASC) first appeared in the spleen where they were detectable after two doses of FVIII (17, Fig. 2a). Their appearance correlated with that of anti-FVIII antibodies in blood plasma (Fig. 2b). Anti-FVIII ASC in bone marrow were detectable after three doses of FVIII (Fig. 2a). These cells had probably formed initially in the spleen and then migrated to the bone marrow. We did not see any formation of anti-FVIII ASC in lymph nodes confirming that the spleen is the

major development location for immune responses against blood-borne antigens. The IgG-subclass distribution of anti-FVIII ASC was similar in spleen and bone marrow and matched the subclasses of anti-FVIII antibodies in blood plasma (Fig. 3, table 1), indicating that both organs contribute to circulating antibodies in the blood. The IgG1 and IgG2a subclasses dominated the anti-FVIII antibody response. After FVIII treatment had terminated, anti-FVIII antibodies persisted for at least 22 weeks (Fig. 2b). The persistence of antibodies correlated with the long-term persistence of anti-FVIII ASC (Fig. 2a). These ASC could be either long-living ASC as described by Slifka et al. (18) and Manz et al. (19) or cells continuously formed by antigen-driven differentiation of memory B cells as described by Ochsenbein et al. (20). Future studies using cell transfer experiments should be able to show which model is best for explaining the maintenance of high titers of anti-FVIII antibodies in hemophilic mice, and possibly also in patients. The outcome of such studies could have considerable implications for creating new strategies aimed at inducing immune tolerance to FVIII.

The development of anti-FVIII antibodies in E-17 mice correlated with the appearance of FVIII-specific CD4+ T cells. Of these, the most prominent type that could be detected were CD4+ T cells producing IFN-γ, followed by T cells producing IL 10 (16, Fig. 4).

The CD40/CD40L interaction is a key event in the initiation of humoral immune responses against T-cell-dependent antigens (review in Ref. 21). Previous studies have shown that a blockade of CD40/CD40L interactions can achieve prolonged survival of allografts in rodents and monkeys (22, 23) and prevent graft-versus-host disease (24, 25) and autoimmunity in rodent models (26, 27). These effects are probably due to tolerance induction in the CD4+ T-cell population (28) and tempt the speculation that anti-CD40L antibodies can induce lasting T-cell tolerance to FVIII in hemophilia A. As anti-FVIII antibody formation is T-cell dependent, inducing FVIII-specific T-cell tolerance should prevent their formation. Using the E-17 mouse model we could show that the blockade of CD40-CD40 ligand interactions prevents the induction of an anti-FVIII immune response (29, Fig. 5). This initial prevention did not, however, induce a permanent tolerance to FVIII (29, Fig. 5). These results confirm earlier data published by Qian et al. (30). Rossi et al. (31) suggested that treating

hemophilic E-16 mice with FVIII accompanied by anti-CD40L antibodies induces lasting immune tolerance to FVIII under certain circumstances, at least in some animals. Their data do not, however, indicate if the immune tolerance would really be long lasting with frequent FVIII treatment. In another study, Qian et al. (32) explored the importance of the B7-CD28 interaction for the anti-FVIII immune response. They were able to show that blocking this interaction completely prevented anti-FVIII antibody development in hemophilic E-16 mice. Initial blocking of the co-stimulatory interaction between B7 and CD28 did not, however, induce lasting immune tolerance to FVIII. It is believed, therefore, that the blockade of a single co-stimulatory interaction might not be sufficient to induce a stable tolerance to FVIII.

Hemophilic FVIII knockout mice certainly resemble the situation in patients in some aspects, but not in others. Sarkar et al. (33) recently showed that E-17 mice produced nonfunctional FVIII heavy chain proteins. Such heavy chain proteins might induce immunological tolerance to epitopes of the heavy chain of murine FVIII. Therefore, the disruption of the FVIII gene in hemophilic E-17 mice might not be severe enough to stimulate the full repertoire of immune responses against murine FVIII. Consequently additional knock-out models, which lack any endogenous FVIII synthesis, have to be developed. Likewise it is essential that a recombinant protein is developed to provide a convenient source of murine FVIII. Even with better knockout models and the availability of murine FVIII, the genome of a mouse is not that of a human. Therefore, we have to be aware that there will always be differences between hemophilic mice and patients with hemophilia A.

4.1.3 NORMAL MICE AND RATS

Before the hemophilic mouse model existed, different groups tried to use normal mice (34) or rats (35, 36) to study FVIII inhibitors. Dazzi et al. (34) did not find any anti-FVIII antibody response after intravenous application of human FVIII in normal mice, but detected anti-FVIII antibodies after intraperitoneal application. This antibody response was dependent on the induction of FVIII-specific T cells. Jarvin et al. (35) and Levin et al. (36) used normal rats and immunized them with human FVIII in adjuvant. This treatment schedule resulted

in the induction of an anti-FVIII immune response that was characterized by neutralizing antibodies that recognized some of the epitopes that were also found in patients with FVIII inhibitors. The application route that induced the anti-FVIII immune response in these studies cannot, however, be compared to that for FVIII in patients. Furthermore, treating normal mice and rats with mouse or rat FVIII should not induce any immune response at all because the murine proteins represent a self-protein. Analytical comparison of the amino-acid sequence of murine and human FVIII has shown that the homology in the functionally important A and C domains is 84-93%, but only 42-70% in the nonfunctional B domain and the acidic regions (14). Therefore, anti-FVIII antibodies developed against human FVIII can be expected to be directed predominantly against the more divergent regions and, accordingly, not to reflect the whole repertoire of possible antibody responses that would be seen in FVIII knockout mice.

Another major disadvantage is the lack of a bleeding syndrome in normal mice. As mentioned above the cross-talk between the immune system and the coagulation system might be important for regulating the immune response to FVIII and should, therefore, be considered in an animal model.

Nevertheless, recent experimental study using normal mice should be mentioned. Chao and Walsh reported the loss of neutralizing anti-FVIII antibodies in normal C57BL/6 mice after sustained expression of human FVIII (37). Antibodies developed within 7 to 14 days of intraportal injection of adeno-associated virus (AAV) carrying human FVIII. Bethesda titers of anti-FVIII antibodies (>100 BU/ml) persisted relatively unchanged for 9 to 10 months. At 10 months after injection of the virus, FVIII inhibitors disappeared and FVIII protein became detectable in the blood. These results suggest that immune tolerance to human FVIII can be induced by sustained expression of human FVIII in a mouse model.

4.1.4 SCID MICE AND SCID/FACTOR VIII KNOCKOUT MICE

Mice with severe combined immunodeficiency (SCID) lack functional B and T cells (review in 38) due to an arrest of B and T lineage committed cells in early development. This arrest is caused by a gene mutation that impairs the

recombination of antigen receptor genes. The SCID-related gene encodes a DNA-dependent protein kinase catalytic subunit and is located in chromosome 16 (39). The SCID mice are unable to produce antibodies or to reject allogeneic skin grafts (38) and, therefore, do not develop a functionally active immune system. Furthermore, spleen cells of SCID mice fail to show a proliferative response to B-cell and T-cell mitogens (38). Other hematopoietic cells such as macrophages and NK cells develop and function normally. Because of their severe immune deficiency, SCID mice are able to accept xenotransplants and can, therefore, be reconstituted with peripheral blood mononuclear cells isolated from human blood donors (38). The major advantage of such a model is the possibility of studying at least part of the human immune system in a mouse model. The major disadvantages are the lack of functional lymphoid organs and difficulty in eliciting primary immune responses.

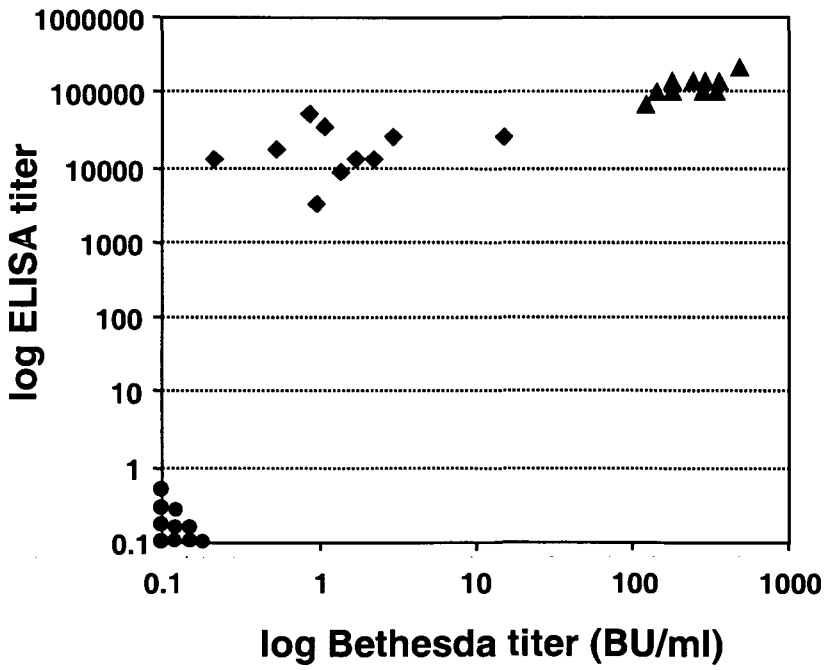
Laulan et al. (40) grafted SCID mice with peripheral blood mononuclear cells from healthy donors or donors with hemophilia A. They injected reconstituted mice intraperitoneally with 100 U (10 μg) of human FVIII. A specific response to FVIII only developed in mice that received blood cells from patients with hemophilia who had FVIII inhibitors and not in those that received cells from healthy donors or patients with hemophilia but no FVIII inhibitors. These results suggest it is possible to induce secondary but not primary immune responses against human FVIII in reconstituted SCID mice. The mice received, however, only one FVIII injection and this was at a dose (100 U per mouse equals about 4000 U/kg) which is not comparable to that used in patients with hemophilia A.

In similar experiments by Vanzieleghem et al. (41) SCID mice were reconstituted with peripheral blood mononuclear cells from healthy blood donors and treated with one intraperitoneal injection of 50 U (5 μg) human FVIII followed by three of 25 U (2.5 μg). All reconstituted mice spontaneously produced anti-FVIII antibodies in the absence of any treatment with FVIII. These antibodies were only detectable after affinity purification. Treating mice with up to four doses of FVIII did not induce an increase in antibody titers detectable after affinity purification. Furthermore, treatment with FVIII did not stimulate antibodies detectable without affinity purification. When SCID mice were reconstituted with peripheral blood mononuclear cells from patients with hemophilia A who had FVIII inhibitors, treating the mice with FVIII induced a

detectable secondary immune response (42). In an attempt to improve the SCID mouse model, Gilles et al. created a SCID/FVIII knockout mouse by cross-breeding SCID mice with FVIII knockout mice (42). Future studies will reveal the advantages and limitations of this new model.

To summarize the results obtained with SCID mice, this model might be suitable for investigating specific questions of secondary anti-FVIII immune responses in an environment that contains parts of the human immune system.

4.1.5 FIGURES

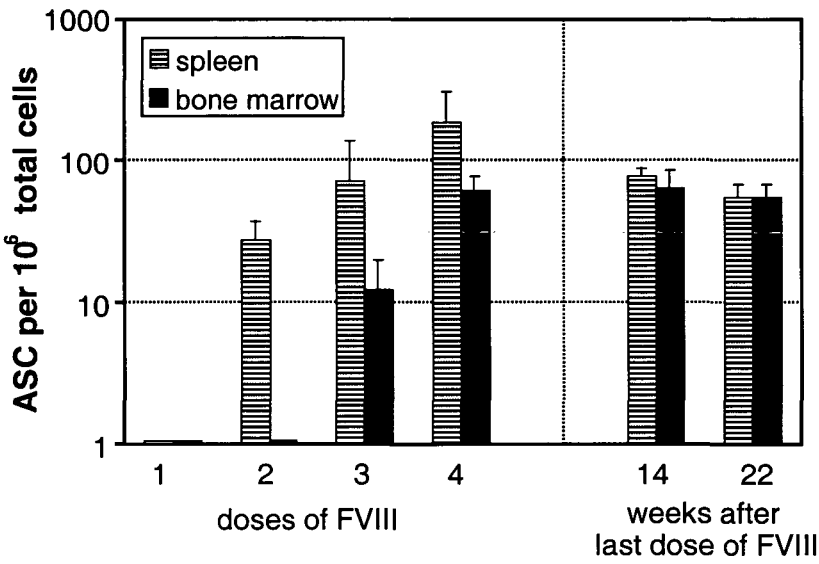


Relation of total anti-FVIII antibody titers (ELISA titer) to titers of FVIII-neutralizing antibodies (Bethesda titer) in plasma obtained from hemophilic mice after one dose (●), two doses (◆) or four doses (▲) of FVIII. Each point represents values for an individual mouse. Blood samples were obtained 1 week after each dose. ELISA titers and Bethesda titers were analyzed as described (16). From Sasgary et al. (16) with permission.

Figure 2

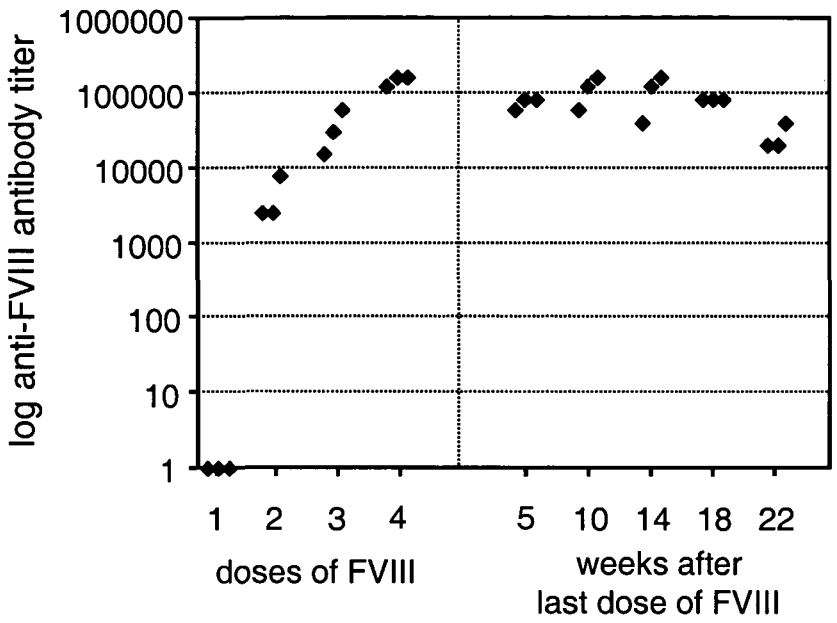
Long-term persistence of anti-FVIII antibody-secreting cells in spleen and bone marrow and of anti-FVIII antibodies in blood plasma of hemophilic E-17 mice after treatment with human FVIII. From Hausl et al. (17) with permission.

Figure 2a



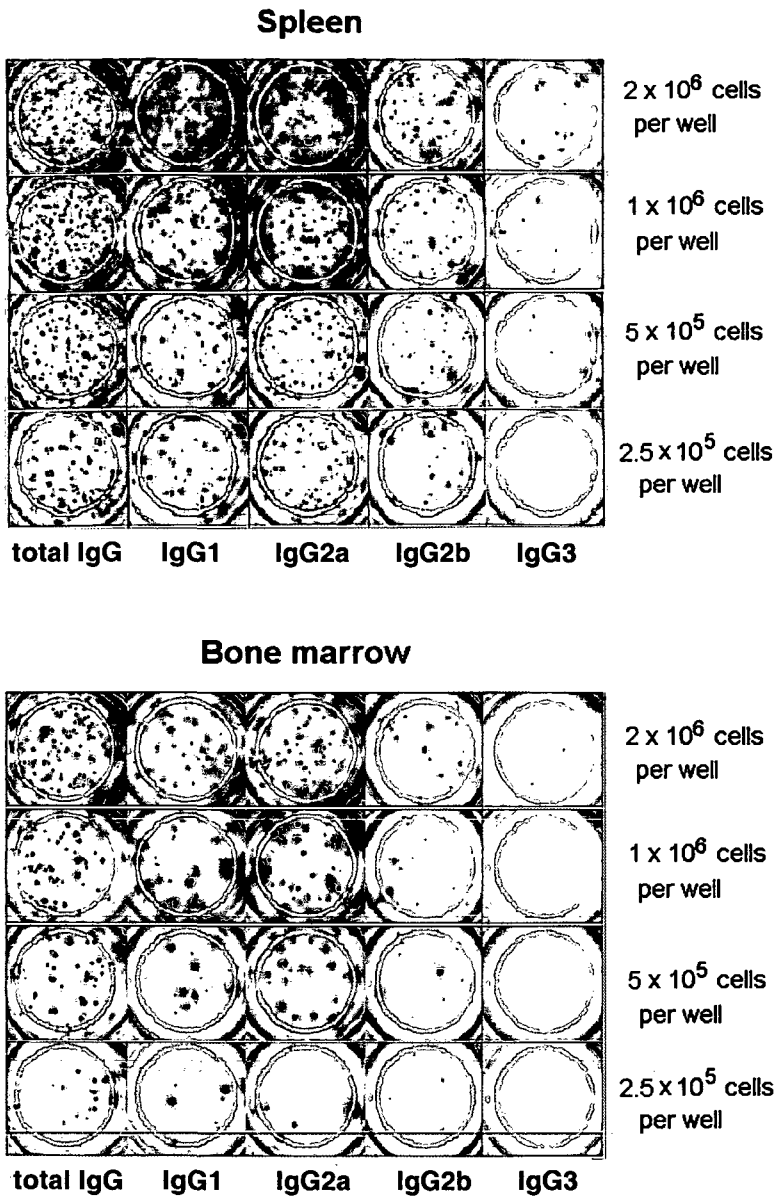
Kinetic of anti-FVIII antibody-secreting cells in spleen and bone marrow of hemophilic mice after one, two, three or four doses of FVIII as well as 14 and 22 weeks after the fourth dose of FVIII. Cells were analyzed by Elispot as shown in Figure 3. Presented are the medium values and standard deviations, calculated from the results obtained with three different cell dilutions.

Figure 2b



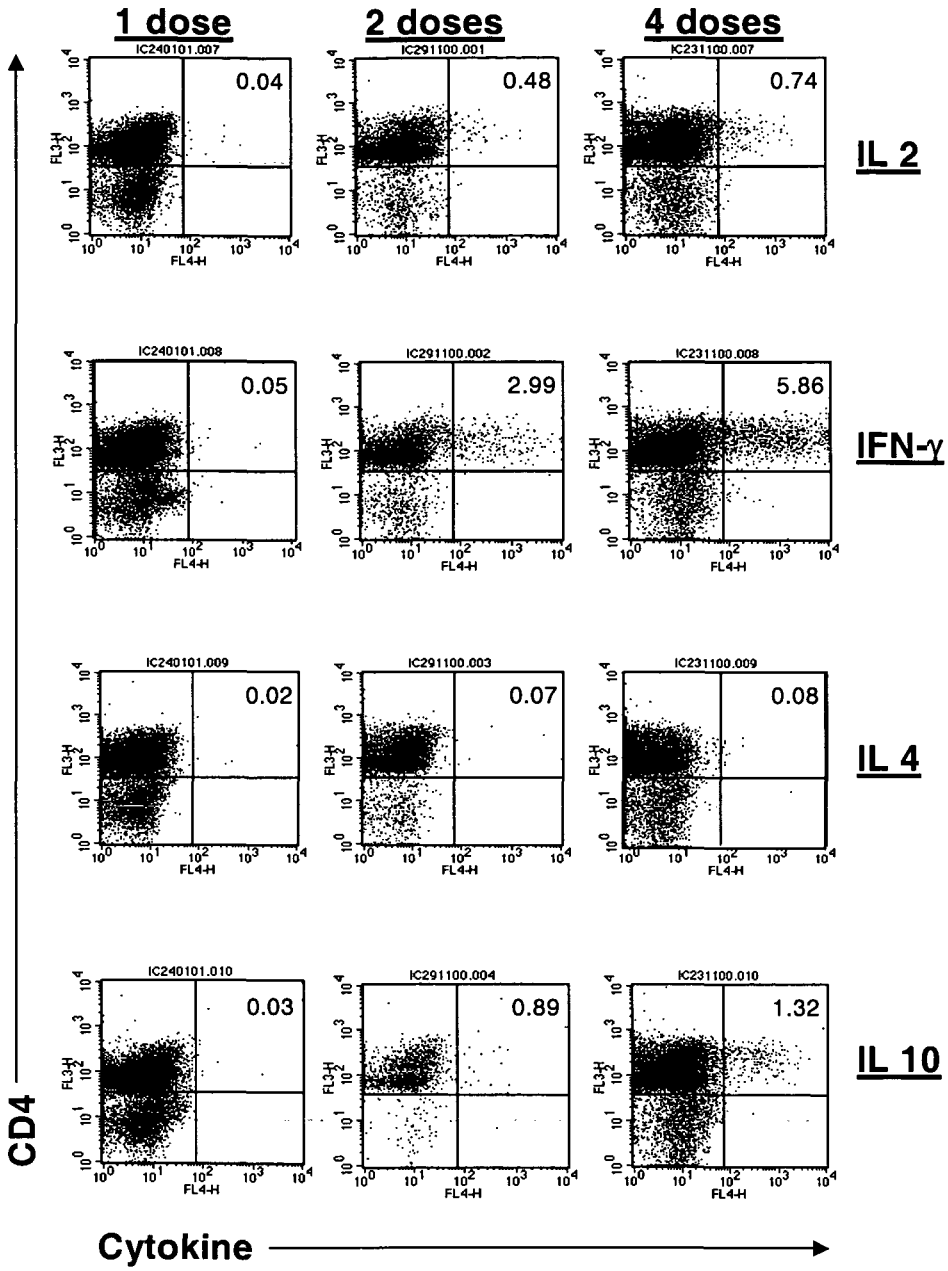
Kinetic of anti-FVIII antibody titers in blood of hemophilic mice after one, two, three or four doses of FVIII as well as 5, 10, 14 and 22 weeks after the fourth dose of FVIII. Anti-FVIII antibody titers were analyzed by ELISA as described in Hausl et al. (17). Each point represents the result obtained from an individual mouse. Three mice were analyzed per time point.

Figure 3



Representative Elispots showing the frequency of anti-FVIII antibody-secreting cells (total IgG) as well as the IgG-subclass distribution of anti-FVIII antibody-secreting cells (IgG1, IgG2a, IgG2b, IgG3) in bone marrow and spleen of hemophilic mice after four intravenous doses of 200 ng FVIII (80 U/kg). Serial dilutions of cell samples were incubated with FVIII immobilized to the solid phase of PVDF-bottomed Multiscreen-IP filtration plates. Each spot represents an individual anti-FVIII antibody-secreting cell. Analyses of antibody-secreting cells were done as described (17). From Hausl et al. (17) with permission.

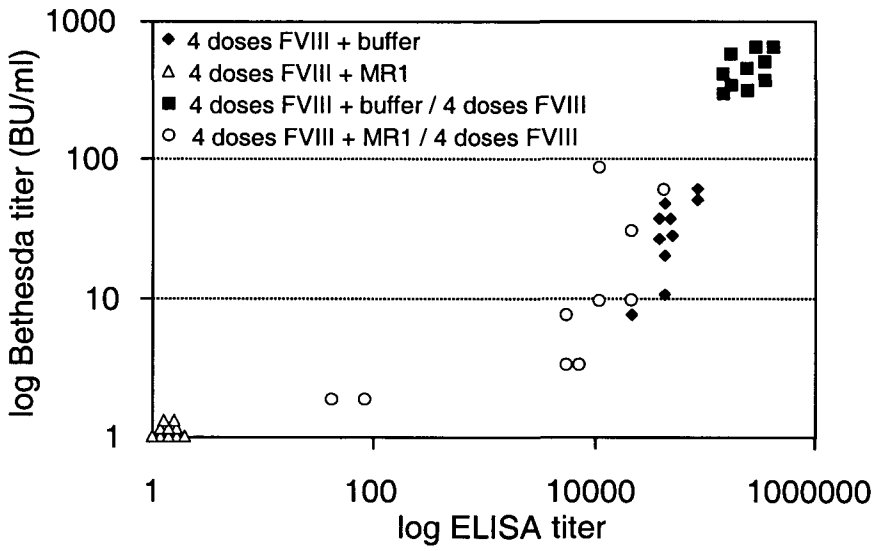
Figure 4



Kinetic of FVIII-specific T cells producing IL-2, IFN-γ IL-4 or IL-10 in the spleen of hemophilic E-17 mice after treatment with one, two or four doses of human FVIII. Dot plots show intracellular cytokine staining in CD4+ T cells after in vitro restimulation of splenic T cells with FVIII. Results shown in the upper right-hand corner of each dot plot represent the percentage of CD4+ T cells that stained positive for the cytokine indicated. The negative cell population (lower left-hand corner) was defined by using cells that were stained

with appropriate isotype-matched negative control antibodies. T cells were isolated, restimulated and stained as described (16). From Sasgary et al. (16) with permission.

Figure 5



Relation of neutralizing anti-FVIII antibodies (Bethesda titer) and total anti-FVIII antibodies (ELISA titer) in hemophilic E-17 mice after treatment with FVIII together with or without anti-CD40 ligand antibody. 200 ng anti-mouse CD40 ligand antibody MR1 were given intravenously 24 hours before treatment with FVIII as described in Reipert et al. (29). Each point represents values for an individual mouse. Mice received the treatments as indicated at weekly intervals. 200 ng FVIII (80 U/kg) were given per dose. All analyses were done as described (29). From Reipert et al. (29) with permission.

Table 1

Median anti-factor VIII antibody titers of different IgG subclasses in hemophilic E-17 mice (n=3)

	Anti-factor VIII antibody titers			
	IgG1	IgG2a	IgG2b	IgG3
One dose FVIII	n.d.	n.d.	n.d.	n.d.
Two doses FVIII	1280	2560	2560	120
Three doses FVIII	40960	40960	15360	640
Four doses FVIII	122700	122700	61440	5120
22 weeks after the fourth dose	20480	81920	5120	640

n.d. – not detectable

IgG subclasses of anti-FVIII antibodies were analyzed as described in Hausl et al. (17). Briefly, PolySorp multiwell plates were coated with FVIII (free from albumin). Plates were incubated with serial dilutions of plasma samples from mice treated with FVIII. IgG subclasses of anti-FVIII antibodies bound to the immobilized FVIII were detected by incubation with isotype-specific, horseradish-peroxidase-labeled secondary monoclonal antibodies (Southern Biotechnologies, Birmingham, AL) and subsequent substrate development. Antibody titers were expressed as the highest dilution of plasma samples showing a positive result (optical density >0.3) in the assay. From Hausl et al. (17) with permission.

4.1.6 DISCUSSION

An animal model suitable for developing new approaches for inducing immune tolerance to FVIII should encompass features that as much as possible resemble those found in patients with hemophilia A while still accommodating sophisticated analysis and manipulation of the ongoing immune response. Hemophilia A is a hereditary X-linked bleeding disorder caused by the incomplete function of circulating clotting FVIII or its total absence due to a mutation in the FVIII gene. The consequent coagulation deficiency is treated with infusions of FVIII concentrates. Depending on the type of gene mutation, the FVIII in these concentrates might be recognized by the patient's immune system as a foreign protein or an altered self-protein. In addition, other genetic factors such as the HLA haplotype are believed to influence the anti-FVIII immune response. Considering this heterogeneous situation, obviously no single animal model can accommodate all the different aspects of the anti-FVIII immune response in patients.

Therefore, we have to be aware that each animal model that we use, and will use in the future, to search for new approaches for inducing tolerance to FVIII has its advantages and its limitations. Furthermore, it must not be forgotten that experimental studies in animal models can only provide new ideas, proof of concepts and strategies for therapeutic approaches and it is the clinical trial that will always be the ultimate tool for leading to therapeutic advances.

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4.2 SINGLE CELL ANALYSIS OF FACTOR VIII-SPECIFIC T CELLS IN HEMOPHILIC MICE AFTER TREATMENT WITH HUMAN VIII

SUMMARY

A multi-parameter flow-cytometry assay was established suitable for analyzing T-cell-specific cell surface markers (CD3, CD4) together with intracellular cytokines on a single cell level. This assay was used to identify the frequency and the kinetic of different populations of factor VIII (FVIII)-specific CD4+ T cells in hemophilic E-17 mice after treatment with human FVIII. A clear temporal correlation was found between the appearance of FVIII-specific CD4+ T cells in the spleen and the detection of anti-FVIII antibodies in plasma. These cells and antibodies were detectable in all experiments after two doses of FVIII and in a few even after a single dose. The IFN-γ producing T cells were the most prominent type of FVIII-specific T cells suggesting Th1-type T cells have an important role in regulating the anti-FVIII immune response in E-17 mice. IL-10-producing T cells were the second most dominant type. They were detectable after two doses of FVIII and increased in frequency after four. Cytokine co-expression studies analyzing IL-10 and IFN-γ in the same cell indicated that there might be at least two types of IL-10 positive T cells, those cells that produce IL-10 only and in addition cells that produce IL-10 and IFN-γ. Furthermore, FVIII-specific T cells producing IL-2 were found in all experiments after two doses of FVIII. In a few experiments IL-4-producing T cells were seen but in most experiments they were not detectable. In contrast, IL-4 could be found in supernatants of in vitro restimulated CD8- spleen cells.

Key words

Hemophilia A, factor VIII, T cells, cytokines, flow cytometry

4.2.1 INTRODUCTION

Patients with severe hemophilia A frequently develop neutralizing anti-factor VIII antibodies after receiving factor VIII (FVIII) replacement therapy. Despite progress in recent years, the mechanisms regulating the immune response to FVIII are still unclear. Recently, we and others have shown that hemophilic E-17 mice (1, 2) have an antibody response to human FVIII similar to that of patients with hemophilia A (3, 4, 5). Intravenous injection of therapeutic doses of human FVIII results in high titers of anti-FVIII antibodies directed against both functional and non-functional parts of the FVIII molecule. Murine anti-FVIII antibodies neutralize FVIII activity following second order reaction kinetic and therefore resemble type I antibodies in human hemophilia A (4, 5). The anti-FVIII antibody response in hemophilic mice is not restricted isotypically and involves all IgG subtypes (4, 5). Qian et al. have shown that the induction of anti-FVIII antibodies in hemophilic E-16 and E-17 mice is T-cell dependent (3). Furthermore, results from Rossi et al. (6) indicate that the regulation of the anti-FVIII antibody response in hemophilic E-16 mice is very complex and might involve both Th1- and Th2-type T cells. Previous findings on FVIII-specific T cells in hemophilic mice have been based on the proliferation capacity and cytokine release of in vitro re-stimulated total spleen cell populations or spleen cell populations depleted of $CD8^+$ cells (3, 6). Although such assays provide valuable information, they do not always reflect the real situation. Recent studies have indicated that the effector function of $CD4^+$ T cells is not necessarily coupled to proliferation (7). Furthermore, identification of subsets of FVIII-specific $CD4^+$ T cells based on the cytokine release of spleen cell populations could provide false positive results because IFN-γ, IL-4 and IL-10 can be secreted not only by T cells but also by non-T cells in the spleen (8, 9, 10). Therefore, I have established a single cell assay based on multiparameter flow cytometry that enables me to detect cell surface antigens together with intracellular cytokines on a single cell level. Using this assay, I re-examined the characteristics and the kinetic of FVIII-specific T cells in hemophilic E-17 mice after treatment with human factor VIII. In particular, we investigated the cytokine profile of FVIII-specific T cells that could form the basis for the development of new strategies aimed at the induction of T-cell tolerance against FVIII.

4.2.2 MATERIALS AND METHODS

Hemophilic mice

All experiments used inbred hemophilic C57BL/6J mice carrying a targeted disruption of exon 17 in the *FVIII* gene. Our colony of hemophilic mice was established with a breeding pair from the original colony (1, 2) crossed into the C57BL/6J background as described previously (11). Mice were male and aged 8–10 weeks at the beginning of the experiments. All studies were carried out in accordance with Austrian federal law (Act BG 501/1989) regulating animal experimentation.

Treatment with human factor VIII and blood sampling

Hemophilic mice received one, two or four intravenous doses of recombinant human FVIII (albumin-free bulk material, Baxter BioScience, Thousands Oaks, CA) at weekly intervals. Each dose comprised 200 ng FVIII (approximately 80 U/kg) diluted in 200 μl of Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, Irvine, UK). Control mice received DPBS only. Spleens and blood samples were obtained at 3 days and at 1 week after the first dose and 1 week after the second and fourth dose. Blood samples were taken by cardiac puncture and added in a 4:1 (v/v) ratio to 0.1mol/L sodium citrate. Plasma was separated by centrifugation and the samples were stored at -20° C until further analysis. All invasive procedures were carried out under anesthesia with pentobarbital (Nembutal, Richter Pharm, Wels, Austria).

Single cell analysis of factor VIII-specific T cells

Spleens from seven to ten mice obtained 3 days or 1 week after the first dose and 1 week after the second and fourth dose were pooled and disaggregated into spleen cell suspensions. $CD8^+$ cells were depleted by magnetic bead

separation using beads coated with the anti-mouse CD8 antibody Lyt 1.2 (Deutsche Dynal, Hamburg, Germany). Erythrocytes were removed by hemolysis using a hypotonic buffer (pH 7.2) composed of 0.15 Mm ammonium chloride, 0.1mM potassium bicarbonate (both from Merck, Darmstadt, Germany) and 0.01mM ethylendiaminetetaacetic acid (Life Technologies, Paisley, Scotland). The remaining $CD8^-$ cells were cultured for 24, 48, 72 or 96 h in RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 10% preselected fetal calf serum, 2 mM L-glutamine (both from Hyclone, Logan, Utah), 25 U/ml penicillin, 25 mg/ml streptomycin (both from Life Technologies, Paisley, Scotland), 1 mM sodium pyruat and $5x10^{-5}$ M ß-mercaptoethanol (both from Sigma-Aldrich, Irvine, UK). Cells were either restimulated with 20 µg/ml FVIII or with the same volume of dilution buffer (DPBS). At 6 h before harvesting cells, 1 µg/ml Golgi-Plug (PharMingen, Heidelberg, Germany) was added to the medium to retain intracellular cytokines in the Golgi apparatus of the cells. After termination of cell culture, B lymphocytes were depleted using magnetic beads coated with an anti-mouse pan B-cell antibody (B220, Deutsche Dynal, Hamburg, Germany). The remaining cells were washed and resuspended in Cellwash (Becton Dickinson, Vienna, Austria). To block nonspecific binding sites, cells were incubated with a mixture of anti-mouse CD16 and anti-mouse CD32 anti-Fc receptor antibodies (Fc-block, Becton Dickinson, Vienna, Austria). Cells were subsequently stained with a peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD4 antibody and a fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3 antibody or with appropriate isotype-matched negative control antibodies. Labeled cells were fixed and permeabilized with CytoFix/CytoPerm and subsequently stained with one of the following four allophycocyanin (APC)-labeled anti-cytokine antibodies: anti-mouse IL-2, anti-mouse IFN-γ, anti-mouse IL-4 or anti-mouse IL-10. For the analysis of co-expression of IFN-γ and IL-10, cells permeabilized with CytoFix/CytoPerm were stained with an APC-labeled anti-IL-10 antibody and a phycoerythrin (PE)-labeled anti-IFN-γ antibody. Samples stained with appropriate isotype-matched negative control antibodies were included as negative controls for staining with anti-cytokine antibodies in all experiments. The fixing agent and antibodies were purchased from PharMingen (Heidelberg, Germany) except when stated otherwise. Stained cells were analyzed by flow

cytometry using a FACS Calibur and CellQuest software (Becton Dickinson, Vienna, Austria). 30,000 cells from each sample after exclusion of cell debris by electronic means were analyzed. Cells were gated for $CD3^+$ cells and then further analyzed for $CD4^+$ cells that stained positive for cytokines.

Experiments A and B (Fig. 7)

I did two separate experiments using the same treatment schedule. In each experiment ten mice received a single dose of 200 ng FVIII, given intravenously. One week after the treatment, spleens and blood samples were obtained from all mice and used for antibody and T-cell assays.

Analysis of cytokines released into supernatants of spleen cells by ELISA

Spleen cells depleted of $CD8^+$ cells (prepared and cultivated as described above) were cultured in the presence or absence of 20 μg/ml FVIII. After 24, 48, 72 or 96 h supernatants were harvested and stored at –70°C until further analysis. IL-2, IFN-γ, IL-4, IL-5 and IL-10 were analyzed using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Detection of total anti-factor VIII antibodies

Titers of anti-factor VIII antibodies were determined by ELISA as described previously (5). Briefly, FVIII was immobilized to the solid phase of PolySorp multiwell plates (Nunc, Roskilde, Denmark). Nonspecific binding sites were blocked with 1% gelatin in DPBS. Plates were incubated with serial dilutions of plasma samples obtained from mice treated with FVIII or DPBS. Anti-factor VIII antibodies, bound to immobilized FVIII, were detected using rabbit anti-mouse

immunoglobulin labeled with horseradish peroxidase (Dako, Glostrup, Denmark) and the substrate *O*-phenylene diamine dihydrochloride (Sigma-Aldrich, Steinheim, Germany). Antibody titers were expressed as the highest dilution of plasma samples showing a positive result (optical density >0.3) in the assay.

IgG subclass determination of anti-factor VIII antibodies

PolySorp multiwell plates were coated with FVIII. Nonspecific binding sites were blocked with 1% gelatin in DPBS. Plates were incubated with serial dilutions of plasma samples from mice treated with FVIII or DPBS. We determined IgG-subclasses of anti-FVIII antibodies by incubation with isotype-specific, horseradish peroxidase-labeled secondary monoclonal antibodies (Southern Biotechnologies, Birmingham, AL). *O*-phenylene diamine dihydrochloride was used as substrate.
Antibody titers were expressed as the highest dilution of plasma samples showing a positive result (optical density >0.3) in the assay.

Detection of neutralizing anti-factor VIII antibodies

Neutralizing anti-FVIII antibodies were detected using the Bethesda assay (12).

4.2.3 RESULTS

Single cell analysis of factor VIII-specific T cells by multiparameter flow cytometry

The combined detection of surface antigens and intracellular cytokines on a single cell level allowed the unequivocal identification of different subsets of FVIII-specific T cells in hemophilic mice after treatment with FVIII. I did a series of methodical studies to define the optimal incubation time for the in vitro restimulation of FVIII-specific T cells. Incubation times of 24, 48, 72 and 96 h were compared. For the analysis of intracellular cytokines, incubation for 48 or 72 h was found to provide the most reproducible results. Although the frequencies of FVIII-specific T cells varied depending on the incubation time used, I did not see qualitative differences between the type of FVIII-specific T cells that were restimulated during 48 and 72 h of incubation. I found, however, a higher degree of nonspecific background staining in non-stimulated T cells after 72 than after 48 h of incubation and therefore decided to use an incubation time of 48 h in all experiments. The results from hemophilic mice treated with four doses of either FVIII or DPBS, indicate the presence of FVIII-specific CD4+ T cells that stain positive for IL-2, IFN-γ or IL-10 (Fig. 1). Both IL-2 and IFN-γ are characteristic for activated Th1-type T cells (13). Whereas IL-10 was originally described as a marker for Th2-type T cells (14) recent studies indicated that IL-10 might be produced by both Th1- and Th2- type T cells (15, 16). Cytokine co-expression studies analyzing IFN-γ and IL-10 in the same cell indicated that there might be at least two types of IL-10 positive T cells; cells that produce IL-10 only and in addition cells that produce IL-10 and IFN-γ. (Fig. 2). T cells producing IL-4 were detected at low frequency (< 0.4% of CD4+ T cells) in a few experiments but were absent in most. To control the staining procedure for intracellular staining of IL-4, I used fixed cells containing T cells that are positive for intracellular IL-4 (MICK-2, PharMingen, Heidelberg, Germany) as a positive control. The proportion of cells that stained positive for IL-4 in these control cells was 26%. CD4+ T-cells in spleen cells obtained from hemophilic mice treated with DPBS (negative controls) did not show any intracellular cytokine staining after in vitro restimulation with FVIII.

Cytokine release into culture supernatants of CD8- spleen cells

I did a series of methodical investigations to define the optimal incubation time for the in vitro restimulation of FVIII-specific T cells. Incubation times of 24, 48, 72 and 96 h were compared. The release of IL-4 was found to be measurable after 48 and 72 h, of IL-2 and IFN-γ after 48, 72 and 96 h and of IL-10 after 72 and 96 h (see example in Table 1). I decided to use an incubation time of 72 h in all experiments.

Kinetic of factor VIII-specific T cells

Factor VIII-specific T cells were detectable in the spleens of hemophilic mice after two doses of FVIII in all experiments (Fig. 3, Fig. 4). T cells producing IFN-γ were the most prominent type of FVIII-specific cells (Fig. 3). The highest frequency of these cells was found after four doses of FVIII and varied between 1.5% and 5.9% of $CD3^+CD4^+$ spleen cells in different experiments. In some experiments, IFN-γ–producing T cells could be detected 1 week after the first dose of FVIII (Fig. 7, Experiment B). In these cases, no FVIII-specific T cells were found at 3 days after the first dose. The second most prominent type of FVIII-specific T cells were those producing IL-10 and these were always detectable after two and after four doses of FVIII but never after just one (Fig. 4). Their frequency varied between 0.2% and 1.4% of $CD3^+CD4^+$ spleen cells after four doses. Factor VIII-specific T cells producing IL-2 were seen after two doses of FVIII and increased in frequency after four doses. Their frequency was found to be between 0.1% and 0.8% of $CD3^+CD4^+$ spleen cells. The presence of FVIII-specific T cells producing IL-2, IFN-γ or IL-10 in the spleen of hemophilic mice treated with FVIII was supported by the presence of these cytokines in the supernatants of spleen cells that were restimulated in vitro with FVIII (Fig. 5). T cells producing IL-4 were seen in some experiments after four doses of FVIII but were not detectable in most experiments. In contrast, IL-4 was detectable in supernatants of in vitro re-stimulated spleen cells obtained from hemophilic mice after two doses or four doses of FVIII (Fig. 5). Interleukin 5 (IL-5) was not found in any experiment (Fig. 5).

Kinetic of anti-factor VIII antibody formation

The kinetic of anti-FVIII antibody formation in hemophilic mice treated with FVIII correlated with the kinetic of the development of FVIII-specific T cells. All mice developed detectable anti-FVIII antibodies after two doses of FVIII that increased in titer after four doses (Fig. 6). In most experiments, no anti-FVIII antibodies were found after the first dose of FVIII. In a few experiments some animals developed anti-FVIII antibodies that were detectable as early as 1 week after the first dose (Fig. 7, Experiment B). In these cases, the appearance of anti-FVIII antibodies correlated with the detection of FVIII-specific T cells producing IFN-γ (Fig. 7, Experiment B).

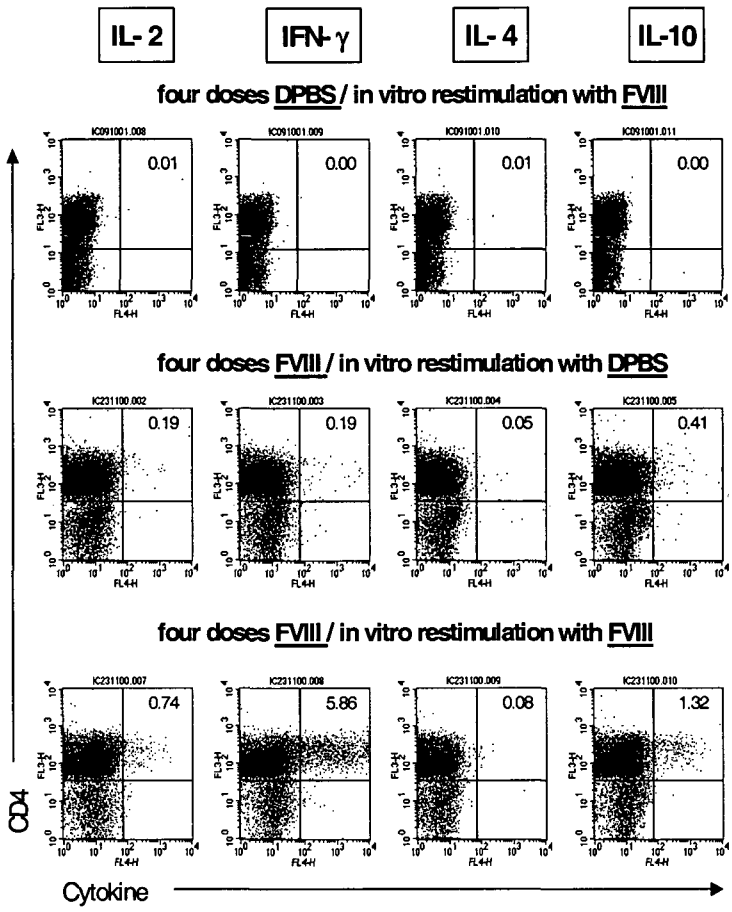
Titers of total anti-FVIII antibodies analyzed by ELISA correlated with titers of neutralizing anti-FVIII antibodies measured by Bethesda assays (Fig. 6).

Isotype distribution of anti-FVIII antibodies

The anti-FVIII immune response in hemophilic mice was not restricted isotypically (Table 2). Anti-FVIII antibodies were composed of all subclasses of IgG, namely IgG1, IgG2a, IgG2b and IgG3.

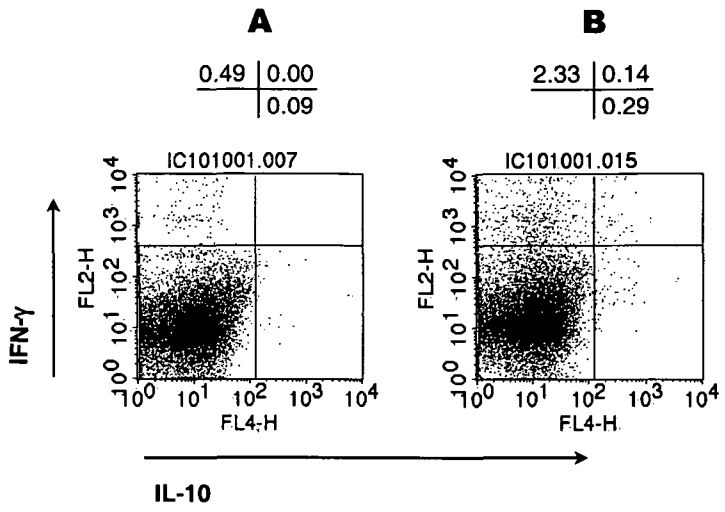
4.2.4 FIGURES

Figure 1



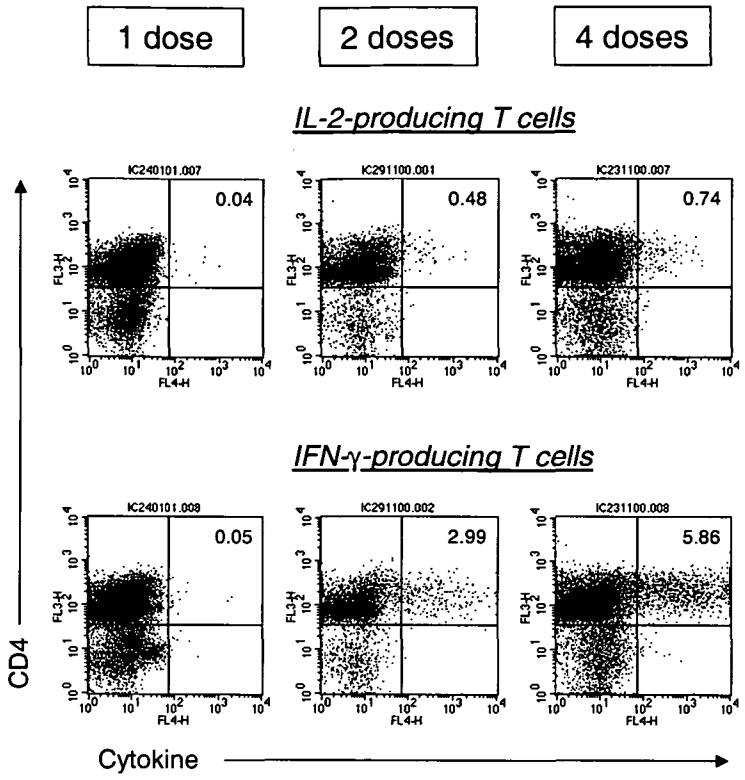
Cytokine pattern of splenic T cells restimulated in vitro as indicated. Cells were obtained from hemophilic mice after four doses of FVIII or DPBS. Dot plots show intracellular cytokine staining in $CD4^+$ T cells. Results shown in the upper right-hand corner of each dot plot represent the percentage of $CD4^+$ T cells that stained positive for the cytokine indicated. The negative cell population (lower left population) was defined by using cells that were stained with appropriate isotype-matched negative control antibodies. T cells were isolated, restimulated and stained as described in materials and methods.

Figure 2



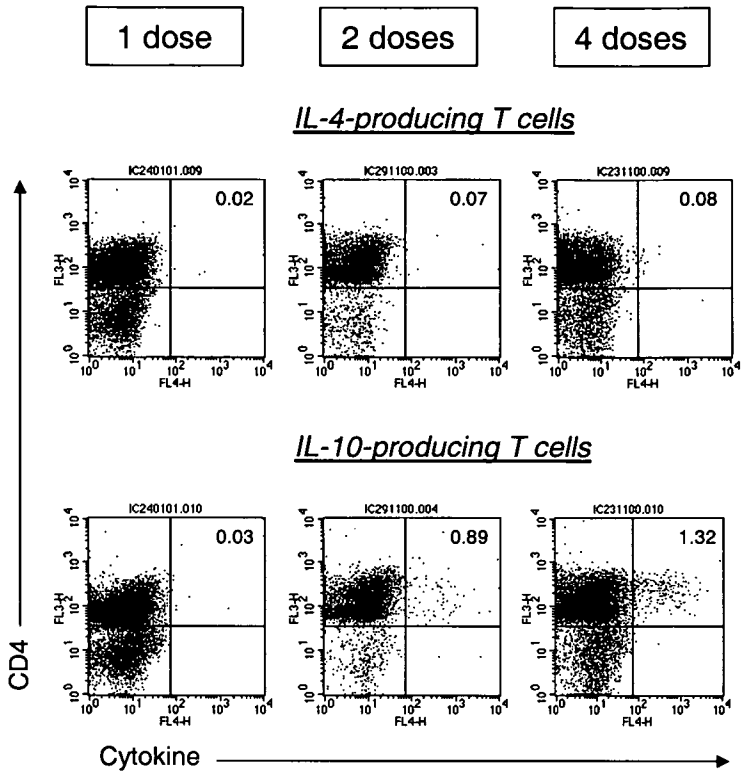
Expression of IL-10 and IFN-γ in splenic $CD3^+CD4^+$ cells, restimulated with medium (A) or FVIII (B) in vitro. Cells were obtained from hemophilic mice after four doses of FVIII. Results shown in the upper right-hand corner of each dot blot represent the percentage of $CD3^+CD4^+$ cells that stained positive for IL-10 and IFN-γ. Results in the upper left-hand corner represent cells that stained positive for IFN-γ only and results in the lower right-hand corner indicate cells that produced IL-10 only. Each dot represents a single cell. T cells were isolated, restimulated and stained as described in materials and methods.

Figure 3



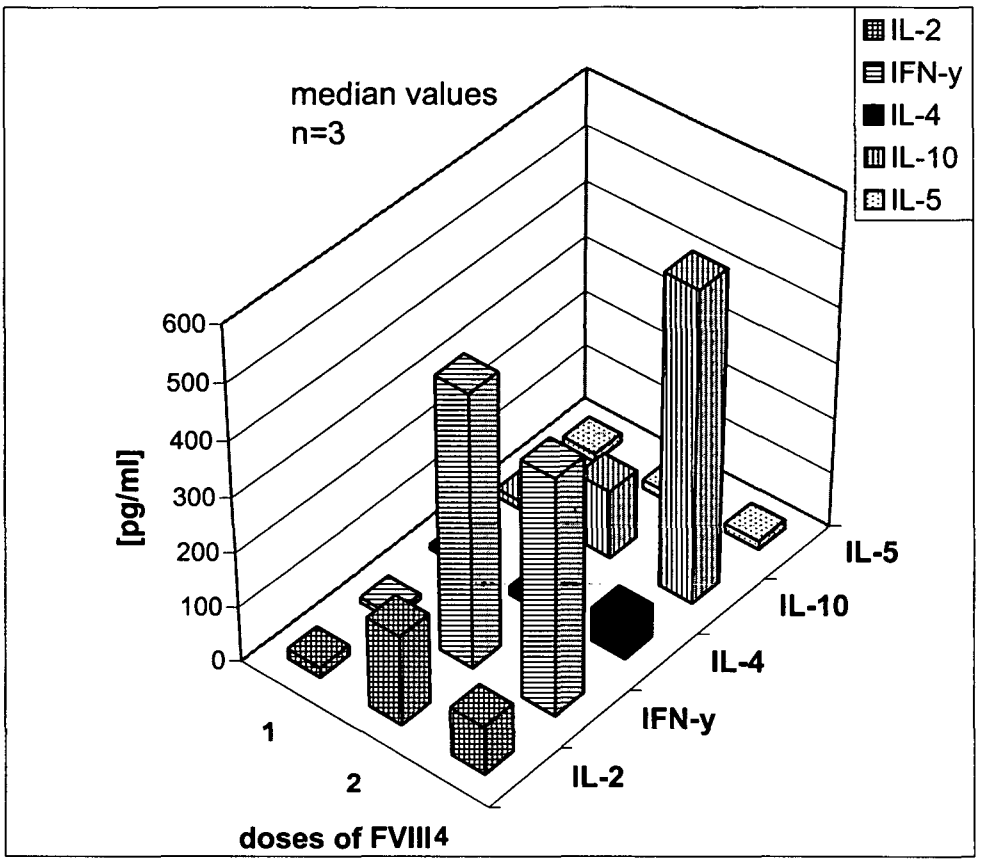
Kinetic of FVIII-specific T cells producing IL-2 or IFN-γ in the spleen of hemophilic mice after treatment with one, two or four doses of FVIII. Dot plots show intracellular cytokine staining in $CD4^+$ T cells after in vitro restimulation of splenic T cells with FVIII. Results shown in the upper right-hand corner of each dot plot represent the percentage of $CD4^+$ T cells that stained positive for the cytokine indicated. The negative cell population (lower left population) was defined by using cells that were stained with appropriate isotype-matched negative control antibodies. T cells were isolated, restimulated and stained as described in materials and methods.

Figure 4



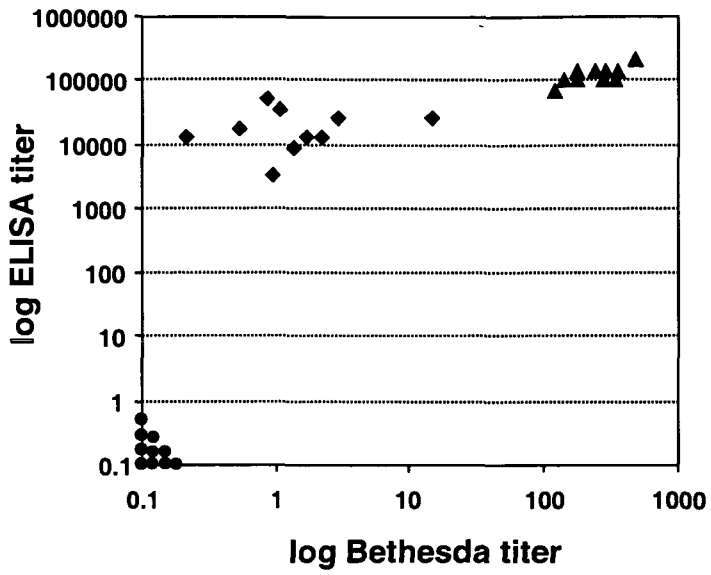
Kinetic of FVIII-specific T cells producing IL-4 or IL-10 in the spleen of hemophilic mice after treatment with one, two or four doses of FVIII. Dot plots show intracellular cytokine staining in $CD4^+$ T cells after in vitro restimulation with FVIII. Results shown in the upper right-hand corner of each dot plot represent the percentage of $CD4^+$ T cells that stained positive for the cytokine indicated. The negative cell population (lower left population) was defined by using cells that were stained with appropriate isotype-matched negative control antibodies. T cells were isolated, restimulated and stained as described in materials and methods.

Figure 5



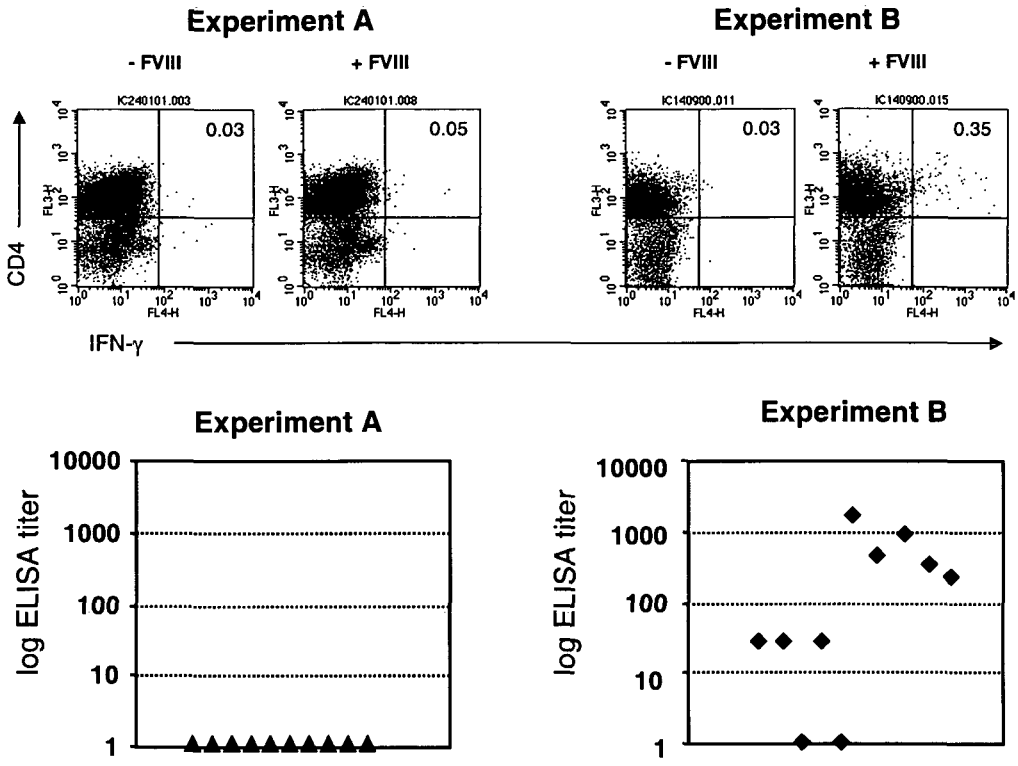
Cytokine release of CD8$^-$ spleen cells after in vitro restimulation with FVIII. Cells were obtained from hemophilic mice after treatment with one, two or four doses of FVIII. One representative experiment is shown. Results are median values obtained from triplicates

Figure 6



Relation of total anti-FVIII antibody titers (ELISA titer) to titers of FVIII-neutralizing antibodies (Bethesda titer) in plasma obtained from hemophilic mice after one dose (●), two doses (◆) or four doses (▲) of FVIII. Each point represents values for an individual mouse. Blood samples were obtained 1 week after each dose

Figure 7



Correlation of anti-FVIII antibodies (ELISA titer) detected in plasma and the presence of FVIII-specific T cells producing IFN-γ in the spleen of hemophilic mice after one dose of FVIII. Two separate experiments (Experiment A and Experiment B) were done using the same treatment schedule (described in materials and methods).

Upper pannel: Dot plots show intracellular staining for IFN-γ in $CD4^+$ T cells after in vitro restimulation of splenic T cells with either FVIII (+ FVIII) or buffer DPBS (-FVIII). Results shown in the upper right-hand corner of each dot plot represent the percentage of $CD4^+$ T cells that stained positive for IFN-γ. The negative cell population in each dot plot (lower left population) was defined by using cells that were stained with appropriate isotype-matched negative control antibodies. T cells were isolated, restimulated and stained as described in Materials and Methods. Spleens were obtained 1 week after treatment with a

single dose of FVIII.

Lower pannel: ELISA titers of anti-FVIII antibodies. Each point represents values for an individual mouse. Blood samples were obtained 1 week after treatment with a single dose of FVIII.

4.2.5 TABLES

Table 1.

Cytokine release of $CD8^-$ spleen cells obtained from hemophilic mice after four doses of FVIII and restimulated with FVIII in vitro for different periods of time (a representative experiment is shown)

time of incubation		**Cytokine release (pg/ml)** (x ± SD, n=3)			
		IL 2	IFN-γ	IL-4	IL-10
48 h	medium control	n.d.	n.d.	n.d.	n.d.
	FVIII-restimulation	23.8 ± 6.1	108.8 ± 18.0	13.7 ± 0.0	n.d.
72 h	medium control	n.d.	n.d.	n.d.	n.d.
	FVIII-restimulation	45.4 ± 4.5	561.8 ± 129.2	7.8 ± 0.3	61.7 ± 7.2
96 h	medium control	n.d.	n.d.	n.d.	n.d.
	FVIII-restimulation	75.8 ± 11.4	594.9 ± 5.4	n.d.	173.4 ± 43.5

n.d. – not detectable

Table 2.

Median anti-factor VIII antibody titers of different IgG subtypes in hemophilic mice (n=10 mice per group)

	Antifactor VIII antibody titers			
	IgG1	IgG2a	IgG2b	IgG3
One dose FVIII	n.d.	n.d.	n.d.	n.d.
Two doses FVIII	10240	7680	5120	560
Four doses FVIII	163840	81920	81920	5120

n.d. – not detectable

4.2.6 DISCUSSION

The identification of subsets of FVIII-specific $CD4^+$ helper T cells producing distinct patterns of cytokines provides valuable information for understanding the regulation of the immune response to FVIII in hemophilic mice. Such identification might, therefore, form a basis for developing new strategies aimed at inducing FVIII-specific tolerance in patients with hemophilia A. Previously, it has been shown that the antibody response to human FVIII in hemophilic mice is not isotypically restricted and involves all subclasses of murine IgG (4, 5). Therefore I concluded that the regulation of anti-FVIII antibody responses in hemophilic E-17 mice was very complex and involved both Th1- and Th2-type T cells. This conclusion is supported by recent findings from Rossi et al. (6). I have now further investigated the kinetic and the characteristics of FVIII-specific T cells using a multiparameter flow cytometric assay.

I analyzed T-cell specific cell surface markers (CD3, CD4) together with intracellular cytokines on a single cell level to identify the frequency of different types of FVIII-specific $CD4^+$ T cells. A clear temporal correlation between the appearance of FVIII-specific $CD4^+$ T cells in the spleen and the detection of anti-FVIII antibodies in plasma was found. Both FVIII-specific T cells and anti-FVIII antibodies were detectable after two doses of FVIII in all experiments (Figs 3, 4, 6). In some experiments, however, FVIII-specific T cells and anti-FVIII antibodies could be detected after a single dose of FVIII (Fig. 7, Experiment B). In these cases, only FVIII-specific T cells producing IFN-γ were found. Therefore, IFN-γ–producing T cells might be particular important for inducing anti-FVIII antibodies in hemophilic E-17 mice. This would be supported by the high frequency of FVIII-specific T cells producing IFN-γ after four doses of FVIII which was found to be between 1.5% and 5.9% of $CD3^+CD4^+$ spleen cells in different experiments. Cells producing IL-10 were the second most prominent type of FVIII-specific T cells in the spleen after those producing IFN-γ. They were detectable following two and four doses of FVIII in all experiments (Fig. 4). Originally IL-10 was described as a product of Th2-type T-cell clones that inhibits cytokine, in particular IFN-γ, production by Th1-type T cells (14). Recent results indicate, however, that IL-10 might be produced by both Th1- and Th2-type T cells (15, 16). Furthermore, findings by Asseman et al. point to IL-10

being an important effector cytokine of regulatory T cells (17). Thus, it is attractive to speculate that the FVIII-specific T cells producing IL-10 are a T-cell population able to down-modulate FVIII-specific T cell responses. To further characterize IL-10-producing T cells I did cytokine co-expression studies analyzing IFN-γ and IL-10 in the same cell. These studies indicate that there might be at least two types of IL-10 positive T cells that produce either IL-10 only or IL-10 and IFN-γ (Fig. 2). Studies are in progress to characterize the functional activity of IL-10-producing T cells in hemophilic E-17 mice. Apart from its effects on activated T cells, IL-10 was shown to stimulate the differentiation of activated B lymphocytes (18, 19). It might, therefore, potentiate the differentiation of FVIII-specific B lymphocytes once they are activated and thereby amplify the anti-FVIII antibody response. In a few experiments FVIII-specific $CD4^+$ T cells that stained positive for IL-4 could be detected, but in most IL-4 staining was not seen. (Fig. 4). In contrast, I found IL-4 in supernatants of in vitro re-stimulated $CD8^-$ spleen cells (Fig. 5). This IL-4 might have been secreted by non-T cells and therefore not reflect the activation of FVIII-specific T cells. Ben-Sasson et al. (8) have previously shown that a non-B, non-T cell population, belonging to the mast cell/basophil lineage, is an important source of IL-4 in the spleen. This cell population releases IL-4 in response to Fc-receptor-mediated signaling.

Summarizing the results presented in this paper, I have shown that the anti-FVIII antibody response to human FVIII in hemophilic E-17 mice is accompanied by a response of FVIII-specific T cells that produce IFN-γ, IL-10 or IL-2. These results indicate the importance of Th1-type T cells for the regulation of the anti-FVIII antibody response in hemophilic E-17 mice.

In general, my results agree with data published by Qian et al. (3) and Rossi et al. (6). Qian et al. found detectable anti-FVIII antibody responses in hemophilic E-16 and E-17 mice after two doses of human FVIII and FVIII-specific T-cell responses (measured by proliferation assays) after one and two doses of FVIII. Rossi et al. used hemophilic E-16 mice and detected anti-FVIII antibodies after two doses of human FVIII. They characterized FVIII-specific T-cell responses by cytokine release of $CD8^-$ spleen cell populations and found IL-2, IL-4, IL-10 and IFN-γ. It is, however, not clear from their results whether these cytokines were released by T cells or other spleen cells. In contrast, Wu et al. (20) treated

hemophilic E-16 mice with human FVIII and did not see any anti-FVIII antibody response before the fourth or fifth injection. Their data on FVIII-specific T cells indicate the involvement of T cells that produce IL-10, IL-4 or IFN-γ. Like Rossi et al., however, their data do not prove that these cytokines are released by T cells.

The division of cytokine-producing T cells into Th1 and Th2 subsets is probably an oversimplified model that might not reflect the diversity and sophistication of T-cell responses in vivo. Recent studies have shown that many T-cell clones do not fit into the Th1/Th2 classification (review in 21, 22). Flow cytometry analysis of single cells allows the individual characterization of T cells and can fully display the heterogeneity of cell populations. Compared with ELISA, the advantage of flow cytometry is that multicolour staining can show exclusive or mutual co-expression of cytokines in individual T cells. Therefore, I am currently in the process of further developing this methodology for the analysis of FVIII-specific T cells in hemophilic mice to obtain a complex picture of the regulation of anti-FVIII immune responses by FVIII-specific T cells.

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4.3 BLOCKADE OF CD40/CD40 LIGAND INTERACTIONS PREVENTS INDUCTION OF FACTOR VIII INHIBITORS IN HEMOPHILIC MICE BUT DOES NOT INDUCE LASTING IMMUNE TOLERANCE

SUMMARY

Patients with severe hemophilia A frequently develop neutralizing anti-factor VIII antibodies after replacement therapy with factor VIII (FVIII). In a search for new strategies to induce immune tolerance against FVIII in these patients, I used a murine model of hemophilia A to investigate the importance of CD40/CD40 ligand (CD40L) interactions for the initiation of the anti-FVIII immune response. I focused my attention in particular on the induction of neutralizing anti-FVIII antibodies and the Th1/Th2 polarization of FVIII-specific T cells. The development of anti-FVIII antibodies was analyzed by ELISA systems (detection of total anti-FVIII antibodies) and Bethesda assays (determination of neutralizing anti-FVIII antibodies). Factor VIII-specific T cells were characterized by mulitparameter flow cytometry and cytokine ELISAs for the detection of cytokine production in splenic $CD4^+$ T cells after in vitro restimulation with FVIII. Hemophilic mice received four doses of FVIII and anti-CD40L antibody MR1 (24 hours before FVIII). Subsequently mice received four doses of FVIII only. The induction of neutralizing anti-FVIII antibodies in hemophilic mice after treatment with human FVIII could be prevented completely by a blockade of CD40/CD40L interactions using MR1. Furthermore, FVIII-specific T-cell responses that included both Th1 and Th2 cells were suppressed when mice were treated with FVIII and MR1. The initial blockade of CD40/CD40L interactions was, however, not sufficient to induce a lasting immune tolerance against FVIII. The immune suppression was abolished and both neutralizing anti-FVIII antibodies and FVIII-specific T cells developed when treatment with FVIII was continued after the omission of MR1. In addition, there were no alterations in the Th1/Th2 polarization induced by the initial blockade of CD40/CD40L interactions.

Key words

Hemophilia A, factor VIII inhibitors, T cells, immune tolerance, CD40 ligand

4.3.1 INTRODUCTION

The induction of neutralizing anti-factor VIII antibodies (factor VIII inhibitors) is a major problem in factor VIII (FVIII) replacement therapy for patients with hemophilia A (1, 2). The highest risk for developing such antibodies is associated with nonsense mutations, large deletions and intrachromosomal recombinations (inversions) in the FVIII gene that can be predicted to cause a complete lack of any endogenous FVIII production in the patient (3-5, HAMSTeRS: The haemophilia A mutation, structure, test, resource site: http://europium.csc.mrc.ac.uk). I therefore used a murine knockout model in which a targeted gene disruption in exon 17 (E-17 mice) of the FVIII gene results in complete FVIII deficiency (6, 7), to explain the mechanisms responsible for the regulation of the immune response to human FVIII. Such hemophilic E-17 mice are similar to hemophilia A patients in their antibody response to human FVIII (8). Intravenous injection of human FVIII results in high titers of anti-FVIII antibodies directed against both functional and non-functional parts of the FVIII molecule. Murine anti-FVIII antibodies neutralize FVIII activity following second order reaction kinetics and therefore resemble type I antibodies in human hemophilia A (8).

Qian et al. have recently shown that the induction of anti-FVIII antibodies in hemophilic mice that are characterized by a disruption of exon 16 (E-16 mice) or exon 17 (E-17 mice) of the FVIII gene is T-cell dependent (9). Furthermore, they were able to modulate the anti-FVIII immune response in E-16 mice by an interruption of co-stimulatory interactions between CD40 and CD40 ligand (CD40L) (10). Interactions between CD40 and CD40L have an essential role in the initiation of B-cell affinity maturation and isotype switching in response to T-cell-dependent antigens (reviewed in Ref. 11). Moreover, evidence has

accumulated that these interactions are essential for the initiation of antigen-specific T-cell responses (12, 13). Previous studies have shown that anti-CD40L antibodies can achieve prolonged survival of allografts in rodents and in monkeys (14-16). This effect is probably due to the induction of lasting tolerance in the $CD4^+$ T-cell population (17). Therefore the importance of CD40/CD40L interactions in the regulation of immune responses to human FVIII in hemophilic mice was further evaluated. In particular, it was considered whether MR1, an anti-CD40L antibody that has been shown to block the CD40/CD40L interactions both in vitro and in vivo (18, 19), influences the Th1/Th2 polarization of FVIII-specific $CD4^+$ T cells and induces a lasting tolerance to FVIII in the $CD4^+$ T-cell population when given 24 hours before FVIII.

4.3.2 MATERIALS AND METHODS

Animals

Our colony of *FVIII* knockout mice (fully inbred C57 BL/6J mice with a targeted disruption of exon 17 of the *FVIII* gene) was established with a breeding pair from the original colony (6, 7) that was crossed into the C57BL/6J background as described previously (20). All mice were male and aged 8-10 weeks at the beginning of the treatment period. Individual mice were identified by tail markings. All studies were done in accordance with Austrian federal law (Act BG 501/1989) regulating animal experimentation.

Treatment Strategies

Factor VIII knockout mice were treated intravenously with 200 ng recombinant FVIII per dose (approximately 80 U/kg FVIII), diluted in 200 μl of Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, Irvine, UK). To evaluate the influence of CD40/CD40L interactions, 200 μg of anti-CD40L antibody MR1,

200 μg of isotype-matched hamster IgG control antibody or DPBS were given in a volume of 200 μl intravenously 24 hours before treatment with FVIII. Mice received four doses of FVIII and antibodies or DPBS at weekly intervals. Subsequently, antibodies and DPBS were omitted and mice received four more doses of FVIII only. All doses of FVIII were given at weekly intervals.

Blood Sampling

Blood samples for the evaluation of ELISA titers were obtained by tail snipping 1 week after the second, fourth and eighth dose. For measurement of inhibitory anti-FVIII antibody titers, mice were killed and bled by cardiac puncture. For this reason, Figure 1 does not indicate sequential data for individual mice.
All invasive procedures were carried out under anesthesia with pentobarbital (Nembutal, Richter Pharm, Wels, Austria). The blood samples were added at a 4:1 (v/v) ratio to 0.1mol/L sodium citrate and plasma was separated by centrifugation. The plasma samples were subsequently stored at -20°C until further analysis.

Factor VIII Preparation

The recombinant human FVIII used throughout the studies was albumin-free bulk material obtained from Baxter BioScience (Thousand Oaks, CA).

Anti-CD40L Antibody and Hamster IgG Control

The hamster anti-mouse CD40L antibody MR1 and the isotype-matched hamster IgG negative control antibody were purchased from BD PharMingen (San Diego, CA). MR1 has been shown to block the CD40/CD40L interactions both in vitro and in vivo (18, 19).

Detection of Total Anti-FVIII Antibodies

Total anti-FVIII antibody titers were measured by ELISA as described previously (21). Briefly, recombinant FVIII was immobilized to PolySorp multiwell plates (Nunc, Roskilde, Denmark). After blocking of non-specific binding sites using 1% gelatin in DPBS, plates were incubated with serial dilutions of plasma samples obtained from mice treated as indicated. Anti-FVIII antibodies, bound to immobilized human FVIII, were detected by incubation with polyclonal rabbit anti-mouse immunoglobulin labeled with horseradish peroxidase (Dako, Glostrup, Denmark) and subsequent substrate development using *O*-phenylene diamine dihydrochloride (Sigma-Aldrich, Steinheim, Germany). Antibody titers were expressed as the highest dilution of plasma samples showing a positive result (optical density > 0.3) in the ELISA assay.

Detection of Inhibitory Anti-Factor VIII Antibodies

Inhibitory (FVIII-neutralizing) anti-FVIII antibodies were detected using the Bethesda assay (22).

Determination of IgG Subclass of Anti-Factor VIII Antibodies

The IgG subclasses of anti-FVIII antibodies were determined as previously described (8). PolySorp multi-well plates (Nunc, Roskilde, Denmark) were coated with 1 μg/ml recombinant FVIII in carbonate buffer, pH 9.0, by overnight incubation at 4°C. After blocking non-specific binding sites using 1% gelatin in DPBS, the plates were incubated with serial dilutions of plasma samples obtained from mice treated as indicated. The IgG-subtype of anti-FVIII antibodies bound to immobilized human FVIII was determined by incubation with isotype-specific, biotin-labeled secondary monoclonal antibodies (PharMingen, San Diego, CA) and subsequent incubation with avidin-horseradish peroxidase (PharMingen, San Diego, CA). Substrate development was done using *O*-phenylene diamine dihydrochloride (Sigma-Aldrich,

Steinheim, Germany). Antibody titers were expressed as the highest dilution of plasma samples showing a positive result (optical density > 0.3) in the ELISA assay.

Medium for In Vitro Restimulation of T cells (complete RPMI medium)

The RPMI 1640 (Hyclone, Logan, Utah) was supplemented with 2 mM L-glutamine (Hyclone, Logan, Utah), 25 U/ml penicillin (Life Technologies, Paisley, Scotland), 25 μg/ml streptomycin (Life Technologies, Paisley, Scotland), 1 mM sodium pyruvate (Sigma-Aldrich, Irvine, UK), 5 x 10^{-5} M β-mercaptoethanol (Sigma-Aldrich, Irvine, UK) and 10% pre-selected fetal calf serum (lot 4722, Hyclone, Logan, Utah).

Characterization of FVIII-Specific T cells by Multiparameter Flow Cytometry

Spleen cells from seven to ten mice treated as indicated were pooled and depleted of $CD8^+$ cells using magnetic beads coated with a monoclonal anti-mouse CD8 antibody (Deutsche Dynal, Hamburg, Germany). The remaining $CD8^-$ cells were then further depleted of erythrocytes by hemolysis using a hypotonic buffer and subsequent correction of ionicity. The hypotonic buffer was composed of 0.15 M ammonium chloride (Merck, Darmstadt, Germany), 1 mM potassium bicarbonate (Merck, Darmstadt, Germany), 0.1 mM ethylenediaminetetraacetic acid (Life Technologies, Paisley, Scotland) and adjusted to pH 7.2. Cells were cultured at 10^6/ml in complete RPMI medium in the presence of either 20 μg/ml recombinant FVIII or DPBS for a total of 48 hours. At 6 hours before harvesting, 1 μg/ml Golgi-Plug (PharMingen, Heidelberg, Germany) was added to the cultures to retain cytokines in the cytoplasm. Cells were then washed and depleted of B cells using magnetic beads coated with a monoclonal anti-mouse pan B (B220) antibody (Deutsche

Dynal, Hamburg, Germany). Cells depleted of B cells were stained with a monoclonal rat anti-mouse CD3 antibody labeled with fluorescein isothiocyanate (FITC) and a peridinin chlorophyll protein (PerCP)-labeled monoclonal rat anti-mouse CD4 antibody. Non-specific binding sites were blocked using a mixture of anti-mouse CD16 and anti-mouse CD32 anti-Fc receptor antibodies. Labeled cells were fixed and permeabilized using CytoFix/CytoPerm (Becton Dickinson PharMingen, Heidelberg, Germany). Subsequently, permeabilized cells were stained with either of three rat anti-mouse cytokine antibodies labeled with allophycocyanin (APC) to stain intracellular cytokines. I used APC-labeled rat anti-mouse interleukin 2, APC-labeled rat anti-mouse interleukin 4 and APC-labeled rat anti-mouse interferon-γ for this purpose. As negative controls for antibody staining, isotype-matched negative control antibodies were included. All samples were analyzed on a FACSCalibur using CellQuest software (both from Becton Dickinson, CA). I collected 30,000 events from each sample after exclusion of cell debris by electronic means. Viable cells were characterized by side scatter and forward scatter and then gated for $CD3^+$ cells. The $CD3^+$ cells were further analyzed for $CD4^+$ cells that stained positive for cytokines. All the antibodies were obtained from Becton Dickinson PharMingen, Heidelberg, Germany unless otherwise stated.

Quantification of Cytokine Release into Supernatants of In Vitro Restimulated T Cells

Spleen cells of mice treated as indicated were depleted of $CD8^+$ cells using magnetic beads coated with a monoclonal anti-mouse CD8 antibody (Deutsche Dynal, Hamburg, Germany). The remaining $CD8^-$ cells were then further depleted of erythrocytes by hemolysis. Cells were cultured at 10^6/well in 24-well plates (Nunc, Roskilde, Denmark) in the presence or absence of 20 μg/ml recombinant FVIII. Cell supernatants were collected after 72 hours and stored at –70°C until further analysis. Interleukin 2 (IL-2), interleukin 4 (IL-4), interferon-γ (IFN-γ) and interleukin 10 (IL-10) in cell supernatants were analyzed using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Statistical Analysis

The unpaired Student's *t* test was used for comparison of means between groups. Differences were considered statistically significant if $P<0.05$.

4.3.3 RESULTS

Induction of Anti-FVIII Antibodies is Blocked by Anti-CD40L Antibody

The importance of CD40/CD40L interactions for the development of anti-FVIII antibodies was shown by analyzing total antibodies (ELISA titers, Figs. 1, 2) as well as neutralizing antibodies (Bethesda titers, Fig. 1) against FVIII. Anti-CD40L antibody completely prevented the development of anti-FVIII antibodies (Figs. 1, 2). Both ELISA and Bethesda titers were suppressed. In contrast, control mice that were treated with either FVIII and buffer or FVIII and an isotype-matched hamster control IgG developed high titers of anti-FVIII antibodies (Fig. 2). Antibodies were not detected in untreated mice by either ELISA or Bethesda assays.

Activation of FVIII-Specific T Cells

Multiparameter flow cytometry analyzing intracellular cytokines in $CD4^+$ T cells and the detection of cytokine release by ELISA (Figs. 3, 4, 5) were used to evaluate the Th1/Th2 distribution of FVIII-specific $CD4^+$ T cells in hemophilic mice after a series of four or eight doses of FVIII. The results showed the presence of $CD4^+$ T cells that stain positive for IL-2 or IFN-γ after in vitro restimulation of $CD8^-$ spleen cells with FVIII (Fig. 3A). Both cytokines are characteristic of activated Th1 cells (23). Moreover, in a few experiments $CD4^+$ T cells that stained positive for IL-4 could be detected (Fig. 4, 0.26% $CD4^+$ cells positive for IL-4). The IL-4 staining was not, however, consistently seen. By comparison, negative controls obtained from mice treated with DPBS did not show any response of $CD4^+$ T cells to FVIII (Fig. 3A). Furthermore, spleen cells obtained from mice treated with FVIII and restimulated with DPBS did not show any response either (Fig. 3A). The results obtained by flow cytometry were supported by the concentrations of cytokines detected in the supernatants of $CD8^-$ spleen cells after restimulation with FVIII (Fig. 3B). In addition to IFN-γ and IL-2, IL-10 was found in the supernatants. By comparison, negative controls obtained from mice treated with DPBS did not show any cytokine release in response to FVIII and neither did spleen cells obtained from mice treated with

FVIII and restimulated with DPBS (Fig. 3B). These findings indicate the FVIII-specific activation of Th1 cells and possibly Th2 cells in hemophilic E-17 mice after treatment with human FVIII.

Blockade of CD40/CD40L Interaction Inhibits the Activation of FVIII-Specific T Cells

The blockade of CD40/CD40L interactions was found to suppress the development of FVIII-specific T cells (Figs. 4, 5A). The $CD4^+$ T cells that stained positive for intracellular IL-2 or IFN-γ after restimulation with FVIII were considerably diminished (Fig. 4). Furthermore, the release of IL-2, IFN-γ and IL-10 into the supernatant of $CD8^-$ spleen cells restimulated with FVIII was significantly reduced (Fig. 5A).

Blockade of CD40/CD40L Interaction Does not Induce Lasting Immune Tolerance to FVIII

To analyze for lasting effects of the initial blockade of CD40/CD40L interaction on anti-FVIII immune response, mice received four doses of FVIII and MR1 and subsequently four doses without MR1. When the anti-CD40L antibody, MR1, was omitted considerable titers of anti-FVIII antibodies developed (Fig. 1). Both ELISA as well as Bethesda titers were detected (Fig. 1). These results clearly showed the FVIII-neutralizing activity of anti-FVIII antibodies developed after the omission of MR1. The data indicate that the initial blockade of CD40/CD40L interactions did not induce a lasting tolerance against FVIII in hemophilic mice.

Blockade of CD40/CD40L Interaction Does not Induce Lasting Alterations in Th1/Th2 Polarization of FVIII-Specific T Cells

Factor VIII-specific $CD4^+$ T cells in spleens of hemophilic mice were analyzed after four doses of FVIII and buffer and an additional four doses of FVIII only and compared with T cells from mice who received four doses of FVIII and MR1 and subsequently four more doses of FVIII only (Figs. 4, 5B). Multiparameter flow cytometric analysis of intracellular cytokines in $CD4^+$ T cells showed the induction of both IL-2 positive and IFN-γ positive cells after restimulation with FVIII in both groups of mice (Fig. 4). Furthermore, IFN-γ, IL-2 and IL-10 were released into supernatants of spleen cells obtained from mice treated with four doses of FVIII and anti-CD40L antibody and an additional four doses of FVIII only and restimulated with FVIII in vitro (Fig. 5B). These results suggest that there is no difference in Th1/Th2 polarization of FVIII-specific $CD4^+$ T cells in the spleen between mice that had initially received the anti-CD40L antibody and mice treated with FVIII and buffer.

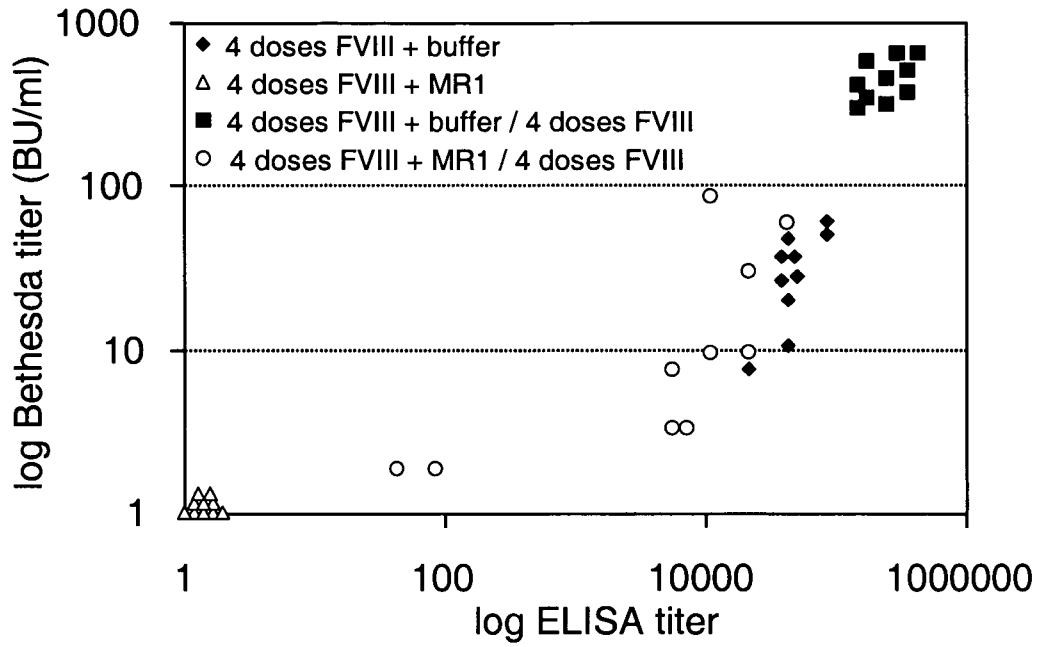
Isotype Distribution of Anti-FVIII Antibodies is not Altered by Initial Blockade of CD40/CD40L Interaction

The ability of B cells to undergo class switching in response to T-cell-dependent antigens requires T-cell help that is mediated at least in part through CD40/CD40L interactions. Therefore it was investigated whether the initial blockade of CD40/CD40L interactions induces lasting effects on the isotype distribution of anti-FVIII antibodies in hemophilic mice.

These results emphasize that the anti-FVIII immune response in E-17 mice is not restricted isotypically (Table 1). Anti-FVIII antibodies are composed of all subclasses of IgG, namely IgG1, IgG2a/b and IgG3. This subclass distribution was not altered by the initial blockade of CD40/CD40L interactions (Table 1).

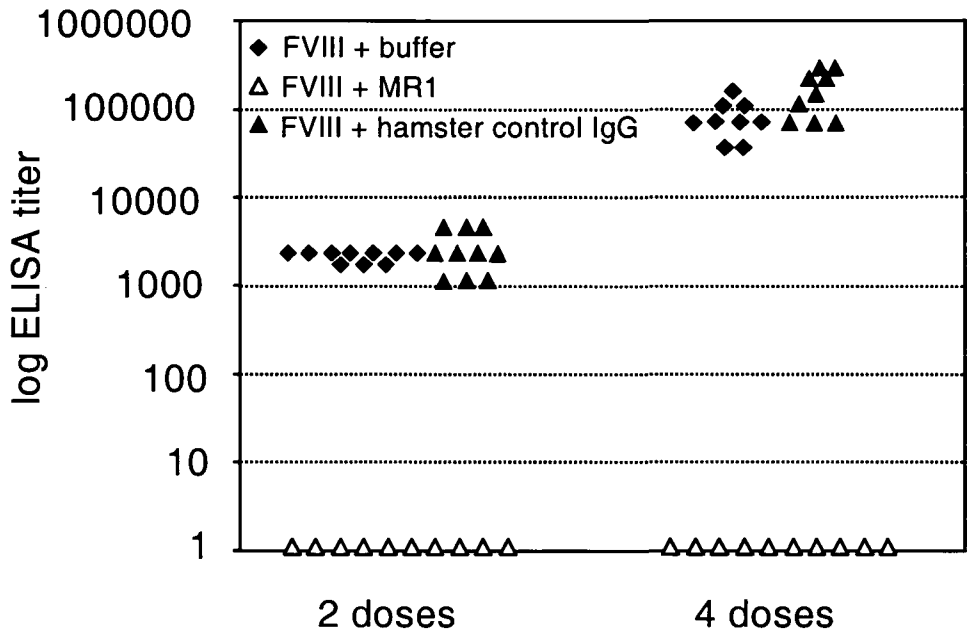
4.3.4 FIGURES

Figure 1



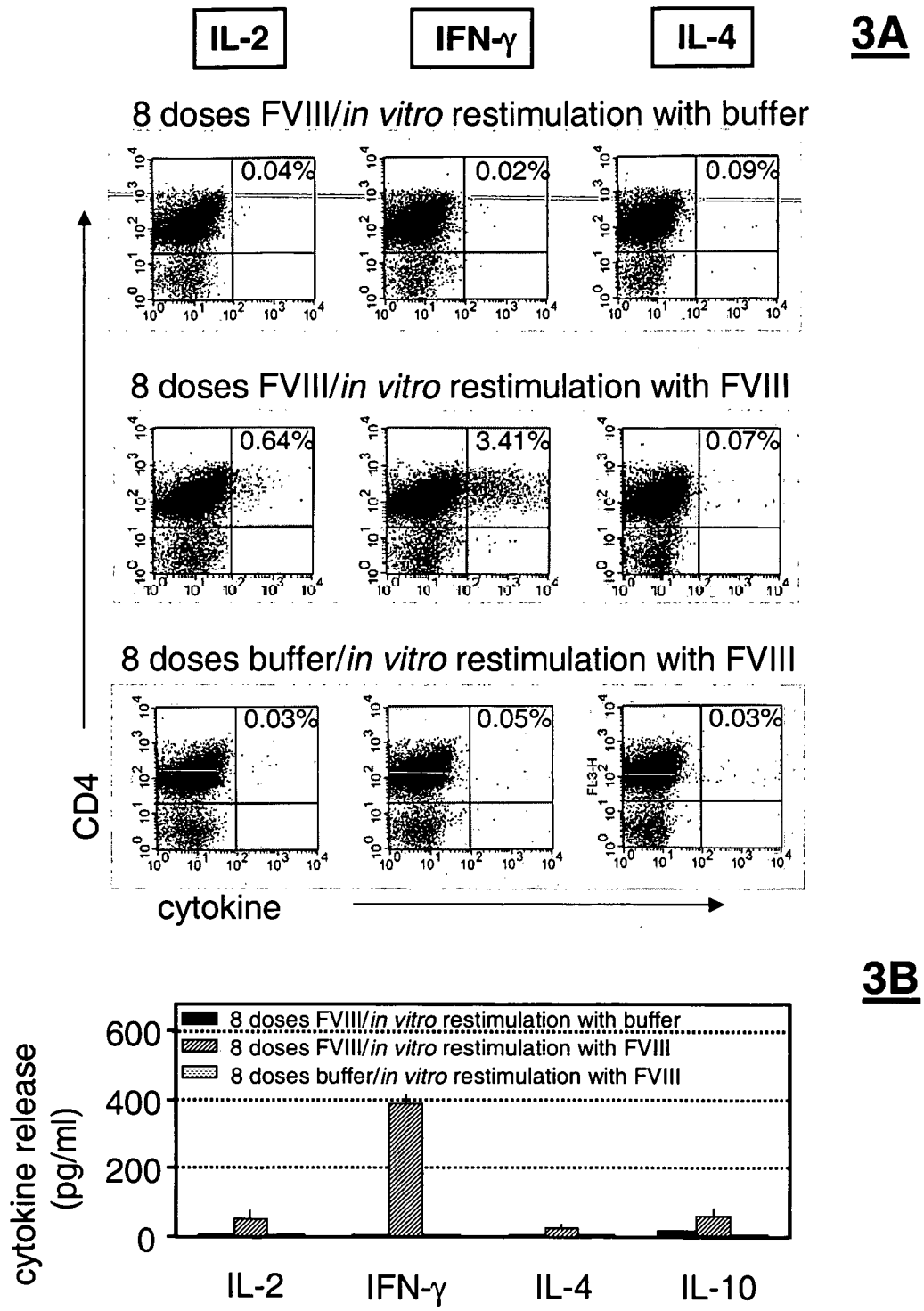
Relation of neutralizing anti-FVIII antibodies (Bethesda titer) and total anti-FVIII antibodies (ELISA titer) in hemophilic E-17 mice after treatment with FVIII. Each point represents values for an individual mouse. Animals were killed 1 week after the fourth treatment or 1 week after the eighth treatment and blood was taken by cardiac puncture. Mice received the treatments as indicated at weekly intervals. 200 ng FVIII were given per dose. The same animals that were used for the preparation of samples shown were also used for the preparation of spleen cell samples shown in figures 4 and 5.

Figure 2



Anti-FVIII antibody formation in hemophilic E-17 mice determined by ELISA. Each point represents an individual mouse after receiving two or four serial treatments as indicated. FVIII was given intravenously at a dose of 200 ng (80 U/kg) per dose at weekly intervals. Blood samples were obtained 1 week after the second and 1 week after the fourth dose.

Figure 3

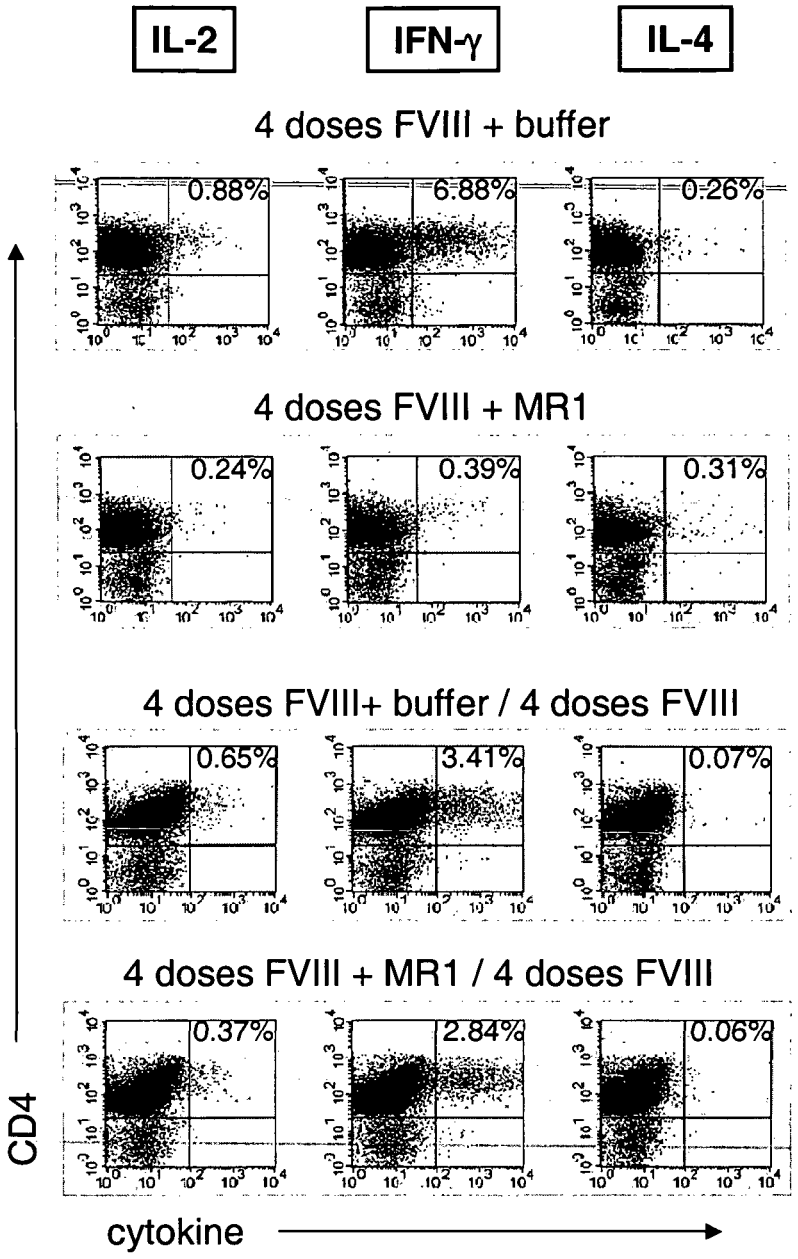


3A:Two-dimensional dot plots showing intracellular cytokine staining in CD4⁺ T cells after restimulation with human FVIII or buffer. Spleen cells were obtained from hemophilic mice treated as indicated. Cells were depleted of

$CD8^+$ cells. $CD8^-$ cells were then restimulated in vitro. Cells were restimulated, stained and analyzed by flow cytometry as described in materials and methods. Dot plots show CD4 compared with cytokine staining. The negative cell population (lower left population) was defined using cells that were stained with appropriate isotype-matched negative control antibodies. The percentage of $CD4^+$ T cells that stained positive for the cytokine indicated (IL-2, IFN-γ or IL-4) are shown in the upper right-hand corner of each dot plot. Samples correspond to samples shown in Figure 3B.

3B: Cytokine release into supernatants of $CD8^-$ spleen cells restimulated with FVIII or buffer. Spleen cells were obtained from hemophilic mice treated as indicated.

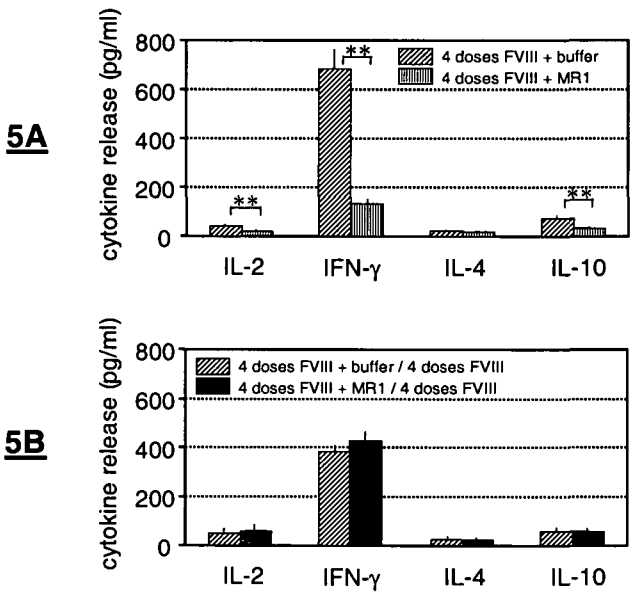
Figure 4



Two-dimensional dot plots showing intracellular cytokine staining in CD4$^+$ T cells after restimulation with human FVIII. CD8$^-$ spleen cells were obtained from mice treated as indicated and restimulated in vitro with FVIII. Cells were restimulated, stained and analyzed by flow cytometry as described in materials and methods. Dot plots show CD4 compared with cytokine staining. The negative cell population (lower left population) was defined using cells that were

stained with the appropriate isotype-matched negative control antibodies. The percentage of $CD4^+$ T cells that stained positive for the cytokine indicated (IL-2, IFN-γ or IL-4) are shown in the upper right-hand corner of each dot plot.

Figure 5



Cytokine release into supernatants of $CD8^-$ spleen cells restimulated with FVIII. $CD8^-$ spleen cells were obtained from hemophilic mice treated as indicated and in vitro restimulated with FVIII.

** significant differences in cytokine release ($P<0.01$)

Table 1.
Median anti-FVIII antibody titers of different IgG-subtypes in hemophilic mice (n=10 mice per group)

Treatment schedule	Anti-FVIII antibody titers		
	IgG1	IgG2a/b	IgG3
4 doses of FVIII and buffer DPBS	20480	1280	1280
4 doses of FVIII and anti-CD40L antibody MR1	negative	negative	negative
4 doses of FVIII and buffer plus 4 doses of FVIII only	163840	10240	20480
4 doses of FVIII and MR1 plus 4 doses of FVIII only	20480	960	1830

negative: no detectable antibody titer

4.3.5 DISCUSSION

The CD40/CD40L interaction is a key event in the initiation of humoral immune responses against T-cell-dependent antigens (review in Ref. 11). Previous studies have shown that a blockade of CD40/CD40L interactions can achieve prolonged survival of allografts in rodents and in monkeys (14-16) and prevent graft versus host disease (24-26) and autoimmunity in rodent models (27-29). These effects are probably due to the induction of tolerance in the $CD4^+$ T-cell population (17). Based on these results, it was tempting to speculate that anti-CD40L antibodies might be able to induce a lasting T-cell tolerance against FVIII in hemophilia A. As the formation of anti-FVIII antibodies is T-cell dependent, the induction of FVIII-specific T-cell tolerance should prevent the formation of anti-FVIII antibodies.

Recently, Qian et al. showed that a blockade of the CD40/DC40L interactions prevented the formation of anti-FVIII antibodies in hemophilic E-16 mice after treatment with human FVIII but did not induce lasting immune tolerance to FVIII (10). The importance of CD40/CD40L interactions for the induction of anti-FVIII immune responses using hemophilic E-17 mice was further evaluated. I focused my attention in particular on the induction of FVIII-specific $CD4^+$ T cells and the Th1/Th2 polarization of these cells. Using a different treatment schedule from Qian et al., I was able to confirm their results and in addition show that (1) the prevention of anti-FVIII antibody formation in hemophilic mice is accompanied by a suppression of FVIII-specific T-cells which is not long lasting (Figs. 4, 5) and (2) the initial treatment of hemophilic mice with blocking anti-CD40L antibodies 24 h before FVIII does not induce lasting alterations in the Th1/Th2 polarization of FVIII-specific T cells (Figs. 4, 5A) and does not alter the isotype distribution of anti-FVIII antibodies (Table 1). I found that after an initial suppression of FVIII-specific T cells, T cells are induced during subsequent challenges with FVIII when the blocking anti-CD40L antibody is omitted (Figs. 4, 5). The difference in results between this study and the studies in models of allograft transplantation, graft versus host disease and autoimmunity might be due to the different nature of the antigens involved. The mechanisms which regulate the induction of immune tolerance against a humoral protein antigen like FVIII might be different from those which activate tolerance in models of

allograft transplantation or autoimmunity. In a recently published paper Rossi et al. (30) suggested that the treatment of hemophilic E-16 mice with FVIII accompanied by anti-CD40L antibodies might induce lasting immune tolerance against FVIII under certain circumstances in some animals. The data published by Rossi et al. do not, however, indicate if the immune tolerance against FVIII is really long lasting under conditions of frequent treatment with FVIII. Rossi et al. used a very specific treatment schedule that is probably not applicable for the treatment of patients. Nevertheless, it would be interesting to evaluate their treatment protocol in hemophilic E-17 mice.

The development of anti-FVIII immune responses in hemophilic E-17 mice treated with human FVIII involves a dominant activation of IFN-γ-producing T cells (Fig. 3). The high percentage of $CD4^+$ T cells that stain positive for intracellular IFN-γ after in vitro restimulation with FVIII (Figs. 3, 4) suggests a bias towards the Th1 class although these results cannot be considered as quantitative. Whether the induction of a surprisingly high percentage of IFN-γ-producing $CD4^+$ T cells after four and eight doses is a unique feature of FVIII or is due to the human origin of the FVIII that was used for these studies in mice needs to be further investigated. Previously, a predominance of IgG1 antibodies during the anti-FVIII immune response in hemophilic mice has been shown and it is therefore concluded that the immune response might be mainly Th2-dependent (8). These results presented here confirm that all IgG-subclasses are involved in the anti-FVIII antibody response (Table 1). The analysis of FVIII-specific T cells shows, however, a possible bias towards Th1 cells (Fig. 3) although the parallel activation of Th2 cells cannot be excluded. Recently published reports by Smith et al. (31) and Faquim-Mauro et al. (32) have indicated that the production of mouse IgG1 is not entirely dependent on Th2 cells but can also be stimulated by Th1 cells. These results on Th1/Th2 polarization of FVIII-specific $CD4^+$ T cells therefore agree with the findings on the IgG-subclass distribution of anti-FVIII antibodies.

It is concluded that the blockade of CD40/CD40L interactions during the treatment of hemophilic E-17 mice with human FVIII completely prevents the development of anti-FVIII antibodies and suppresses the induction of FVIII-specific T cells. The initial blockade of co-stimulatory interactions is, however, obviously not sufficient to induce a lasting immune tolerance against FVIII. The

initial immune suppression is abolished after the omission of the blocking anti-CD40L antibody in subsequent challenges with FVIII.

Further investigations are in progress to find new strategies that will facilitate the induction of FVIII-specific immune tolerance in patients with FVIII inhibitors.

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5. DISCUSSION

These and other studies in animal models of murine hemophilia A demonstrated that the induction of anti-FVIII antibodies is T-cell dependent and involves a complex interaction between different subsets of factor VIII-specific helper T cells that might stimulate or down-modulate the anti-factor VIII immune response (1-5). My data on the kinetics of factor VIII-specific T cells revealed an unexpectedly high percentage of IL-10 secreting cells. Cells that secrete IL-10 have been shown to play an important role in T cell regulation and are negative regulators of dendritic cell maturation (6, 7). My current efforts are therefore focused on a further characterization of IL-10 secreting cells, especially on their functional importance. Furthermore I investigate the role of regulatory T cells and I am trying to explain their importance in the regulation of anti-factor VIII immune response.

As mentioned already in the introduction part, DCs play a central role in controlling immune responses, as initiators of either T cell activation or T cell tolerance. As the functional activity of DCs, and therefore the outcome of the immune response, is in part determined by their maturation state I am currently working on generating semi-mature DCs from bone marrow precursor cells for immune tolerance induction in our hemophilic mice. These in vitro generated semi-matured DCs are primed with recombinant human factor VIII and TNF-α and transferred into naïve E-17 mice. Afterwards mice are immunized and the outcome of the cellular immune responses is characterized. Preliminary results indicate that this approach does not seem to be suitable to induce factor VIII-specific immune tolerance. The injection of semi-mature DCs loaded with factor VIII does not seem to induce an alteration in the pattern of factor VIII-specific T cells induces by treatment of mice with factor VIII. Additionally, no reduction in inhibitor formation or any change in IgG subclass distribution is detectable. The main problem with this approach is that one cannot predict the maturation state of DCs after in vitro generation. Furthermore, the influence of the factor VIII antigen on the maturation of the DCs, the time of priming with factor VIII and the optimal time points to start with immunization after transfer is not known exactly and has to be studied in detail if this strategy is to be used in future.

Specifically targeting factor VIII antigen to immature DCs using an anti-DEC-205 mAb (a DC-restricted endocytic receptor), as described by Mahnke et at (8), might be more successful. Anti-DEC-205 antibody linked to antigen was shown to be efficiently internalized and delivered to antigen processing compartments in DCs without inducing maturation. This offers a new therapeutic opportunity for tolerance achievement via inducing regulatory T cells in vivo.

Another strategy to alter the immune response to FVIII that I currently investigate is the alteration of the local cytokine environment immediately prior to immunization. The development of $CD4^+$ T cells into either Th1 or Th2 cells determines the outcome of an immune response, and is primarily directed by cytokines. By blocking Th1 associated cytokines with specific antibodies and administration of Vitamin D_3 we try to skew the Th1 driven cytokine response in hemophilic mice towards a Th2 driven response. Vitamin D_3 in its active form, 1α, 25-Dihydroxyvitamin D_3, is a potent immunomodulatory agent and has been shown to affect Th cell polarization by inhibiting Th1 (IFN-γ production) and augmenting Th2 development (IL-4, -5, and -13 production) (9) as well as by suppressing DC maturation (10). Preliminary results of my investigations indicate that the anti-factor VIII immune response in hemophilic E-17 mice can indeed be shifted towards a Th2 cell response as a significant increase in IL-10 secreting cells and an increase in IL-4 secreting cells together with a decrease of IFN-γ producing cells is seen after application of factor VIII together with vitamin D_3.

Recently a study in hemophilia A patients with and without FVIII-inhibitors was started to identify and characterize FVIII-specific T cell subsets in hemophilia A patients. The identification and functional characterization of different factor VIII-specific T cell subsets in patients will help to understand the regulation of the induction and maintenance of factor VIII inhibitors. Obtained results will form the basis for developing new strategies for inducing immune tolerance against factor VIII in hemophilia A patients. Furthermore, it is expected that the results could be used to develop diagnostic tools to monitor the state of immune tolerance during immune tolerance therapies.

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6. PUBLICATIONLIST

6.1 MANUSCRIPTS

6.1.1 Birgit M Reipert, Maria Sasgary, Christina Hausl, Elisabeth Maier, Rafi U Ahmad, Peter L Turecek and Hans P Schwarz (2003): Murine models for the study of factor VIII inhibitors. *Hematologica, in press*

6.1.2 Sasgary, M. et al. Single cell analysis of factor VIII-specific T cells in hemophilic mice after treatment with human factor VIII. *Thromb.Haemost 2002; 87/2: 266-72*

6.1.3 Reipert BM, Sasgary M, Ahmad RU, Auer W, Turecek PL, Schwarz HP. "Blockade of CD40/CD40 ligand interactions prevents induction of factor VIII inhibitors in hemophilic mice but does not induce lasting immune tolerance." *Thromb.Haemost 2001; 86/6: 1345-52*

6.1.4 Hausl, C., M. Sasgary, R. U. Ahmad, H. P. Schwarz, E. Muchitsch, P. L. Turecek, F. Dorner, and B. M. Reipert. 2003. High dose factor VIII inhibits restimulation of factor VIII-specific memory B cells; *Manuscript in preparation*

6.1.5 Marie T Stellamor, Birgit M Reipert, Matthias Poell , Maria Sasgary, Siegfried Reipert, Klaus Zimmermann, Fritz Scheiflinger, Hartmut Ehrlich Friedrich Dorner and Hans P Schwarz . 2003 Human polyspecific IgG preparations (IVIGs) inhibit Fas-ligand-induced apoptosis. *Manuscript submitted to J Immunol.*

6.1.6 Thon V, Wolf HM, Sasgary M, Litzman J, Samstag A, Hauber I, Lokaj J, Eibl MM. Defective integration of activating signals derived from the T cell receptor (TCR) and costimulatory molecules in both CD4+ and CD8+ T lymphocytes of common variable immunodeficiency (CVID) patients. *Clin Exp Immunol. 1997; 110 (2): 174-81*

6.1.7 Fischer MB, Wolf HM, Hauber I, Eggenbauer H, Thon V, Sasgary M, Eibl MM. Activation via the antigen receptor is impaired in T cells, but not in B cells from patients with common variable immunodeficiency. *Eur J Immunol. 1996; 1: 231-7.*

6.2 ABSTRACTS

6.2.1 Sasgary M, Ahmad RU, Schwarz HP, Turecek PL and Reipert BM. Dominance of IFN-g and IL-10-producing T cells in the regulation of the anti-factor VIII (FVIII) immune response in hemophilic mice treated with FVIII. *FASEB J 2002; 16, A719 (Abstract)*

6.2.2 Sasgary M, Ahmad RU, Schwarz HP, Turecek PL and Reipert BM . Kinetics of factor VIII-specific T cells in hemophilic mice treated with human factor VIII. *Thromb Haemost 2001; 86, Suppl, P2921 (Abstract).*

6.2.3 Reipert BM, Sasgary M, Ahmad RU, Turecek PL, Schwarz HP. Blockade of CD40/CD40 ligand interactions inhibits the activation of factor VIII-specific T-cells in hemophilic mice. *FASEB J 2001; 15, A349 (Abstract).*

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6.2.6 Stellamor MT, Reipert BM, Poell M, Reipert S, Sasgary M, Schwarz HP. Biological activity of antibodies against members of the TNF receptor superfamily that are present in normal human IgG. *FASEB J 2002; 16, A1247 (Abstract)*

6.2.7 Stellamor MT, Reipert BM, Sasgary M, Reipert S, Schwarz HP. Modulation of apoptosis by anti-Fas antibodies present in human plasmatic IgG *Immunology 2002; 104, Suppl. 1, 73 (Abstract)*

7. CURRICULUM VITAE

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1999-06/99	BAXTER AG, Department: Qualtiy assurance Implementation of change control process
from 07/99	BAXTER AG, R&D, Department Immunology Immunological studies on Hemophila A and inhibitor development, Characterization of anti-Fas Antibodies in intravenious immunglobuline (IVIG)
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