DISSERTATION

Modulation of Factor VIII-specific Memory B cells by Toll-Like Receptor Ligands

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Kurzfassung

Haemophilie A ist eine Blutgerinnungsstörung, welche durch das Fehlen von funktionellem Gerinnungsfaktor VIII (FVIII), einem wichtigen Protein der Blutgerinnungskaskade, charakterisierst ist. Die Standardbehandlung von Haemophilie A Patienten besteht in der intravenösen Substitution mit plasmatisch oder rekombinant gewonnenen FVIII Produkten. Eine schwerwiegende Komplikation in der Behandlung stellt die Bildung von neutralisierenden Antikörpern gegen FVIII dar. Sobald eine solche Immunantwort etabliert ist, werden die interferierenden Antikörper durch memory B Zellen ständig ergänzt und machen so eine Substitutionstherapie unwirksam. Eine Aufklärung der Mechanismen, die diese Zellen kontrollieren, stellt somit den Schlüssel für neue Therapieansätze dar.

In der vorliegenden Arbeit wurde der Einfluss von Toll-Like Rezeptoren (TLR) auf die Restimulation von FVIII spezifischen memory B Zellen untersucht. Es konnte im Mausmodell gezeigt werden, dass der Großteil der untersuchten Liganden für TLR einen deutlichen Einfluss auf die Restimulation von memory B Zellen hat. Besonders Liganden für TLR7 und TLR9 zeigen einen großen bi-phasischen Einfluss auf memory B Zellen: während sie bei niederen Dosen die Restimulation verstärken, zeigen sie bei höheren Dosen einen inhibierenden Effekt, *in vitro* ebenso wie *in vivo*. Weiters haben TLR Liganden die Fähigkeit, die bei hohen Dosen von FVIII beobachtete Inhibierung der Restimulation von FVIII spezifischen memory B Zellen aufzuheben. Alle beschriebenen Eigenschaften von TLR Liganden traten nur unter gleichzeitigem Vorhandensein des spezifischen Antigens FVIII auf. Weiters konnte gezeigt werden, dass die Effekte aufgrund direkter Interaktion von Ligand und dessen Rezeptor zustande kommen.

Zusammenfassend stellen die gefundenen Effekte einen weiteren Baustein im vollständigen Verständnis der Bildung von Antikörpern gegen FVIII dar, welches für die Entwicklung effektiverer Therapien unerlässlich ist.

1. Abstract

Hemophilia A is a bleeding disorder caused by the lack of functional coagulation Factor VIII (FVIII), an important protein of the coagulation cascade. The standard treatment of Hemophilia A patients is intravenous replacement therapy with plasmatic or recombinantely derived FVIII.

The formation of neutralizing antibodies to Factor VIII (FVIII) is a major complication in the treatment of hemophilia A patients. Once an immune response to FVIII has evoked, the pool of interfering specific antibodies is constantly replenished by memory B cells and makes treatment ineffective. The understanding of the mechanisms controlling these cells is the key for possible effective new treatments.

In the present thesis we elucidate the critical influence of Toll-like receptors (TLR) on FVIII-specific memory B cell re-stimulation. We could demonstrate that most examined ligands for TLR show a significant influence on the memory response in a hemophilic mouse model, in vitro as well as in vivo. Especially ligands for TLR7 and TLR9 affect FVIII-specific memory B cells in a dose-dependent, bi-phasic manner: while they strongly amplify their re-stimulation at lower doses, they show a significant inhibiting effect at higher doses, in vitro as well as in vivo.. Furthermore, TLR ligands are capable of overriding the known suppressive properties of high doses of FVIII to memory B cell response. All observed properties of TLR ligands were dependent on the concurrent presence of the specific antigen FVIII and were demonstrated to be effective due to the specific interaction of the TLR ligand with its corresponding receptor.

We conclude that our findings are a further step to better characterize the poorly known mechanisms of inhibitor formation in Hemophilia A, whose understanding is a prerequisite for more effective therapies.

2. Introduction

2.1. Hemophilia A

2.1.1. What is Hemophilia A?

Hemophilia is a group of bleeding disorders caused by the mutation of one of the proteins (coagulation factors) in the blood clotting cascade. This leads to a malfunction of the coagulation system which is needed to stop bleeding after a blood vessel has been injured.

Hemophilia A is the most common variant, caused by an inherited or spontaneous mutation of the coagulation Factor VIII (FVIII). The gene for FVIII is located on the X-chromosome. Hemophilia A belongs to the X-linked recessive hereditary diseases, hence mostly affecting men, with a prevalence of about 1 out of 5,000 to 10,000 men.

Mutations can result either in complete absence of FVIII protein or in a mutated variant of FVIII with no or decreased functionality. The residual functionality of FVIII defines the severity of the disease, which can range from prolonged time for blood coagulation upon injuries in mild cases up to spontaneous, long lasting bleedings without external impact. According to the residual functionality of FVIII, Hemophilia A is clinically classified as follows: [1, 2]

FVIII – Activity in Plasma	**Classification**	**Symptoms**
<1%	severe	Spontaneous joint and muscle bleeding; bleeding after injuries, accidents, and surgery
1 - 5%	moderate	Bleeding into joints and muscles after minor injuries; excessive bleeding after surgery and dental extractions
5 - 30%	mild	Spontaneous bleeding does not occur; bleeding after surgery, dental extractions, and accidents

Table 1: Classification of Hemophilia A

2.1.2. Coagulation Cascade

The blood coagulation pathway consists of a complex proteolytic cascade which involves 13 proteins (coagulation factors) as well as cellular components (e.g. platelets).

2.1.2.1. Haemostasis and coagulation system

Haemostasis describes the overall system of the body to control bleeding of injured blood vessels. It consists of three major parts: first, the constriction of the damaged blood vessel to mechanically reduce the blood flow. Second, an initial mechanical occlusion of the damage by blood cells called platelets or thrombocytes (=primary hemostasis). And finally the formation of a fibrin clot as a result of the blood clotting or coagulation system .

This coagulation system is designed as a proteolytic cascade in which more than a dozen coagulation factors are involved. Each enzyme of the pathway is present in the blood in its inactive precursor form (=zymogen) which on activation undergoes proteolytic cleavage, resulting in the release of the active factor from the precursor molecule. The complexity of the coagulation pathway offers the opportunity for a series of positive and negative feedback loops which control the clot formation process. The ultimate goal of the pathway is to transform liquid blood into a blood clot (thrombus). Normal human haemostasis is provided by two independently functioning coagulation pathways: the extrinsic and the intrinsic pathway, that converge on a single common final pathway resulting in a clot.

2.1.2.2. The intrinsic pathway

The intrinsic pathway, also called contact activation pathway, is initiated in the absence of external trauma by contact of blood with a negatively charged surface such as exposed collagen on the surface of a damaged blood vessel, in vivo, or glass or particulate material such as kaolin or urate crystals, in vitro. Factor XII is activated on interaction with such negatively charged surfaces in the presence of prekallikrein, high-molecular-weight kininogen (HMWK) as well as calcium ions and phospholipids: the co-action of those

factors results in conversion of prekallikrein to kallikrein, which in turn activates factor XII to factor XIIa. FXIIa hydrolyses prekallikrein to more kallikrein, setting up a positive feedback loop. FXIIa hereupon activates factor XI to XIa in the presence of Ca^{++}, furthermore leads to the release of bradykinin (a polypeptide with potent vasodilator and pain-producing action) from HMWK. Factor XIa, again in the presence of Ca^{++} (or of factor VIIa from the extrinsic system), activates factor IX to factor IXa. Factor IXa then cleaves a bond in factor X to change it to its active form, factor Xa. A prerequisite of this activation is the formation of the tenase complex (VIIIa, IXa, X and Ca^{++}) on the surface of activated platelets, initiating the common final pathway. [3]

2.1.2.3. The extrinsic pathway

The extrinsic pathway, also called tissue factor pathway, is the second route for the activation of the clotting cascade. It provides a very rapid response to tissue injury, generating activated factor X almost instantaneously, compared with the seconds, or even minutes, required for the intrinsic pathway to activate factor X. There are two components unique to the extrinsic pathway: tissue factor (or factor III), and factor VII. Tissue factor is present in most human cells bound to the cell membrane, and is a cofactor in the factor VIIa-catalyzed activation of factor X: Once activated, tissue factor binds rapidly to factor VII, which is then activated to form a complex of tissue factor, VIIa, calcium and a phospholipid, and this complex then rapidly activates factor X.[4]

The intrinsic and extrinsic pathways are linked mainly at two points: on the one hand, Factor Xa is able to activate FVII. On the other hand, FVIIa together with tissue factor can activate factor IX.

The intrinsic and extrinsic systems converge at factor X to a single common pathway.

2.1.2.4. Common Pathway

The common pathway comprises the final steps necessary for the formation of a blood clot. Activated Factor Xa, together with activated Factor Va in the presence of Ca++, forms the prothrombinase complex on phospholipids membranes (e.g. on platelets or endothelial cells). FXa is then able to effectively cleave Prothrombin (Factor II) to its active form Thrombin (Factor IIa). Thrombin is a multipotent protein with the main function to cleave Fibrinogen to Fibrin (Factor Ia), a fibrillar protein which finally forms the matrix of the blood clot. [5]

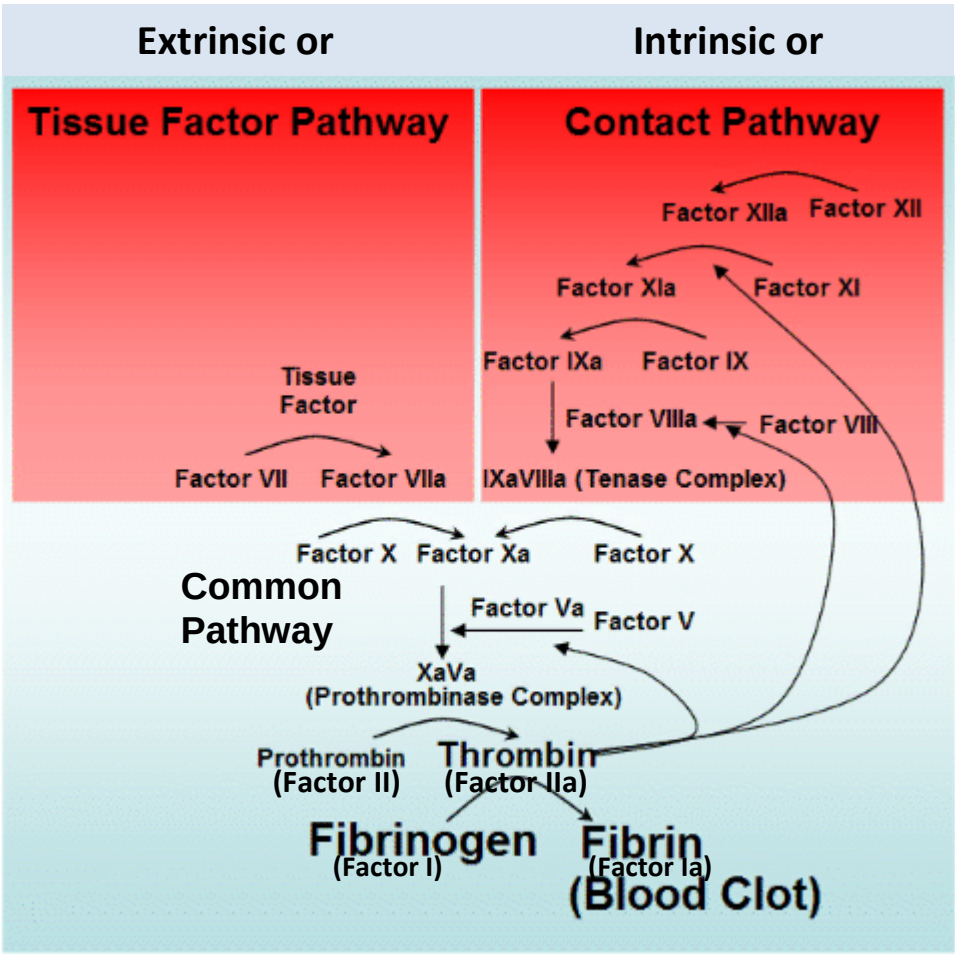


Figure 1: Intrinsic and extrinsic pathway of blood coagulation

Source: www.cardiovascularweb.com, modified

2.1.2.5. Factor VIII (FVIII)

FVIII is a glycoprotein, which is synthesized and released into the circulation by the liver. Additional production sites do exist, but are not yet identified.[6] The gene encoding for FVIII was first discovered on the X-chromosome in 1984 [7] and has a size of about 180 kb. The gene is divided into 26 exons which encode a polypeptide chain of 2351 amino acids. This includes a signal peptide of 19 and a mature protein of 2332 amino acids.[8] The mature FVIII protein is arranged in distinct domains: A1-A2-B-A3-C1-C2. Before the release into the circulation, the protein is proteolytically cleaved into a heavy chain, consisting of A1-A2-B domain and a light chain, consisting of A3-C1-C2 domain.[9] However, the heavy and light chain remains non-covalently associated as a heterodimer through the A1 and A3 domain in a metal-ion-dependent manner. Immediately after its release into the circulation, the heterodimer interacts with Von Willebrand factor, to form a tight, noncovalent complex. Von Willebrand factor plays a dual role: on the one hand, it acts as protective protein to prevent FVIII from early proteolysis. On the other hand it serves as carrier protein to concentrate FVIII on sites of vascular injury due to its property to also bind to subendothelial matrix proteins and adherent platelets.[10] The light chain of FVIII contains the binding sites for von Willebrand factor. The average concentration of FVIII in healthy individuals of about 200 ng/mL blood is sufficient to ensure proper hemostasis. However, FVIII has a very short half-life of about eight hours and therefore FVIII requires permanent replenishment.[10]

Regarding the relatively large size of the FVIII gene with 180kb, the various mutations described are not astonishing. The mutation types of the FVIII gene resulting in less or nonfunctional FVIII leading to Hemophilia A are quite well investigated and can be classified into four major categories: 1) gross gene rearrangements, 2) single DNA base substitutions (point mutations), 3) deletions (ranging from one base-pair up to the entire gene and 4) insertions of DNA of varying size. Almost 50% of the described mutations leading to Hemophilia A are various point mutations, causing “missense” or “nonsense” mutations. The second very common mutation type, contributing to another approx. 35% of Hemophilia A cases, is a gene rearrangement known as “intron-22 inversion”. The intron 22 of the FVIII gene includes a sequence

that has similarity to two sequences distal from the FVIII gene. By intrachromosomal homologous recombination, one of these outside regions forms a crossing-over structure with the corresponding element within intron 22, resulting in an inversion of exons 1–22 with respect to exons 23–26 of the F8 gene and therefore unfunctional gene. [11 12 13.]

2.1.3. Treatment of Hemophilia A patients – Replacement Therapy

Before the early 1960s, when the only treatment for bleeding episodes was the infusion of blood or frozen plasma from healthy donors, patients with severe hemophilia A had a life expectancy of only 25 years. Over the last five decades advances in treatment have permitted a near-normal lifestyle and life-span for many individuals with hemophilia. The first milestone was the discovery that FVIII can be concentrated by cryoprecipitation of plasma.[14] The introduction of such FVIII concentrate products for replacement therapy allowed treatment at home and largely replaced the need for transfusion of whole blood or plasma in the hospital. Unfortunately, a major side effect of the treatment was the high likeliness of infection with blood born pathogens. As the benefits of the treatment were emphasized, the risk of infections like hepatitis was accepted as unavoidable.

This view changed dramatically when it was recognized that the pooled coagulation-factor concentrates transmitted HIV. In the early 1980s about 70% of hemophiliac patients were infected with HIV.[15]

Many efforts were made to eliminate the threat of virus infections due to the use of plasma preparations. This started with the introduction of virus-eliminating production steps like heating or detergent treatment and reached its current peak level with the recombinant production of highly purified coagulation factors by genetic engineering without any compounds of human blood.

The improvements in the production of high quality FVIII concentrates also enabled the possibilities for new treatment regimens for hemophilia A patients. Initially, the focus was put on the cessation of acute bleeding phases “on demand” with the treatment of FVIII concentrates. Soon,

physicians tried to prevent patients from bleeding episodes prophylactic by maintaining a minimal but sufficient FVIII level in the circulation through regular FVIII infusion (usually three times a week). This prophylaxis treatment regimen has proofed over the years to be very effective in preventing hemophiliacs from long-term damages like joint deformations. However, the disadvantages of prophylaxis treatment, namely the high costs and the inconvenience for the patient caused by the frequent infusions, keep the controversial discussion on the optimal treatment regimen vivid.

2.1.4. Treatment of inhibitor patients – Immune Tolerance Induction Therapy (ITI Therapy)

Although most hemophilia patients can use replacement products repeatedly without problems, one of the most problematic complications of treatment is the development of inhibitors to FVIII. [16] [17] These are typically IgG antibodies that neutralize the coagulant effects of replacement therapy. Inhibitors to FVIII occur approximately in 20-30% of severe hemophilia A patients. Although it is known that the interaction of environmental, genetic and immunologic factors contribute to the development of inhibitors [12], the pathophysiology of antibody development has not been completely elucidated.

Studies designed to avoid or modify the immune response and to prepare recombinant factor VIII proteins with reduced immunogenicity are under investigation.

The development of inhibiting antibodies makes prophylaxis treatment ineffective. Furthermore, it raises enormous complications in the controlling of a bleeding event, for patients as well as for physicians.

The treatment of bleeding episodes for patients with inhibitors depends on the inhibitor titer. Low-titer inhibitors can be overwhelmed with FVIII: in case of a serious hemorrhage the goal is to give large enough doses of factor VIII, sufficient to neutralize the inhibitory antibody and provide additional factor VIII to circulate and induce coagulation.

For patients with high-titer inhibitors, other strategies have to be considered, of which the most effective and used are 1) FVIII bypassing agents like

activated prothrombin complex concentrates (FEIBA VH®) or 2) recombinant FVIIa (NovoSeven®). The use of porcine FVIII would be another alternative. Accompanying measures can be immunosuppressive agents like prednisone or cyclophosphamide, the synthetic hormone Desmopressin, the administration of intravenous gammaglobulins and plasmapheresis.[18 19 20]
The disadvantage of these alternative strategies is the need of an experienced hemophilia treatment centre in case of a bleeding. Often more than one approach is needed before bleeding is arrested.[21] The reduced ability to control bleeding especially into the joints, can lead to earlier development of arthritis. Altogether this are less than satisfactory conditions in ensuring good long-term quality of life for patients. Moreover, the treatment costs for such patients are immense.[22 23]

Therefore, the ultimate goal in treating inhibitor patients is the elimination of the inhibitory antibody entirely, allowing the recommencement of FVIII replacement therapy. This can be reached via immune tolerance induction (ITI) therapy, which was first reported to be successful by Brackmann and Gormsen in 1977. [24] In the last 30 years several protocols have been established on this treatment strategy.[25] What all of them have in common is the administration of FVIII at very short time intervals over a longer period. Presently the three mainly used protocols are :

- The Bonn Protocol [24]
Patients are treated with high doses FVIII (100-200 units/kg body weight), usually administered twice a day.

- The Van Creveld Protocol [26]
Patients are treated with low dose regimen. The treatment schedule consist of a “neutralizing dose” of 25-50 units/ kg body weight twice a day for 1-2 weeks and a “tolerizing dose” of 25 units/ kg body weight every other day.

- The Malmö Protocol [27] [28]

The Malmö protocol is not as extensively used as the other two. The protocol combines the treatment of high dose FVIII with cyclophosphamide, high dose immunoglobuline and protein A immunoadsorption.

The Bonn Protocol and the Van Creveld Protocol have a success rate of about 87%, whereas the success rate for the Malmö Protocol was reported to be in a range of 59-83%. [27] [28]

However, the reported success rates are not directly comparable due to different setup of the investigations and definitions of success.

Several attempts have been made to objectify the comparison of results from different protocols, mainly by retrospective surveys like the International Immune Tolerance Registry [29] , the North American Immune Tolerance Registry [30] and the German [31] and Spanish [32] registries with an attempt to collect data on ITI treatment worldwide and observe success rates of Immune Tolerance Induction.

Currently, an international randomized multicenter prospective trial is ongoing to elucidate the prerequisites and proceedings for most effective ITI therapy.[33]

However, even with an overall success rate of ITI therapy of about 75%, there is still a large number of Hemophilia patients with ineradicable inhibitor formation whose medical needs are not satisfyingly met.

2.2. Immune response to a protein antigen

The reasons, why certain hemophilia patients develop antibodies to FVIII and the mode of action of immune tolerance induction are still not completely understood and fields of heavy investigations. However, the key for finding the answers to the open questions is certainly the proper understanding of the underlying mechanisms of the immune system.

2.2.1. T-cell dependent antibody responses

The key players in the development of antibodies to a protein are B and T lymphocytes (T and B cells) together with the assistance of professional antigen presenting cells (APC), like dendritic cells.

Dendritic cells (DCs)

The principal process of the development of a primary immune response to an antigen in form of development of antibodies starts when dendritic cells encounter a potential antigen.

Dendritic cells emerge within the bone marrow and migrate via the blood stream to tissues throughout the body, where they rest in tissues of the periphery as immature DCs.[34] DCs express various receptors for the detection of antigen, including TLRs (see Chapter 2.3). Upon encounter with antigen, e.g. with a foreign protein during infection, dendritic cells in the periphery capture and uptake the antigen and travel from the site of infection to the lymph nodes. The uptake of antigen leads to the maturation of DCs, which includes several significant modulations: Mature DCs turn into very effective antigen-presenting cells (APCs): the cell digests the uptaken antigen and presents the obtained peptide fragments on its surface, complexed with either MHC Class I or MHC class II molecules Such peptide-MHC complexes are recognized by T cells. Furthermore, during maturation of DCs the surface receptors for antigen recognition are downregulated, whereas other surface proteins which are important for the interaction with T cells are upregulated (eg B7-1/B7-2 and ICOS-L). Mature DCs also secrete cytokines, e.g. IL-12, which play an important role in the activation of T cells.

T lymphocytes

The activation of naive T cells in response to antigen, and their subsequent proliferation and differentiation, constitutes a further step in primary immune response.

For primary activation, the most potent activators of naive T cells are mature dendritic cells. From the several existing subsets of T cells, two major compartments are helper T cells (T_H cells) and cytotoxic T cells (T_C cells). They can be distinguished by their expression of characteristic surface molecules and their binding selectivity to MHC molecules: T helper cells express CD4 surface molecules and bind selectively to MHC class II. In contrary, cytotoxic T cells express CD8 surface molecules and bind selectively to MHC class I. All T cells possess a receptor that recognizes peptide-MHC complexes, the T cell receptor (TCR). $CD4^+$ T cells are immune response mediators and play an important role in establishing and increasing the capabilities of adaptive immune response.
Before being able to carry out this role, naïve T cells need activation first.

The primary activation of $CD4^+$ T cells requires at least two signals. The binding of peptide-MHC complexes to the T cell receptor (TCR) and to the CD4 co-receptor provides the first signal.[35] The second signal involves so called co-stimulatory molecules.

One of the best defined co-stimulator is the T cell surface molecule CD28[36] which binds to B7-1 (CD80) and B7-2 (CD86), that are expressed on professional antigen presenting cells (APCs).
Upon activation by this two signals, the T cells starts to proliferate, giving rise to a clone of a large number of cells that all bear the same receptor for antigen. Additional signals given by cytokines released from the APC have influence on the differentiation of the CD4 T cell into one of the different effector subsets. Of these subsets, the compartments of T_H1 and T_H2 cells are especially important for the next step in the development of antibodies, which is the activation of B cells.

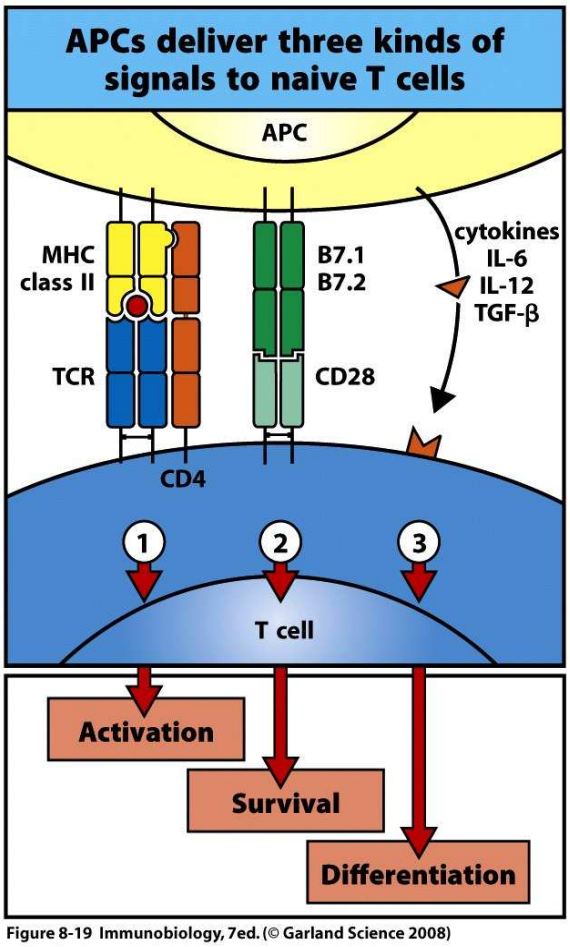


Figure 2: Activation of T cells: Interactions between antigen presenting cell (APC) and T cell.

Binding of the foreign-peptide:self-MHC complex by the T-cell receptor and, in this example, a CD4 co-receptor, transmits a signal (arrow1) to the T cell that antigen has been encountered. Effective activation of naïve T cells requires a second signal (arrow 2), the co-stimulatory signal, to be delivered by the same antigen-presenting cell (APC). IN this example, CD28 on the T cell encountering B7 molecules on the APS delivers signal 2, whose net effect is the increased survival and proliferation of the T cell that has received signal. ICOS and members of the TNF receptor family may also provide co-stimulatory signals. For CD4 T cells in particular, different pathways of differentiation produce subsets of effector T cells that carry out different effector responses, depending on the nature of a third signal (arrow 3) delivered by the APC. Cytokines are commonly, but not exclusively, involved in directing this differentiation .

Source: Janeway's Immunobiology, 7th Ed., 2008, Garland Science

B lymphocytes

The main effector function of B cells is, after terminal differentiation into a plasma cell, the production of antigen-specific antibodies.

B cells attain full maturity in the bone marrow, subsequently they leave the marrow, enter the circulation and populate the lymphoid organs. The mature cells are called naïve B cells. Their function is to recognize antigens and initiate adaptive immune responses.

Similar to the situation in T cells, also B cell activation requires two independent signals.[37] Usually these two signals are the binding of the antigen to the B cell receptor (BCR) and secondly the interaction with an helper T cell which was activated by the same antigen than the B cell, a so called cognate T cell.

The activation of antigen-specific B cell is initiated by the binding of antigen to membrane immunoglobulin (Ig) molecules, which are the antigen receptors of mature, naïve B cells (BCR). The BCR is of the same antigen-specificity than the antibodies that will finally be produced by this cell. However, due to the different nature of BCR and TCR, antigens are recognized in different ways: while BCR recognizes epitopes on an intact whole antigen, the TCR only recognizes the same antigen when it is previously processed and presented in form of small peptides on the MHC molecule of an antigen presenting cell to the TCR.

The B cell receptor has two important tasks in B cell activation: On the one hand it provides the first signal for activation of the cell when it binds antigen. On the other hand it internalizes the antigen, processes it and displays the resulting peptide fragments on the B cell surface bound to MHC class II molecules, similar to dendritic cells and making B cells to effective antigen presenting cells.

Activated helper T cells are now capable of recognize the peptide-MHC complex and deliver the second activating signals to the B-cell.

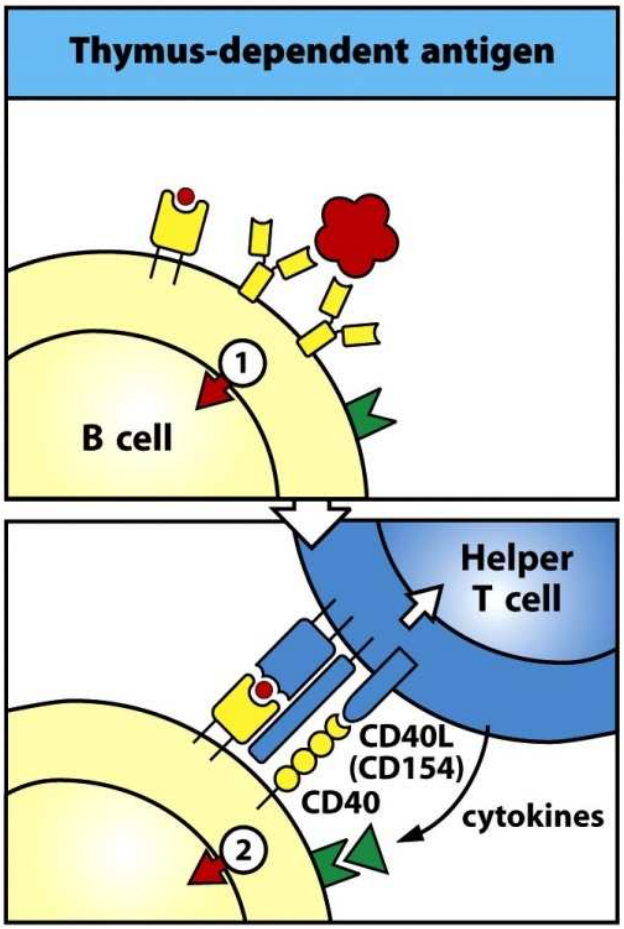


Figure 9-2 Immunobiology, 7ed. (© Garland Science 2008)

Figure 3:Two signals are required for B-cell activation.

The first signal is delivered through is antigen receptor (top panel).The second signal is delivered by an activated helper T cell.The specific interaction of an antigen-binding B cell with a helper T cell leads to the expression effector molecules by the T cell , like the B-cell stimulatory molecule CD40-Ligand on the helper T-cell surface and B-cell stimulatory cytokines, The interaction between CD40 on B cells and CD40-Ligand on T cells plays an important role for B cells in the initiation of B cell affinity maturation and isotype switching in response to T cell dependent antigens.

Source: Janeway's Immunobiology, 7th Ed., 2008, Garland Science

An activated B cell undergoes affinity maturation, clonal expansion isotype switching and differentiation

These maturation steps take place in the germinal centers of the secondary lymphoid organs and maximize quality and quantity of the antibody formation. Affinity maturation is achieved through somatic hypermutation of the V-regions or immunoglobulin genes and thereof resulting point mutations in the variable region of the antibody until highest possible affinity is reached by positive selection: B cells that bind antigen with high affinity are selected to survive. B cells that do not express high affinity receptors for antigen undergo programmed cell death.

The class switch from the rather low affinity IgM to the high-affinity IgG, IgA or IgE type has major effects on the effector function of the antibodies During clonal expansion, the survivors of this antigen-driven selection process undergo further cycles of replication to produce still more cells of the same antibody specificity.
Once these processes are completed, the activated B cell chooses – by mechanisms which are so far largely unknown - between two pathways. Either it differentiates into an antibody-producing plasma cell or into a memory B cell.

Plasma cells

Many of the B cells differentiate into antibody-secreting plasma cells that are morphologically distinct B cells committed to abundant antibody production. Plasma cells develop in lymphoid organs and subsequently migrate to the bone marrow, where they are supposed to persist for long periods and continuously produce antibodies. Plasma cells are rarely found in the peripheral blood. They comprise from 0.2% to 2.8% of the bone marrow white cell count.[38] A single B cell may give rise to ~4000 antibody secreting plasma cells, within a week.

Memory B cells

Memory B-Cells are responsible for the secondary immune response. Responses to second and subsequent exposures to the same antigen, are usually more rapid, larger and often qualitatively different from the primary immune response. The secondary response is dependent on a population of long-lived B memory cells. These cells are generated in lymphoid tissue after B cell activation and proliferation and reside in the bone marrow, lymph nodes and spleen where they are supposed to have long life span. They express high affinity surface immunoglobulins which enable them to be activated by lower levels of antigen than naive B cells.

Immunological memory for a given antigen can be carried for many years by long-lived B cell clones.

2.2.2. T-cell independent antibody responses

Although peptide-specific helper T cells are required for B cell responses to protein antigens, many microbial constituents, such as bacterial polysaccharides, can induce antibody production in the absence of helper T cells. These microbial antigens are known as thymus-independent or T cell independent antigens because they can induce antibody responses in individuals who have no T cells. There are two ways in which the second signal required to activate B cell and thus for antibody production can be provided.[39]

One way is a direct activation by receptors allocated to the innate immune system, e.g. Toll-Like Receptors (TLR). These receptors are capable of recognising common, widely distributed microbial constituents, also called PAMPs (pathogen-associated molecular pattern) and are therefore members of the group of Pattern Recognition Receptors (PRRs). An important example of such a PAMP that is recognized via TLR is the lipopolysaccaride (LPS) from the outer membrane of gram-negative bacteria often referred to as Endotoxin. At very high concentrations such molecules cause the proliferation and differentiation of most B cells regardless of their antigen

specificity; this is known as polyclonal activation, giving such substances also the name B cell mitogens.

A second way for T-cell independent B cell activation exists with molecules that have highly repetitive structures, such as bacterial capsular polysaccharides. Such antigens act by simultaneously cross-linking a critical number of B cell receptors of mature B cells specific for the antigen. There is also evidence, that with the help of dendritic cells, a class switch from IgM to IgG can occur independently from T cells.

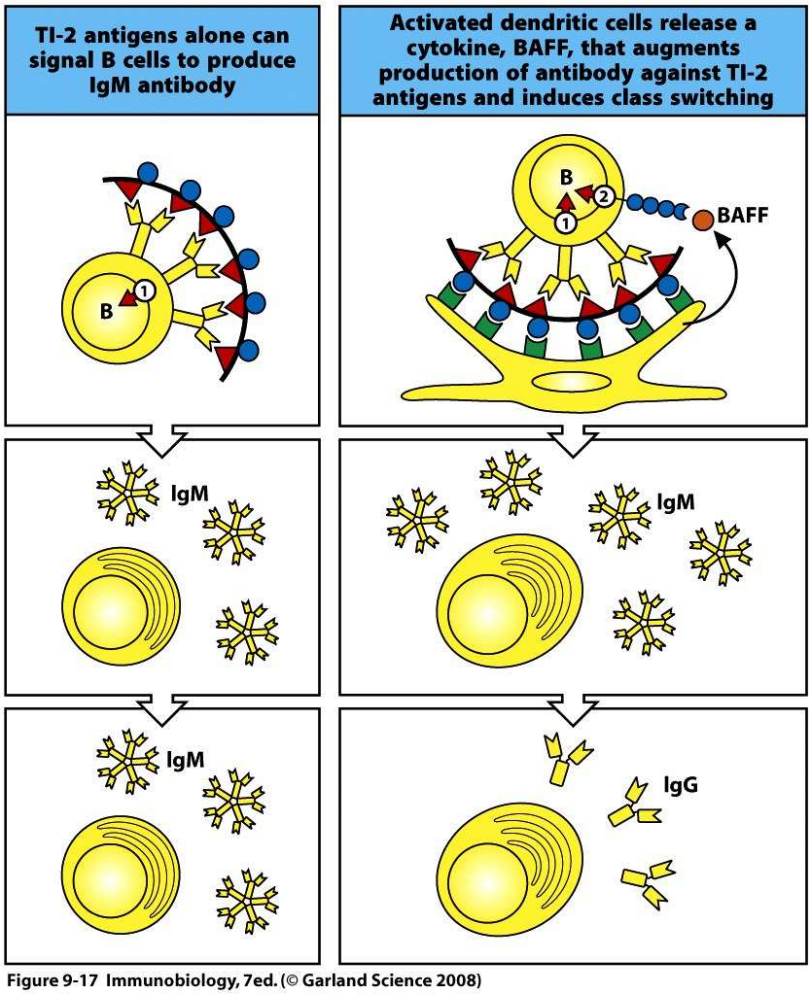


Figure 4: B-cell activation by thymus-independent type 2 antigens (TI-2 antigens).

Multiple cross-linking of the B-cell receptor by TI-2 antigens can lead to IgM antibody production (left panels).

There is evidence that in addition cytokines greatly augment these responses and lead to isotype switching as well (right panels).

Source: Janeway's Immunobiology, 7th Ed., 2008, Garland Science

2.3. Toll-Like Receptors

2.3.1. Introduction on Toll-Like Receptors

Recognition of microbial infection and initiation of host defense responses is of utmost importance to the organism and controlled by multiple mechanisms. The defense mechanisms of the immunological system can generally be divided into two main groups: Innate immunity (also called native immunity) consists of inherited cellular and biochemical defense mechanisms that are invariable and in place even before infection. In contrast, adaptive is specific for different microbial and nonmicrobial antigens and is increased by repeated exposures to the same antigen (immunological memory).[40]

Toll-Like Receptors are members of the innate immune system. At the end of the 20th century, Toll was shown to be an essential receptor for host defense against fungal infection in Drosophila, which only has innate immunity.[43] One year later, a mammalian homolog of the Toll receptor, now termed Toll-Like Receptor (TLR), was shown to induce expression of genes involved in inflammatory responses. These findings have made innate immunity a very attractive subject of research, and in recent years there has been rapid progress in our understanding that the innate immune system possesses a skilful system that senses invasion of microbial pathogens by TLRs. Furthermore activation of innate immunity is a critical step to the development of antigen-specific acquired immunity.[41]

As mentioned earlier, TLRs belong to the class of pattern recognition receptors (PRR) which are capable of recognising a limited set of highly conserved molecular patterns (PAMPs) that are unique to the microbial world and invariant among entire classes of pathogens.[42]

The TLR-familiy is the best characterized class of PRRs in mammalian species and consists of at least 11 members. On comparison of human and murine TLRs, TLR1-9 are conserved between the human and mouse. TLR10 in functional in the human and non-functional in mouse, in case of TLR11 it is the other way round.[43] Additionally, TLR12 and TLR13 are known in the murine system.[44]

2.3.2. Specificity of TLRs

TLRs are capable to detect multiple PAMPs, however each TLR with specificity to a certain subclass: for example, TLR4 recognises LPS; TLR5 recognises for flagellin (a compound specific to bacterial flagellae), TLR7 is activated by single stranded viral RNA.

TLRs 1,2,4,5 and 6 specialize in the recognition of mainly bacterial products that are unique to microbes and not made by the host.

TLRs 3,7,8 and 9 in contrast specialize in recognition of nucleic acids, which are not specific to the microbial world. In this case, self/non-self discrimination is mediated not so much by the molecular nature of the ligands as by their accessibility to the TLRs: These TLRs are localized to intracellular compartments and detect nucleic acids in lysosomes. Because the host's nucleic acids are not normally accessible in these compartments, they do not trigger TLRs.

2.3.3. Signaling pathways of TLRs

TLRs represent transmembrane-signaling receptors. Their extracellular domain includes a repetitive structure rich in leucine residues, called leucine-rich repeat (LLR), that is involved in the ligand recognition. The intracellular region contains a common structure in both, TLR as well as Interleukin-1 (IL-1) receptor family members, therefore called Toll/IL-1 receptor homologous (TIR) domain, which is essential for signal transduction. Generally, activation of TLRs leads to the activation of several transcription factors, including NF-κB and IRFs. Subsequently, expression of a variety of immune response genes is induced.[44]

Two major signaling pathways via TLR can be distinguished, characterised by the first adaptor molecule downstream from the TIR-domain, which starts the subsequent signaling cascade. One of those adaptor molecules is MyD88, the second one is TRIF.

It is not surprising that he first pathway discovered was the MyD88 dependent cascade, as it is analogous to the well described signaling pathway in IL-1 receptors. MyD88 can adapt to the TIR-domains of all TLRs

except of TLR3.[44]. Activation of the MyD88 cascade leads eventually to an activation of transcription factor NF-κB, resulting in the production of pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-8, TNF-α). Recently it was discovered that stimulation of TLR7 or TLR9 in plasmacytoid dentritic cells results in activation of an unique MyD88 dependent pathway leading to induction of Typ I Interferons (IFN-α/IFN-β).[41] They are therefore also called Type I IFN producing cells.[45]

The second major pathway, which is accordingly named MyD88 independent pathway or TRIF pathway, is used by TLR3 and can also be induced by TLR4 alternatively to the MyD88 dependent pathway. It was discovered by studying MyD88-deficient mice, which still showed the capability of responding – albeit slower - to LPS, indicating a second possible way of activation. Induction of the TRIF-pathway leads finally also to the activation of either NF-κB and pro-inflammatory cytokines or to the secretion of Typ I Interferons, mainly IFN-β.[41]

A very recent discovery is the feature of TLR4 to traffic into the cell upon activation and act as intracellular receptor. This could explain its ability of inducing the TRIF pathway, analogous to intracellular receptor TLR3.[46] [47]

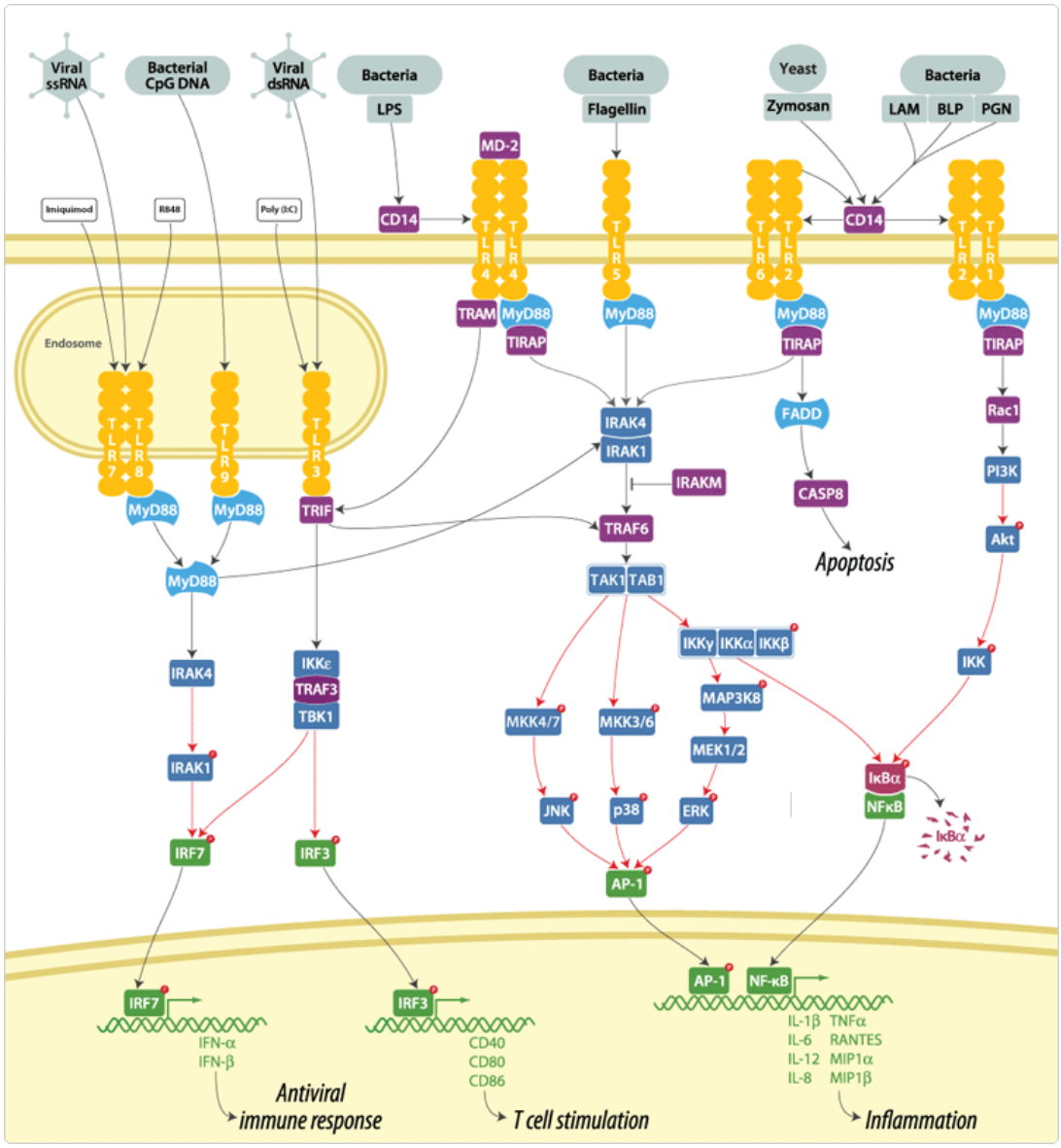


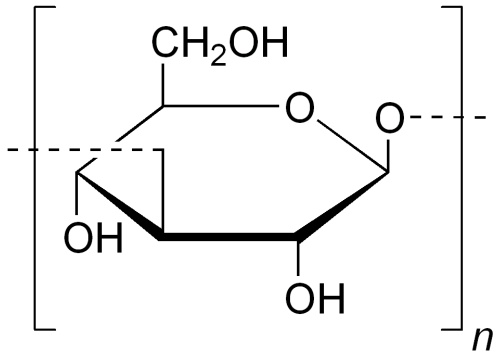
Figure 5: Overview Toll Like Receptors (TLRs)

Source: www.invitrogen.com

2.3.4. Inidividual properties of TLRs

2.3.4.1. TLR2

TLR2 is expressed extracellularily has the capability of recognizing PAMPs from various microbes. Important examples are lipoproteins from Gram-negative bacteria, peptidoglycans and lipoteichoic adic from Gram-positiv bacteria and also the glucan zymosan which is a cell wall compound of fungi such as Saccharomyces sp.



β-1,3

Figure 6: Chemical Structure of Zymosan

Glucans, like Zymosan, are polysaccharides consisting of D-glucose monomers linked by glycosidic bonds. The many different types of glycosidic bonds allow a large variety of resulting molecules. Zymosan is characterized by β-1,3-bonds.[48]

Source of structure formula: http://en.wikipedia.org/wiki/Zymosan

The mechanism by which TLR2 recognizes such a surprising wide variety of microbial components is now explained by the fact that TLR2 cooperates with other TLRs such as TLR1 and TLR6 to discriminate between the specific patterns.[41]

2.3.4.2. TLR3

TLR3 is expressed intracellularily and recognises double-stranded RNA (dsRNA) which is produced by most viruses during their replication. TLR3 differs from the other TLRs in that it has an unique signaling cascade independent from the adaptor molecule MyD88, resulting in the induction of type I interferons (IFN-α/IFN-β). These chemokines exert antiviral and immunostimulatory activities, including the transcription of some IFN-inducible genes and maturation of dendritic cells.[41] Furthermore, TLR3 has been shown to promote crosspresentation of virus in DCs through viral dsRNA-mediated activation of the DCs.[49] Thus TLR3 is crucial in the detection and defense of viral infections.

Poly I:C (polyinosinic:polycytidylic acid) is known to trigger TLR3 and was used for our experiments.Poly I:C is a mismatched double-stranded RNA with one strand being a polymer of inosinic acid, the other a polymer of cytidylic acid. It is structurally similar to dsRNA and can therefore be considered as a synthetic analog of double-stranded RNA. [50]

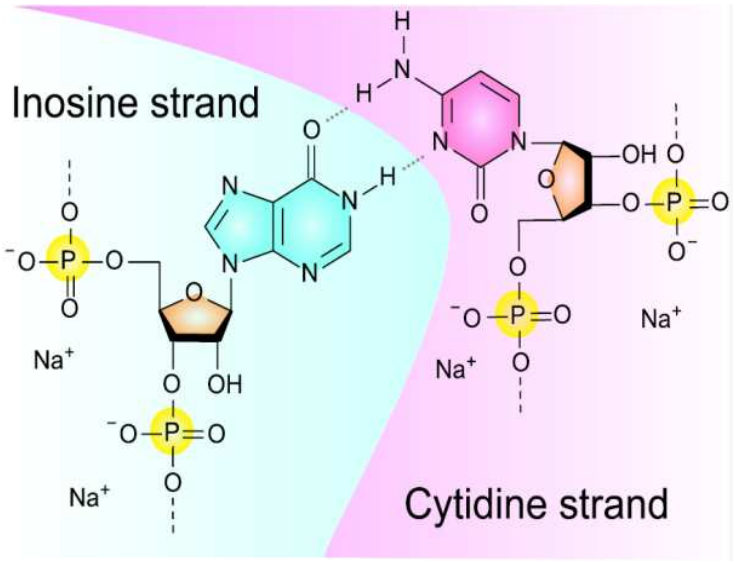


Figure 7 Structure of Poly (I:C)

Source: http://en.wikipedia.org/wiki/Poly_I:C

2.3.4.3. TLR4

TLR4 is an extracellular receptor with a recently detected ability of trafficking to intracellular endosomal compartments. TLR4 is one of the best examined and described TLRs due to its important function in detecting Lipopolysaccharide (LPS). LPS (also referred to as Endotoxin) is a major component of the outer membrane of Gram negative bacteria and shows potent immune-stimulatory activity. Excessive activation of monocytes and macrophages by LPS leads to endotoxic shock, a systemic disorder with a high mortality rate in humans and thus of great medical interest.
Despite of LPS, TLR4 seems to be implicated in immunological reactions to endogenous ligands, such as heat shock proteins or hyaluronic acid. [41]

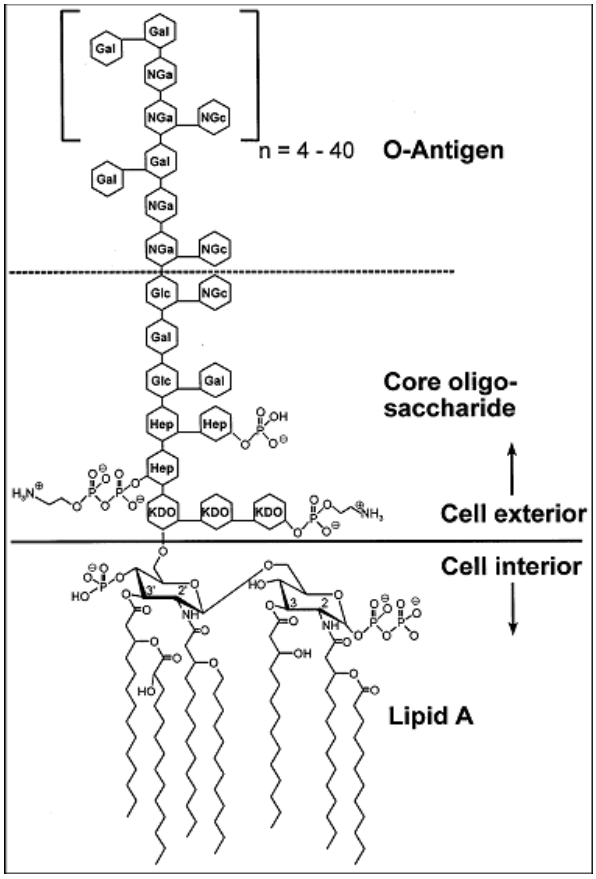


Figure 8: Chemical structure of endotoxin from E. coli O111:B4
(Hep) L-glycerol-D-manno-heptose; (Gal) galactose; (Glc) glucose; (KDO) 2-keto-3-deoxyoctonic acid; (NGa) N-acetyl-galactosamine; (NGc) N-acetyl-glucosamine.
Source: Ohno and Morrison [51]

2.3.4.4. TLR5

TLR5 recognises monomeric flagellin, an evolutionary highly conserved constituent of bacterial flagella.[52] Flagellin is a protein that arranges itself in a hollow cylinder to form the filament in bacterial flagellum. It is the principal substituent of bacterial flagellum, and is present in large amounts on nearly all flagellated bacteria.

As known so far, TLR5 is very specialized and is not being triggered by any other component. However, it is responsible for the immune response of such prominent pathogens as Salmonella typhi (in humans) /Salmonella typhimurium (in mice) and Legionella pneumophila.

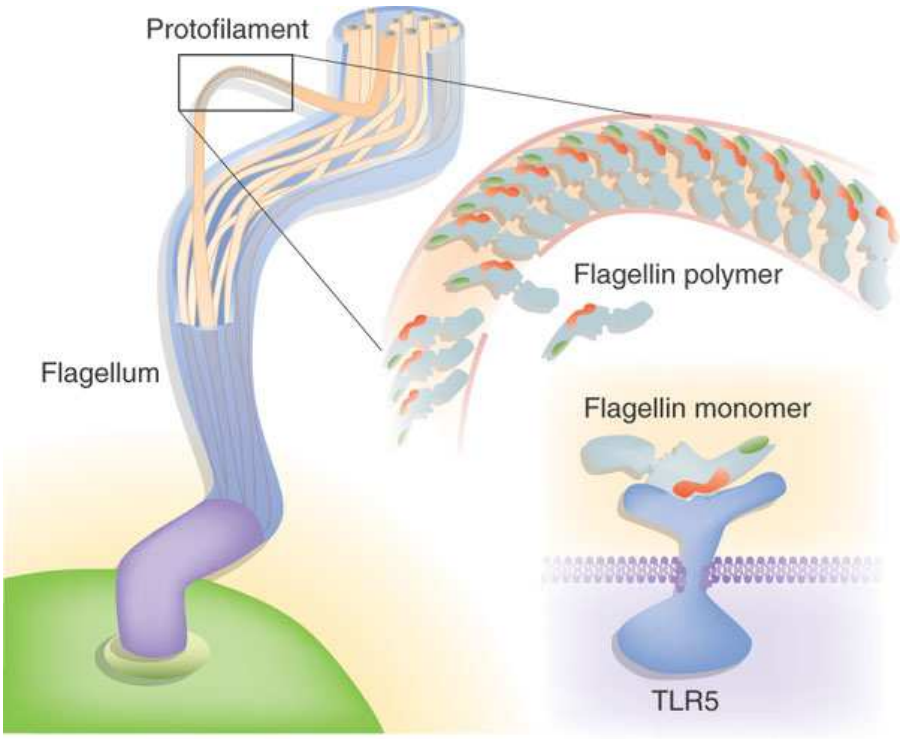
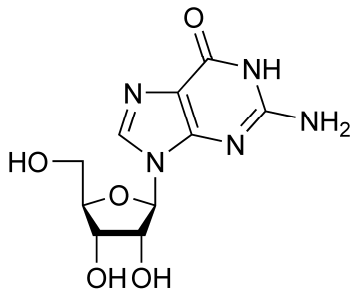


Figure 9: Structure of Flagellin

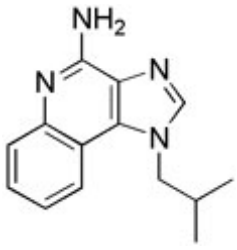
Source: Reichhart J-M [53]

2.3.4.5. TLR7

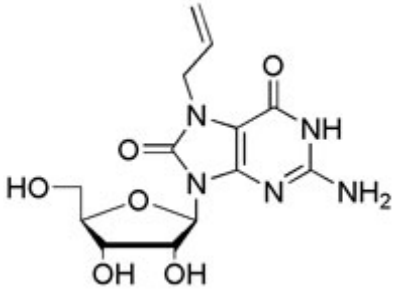
TLR7 was first shown to be involved in the immune response to synthetic compounds, namely Imidazoquinolines (double cyclic organic molecules), that are meanwhile approved for treatment of diseases associated with viral infection like genital warts caused by human papillomavirus. Subsequently, TLR7 has been shown to recognize guanosine- or uridine-rich single stranded RNA (GU-rich ssRNA) from viruses, f.e. HIV or influenza.[54] [55] The reason for discrimination of viral ssRNA and self-derived host ssRNA lies in the localization of TLR7 in the endosomal membrane, where host ssRNA is not present under non-pathologic conditions.[56]



Guanosine



Imiquimod



Loxoribine

Figure 10: Chemical Structures of Guanosine, Imiquimod and Loxoribine

Source: www.invivogen.com

2.3.4.6. TLR9

TLR9 is essential for the recognition of the CpG motif of bacterial and viral DNA. CpG motifs contain a cytosine “C” followed by a guanine "G". The "p" refers to the phosphodiester backbone of DNA. In vertebrates, the frequency of CpG motifs is severely suppressed and the cysteine residues of the CpG motifs are highly methylated, which leads to loss of immunostimulatory acitivity.[57] A further reason - similar to TLR7 - for discrimination of foreign and self-derived host DNA lies in the localization of TLR9 in the endosomal membrane, where host DNA is not present under non-pathologic conditions.[56]

Recently, CpG oligonucleotides were further characterised and divided into three classes, namely CpG-A, CpG-B and CpG-C, which differ in their sequences as well in their mode of action. A-class CpG-ODN are especially potent at inducing IFN-α productition by plasmacytoid dendtritic cells (pDCs). B-class CpG ODNs are potent B cell activators resulting in increased MHC II expression, secretion of immunoglobins and B cell proliferation. Finally, C-Class CpG ODNs induce both A-class and b-class signaling effects[57,58]. For our experiments we used CpG oligodeoxynucleotides (ODN) of the CpG-B class.

5’- tcc atg a**<u>cg</u>** ttc ctg a**<u>cg</u>** tt -3’ (20 mer)

Figure 11: Nucleotide sequence of ODN 1826

The relevant CpG parts are printed in bold and underlined

Source: www.invivogen.com

2.4. Objective of the project

With regard to the nonsatisfying situation in the treatment of patients suffering from Hemophilia A with inhibitor development, a complete understanding of the immunological processes leading to inhibitor formation is a prerequisite for more effective treatments and consequently improved quality of live for patients.

The discovery of TLRs just a decade ago and the still increasing knowledge of their importance for the immunological system raises the question, whether TLRs also play a significant role in the case of Inhibitor development in Hemophilia A.

There is clear evidence from clinical experience, that innate immune response does show direct effects on FVIII inhibitors. As an example, when patients who undergo an immune-tolerance induction therapy suffer from a concomitant bacterial or viral infection, FVIII inhibitors show an increase. Furthermore there is the recommendation of not stimulating the immune system during ITI therapy, e.g. by vaccination, as this also has an negative effect on the outcome of the therapy. However the mechanisms behind those effects are mostly unknown. Based on the current knowledge, TLRs are possible candidates as link between innate immune system and antibody development.

The aim of this project was therefore to provide a profound and systematic insight on the influence of TLRs in the development of antibodies to FVIII in Hemophilia A. A well characterised mouse model of Hemophilia A was used to perform in vitro as well as in vivo studies. Special focus was put on FVIII specific memory B cells, as these are the key players in an established antibody reaction: upon reencounter with their specific antigen and further stimuli, they differentiate into plasma-cells which have the capability of producing vast amounts of antigen-specific antibodies. Means to control of FVIII specific memory B cells would be a major achievement in the treatment of Hemophilia A patients with FVIII inhibitors.

3.1. Modulation of factor-VIII-specific memory B cells

3.1.1. Abstract

Introduction: The development of inhibitory antibodies against factor VIII (FVIII) is the major complication in patients with hemophilia A who are treated with FVIII products. Memory B cells play an essential role in maintaining established antibody responses. Upon re-exposure to the same antigen, they are rapidly re-stimulated to proliferate and differentiate into antibody-secreting plasma cells (ASC) that secrete high-affinity antibodies. It is, therefore, reasonable to believe that memory B cells have to be eradicated or inactivated for immune tolerance induction therapy to be successful in patients with hemophilia A and FVIII inhibitors.

Aim: Develop strategies to prevent FVIII-specific memory B cells from becoming re-stimulated.

Methods: We tested the blockade of co-stimulatory interactions, different concentrations of FVIII and ligands for toll-like receptors (TLR) to modulate FVIII-specific muirne memory-B-cell re-stimulation *in vitro* and *in vivo.*

Results: Blockade of B7-CD28 and CD40-CD40 ligand interactions prevented FVIII-specific murine memory B cells from becoming re-stimulated by FVIII *in vitro* and *in vivo*. Furthermore, high concentrations of FVIII blocked re-stimulation of FVIII-specific murine memory B cells. However, triggering of TLR7 amplified re-stimulation by low concentrations of FVIII and prevented blockade by high concentrations of FVIII.

Conclusions: We established a 6-day *in vitro* culture system that enabled us to study the regulation of FVIII-specific murine memory-B-cell re-stimulation. Using this system, we could define important modulators that either amplify or inhibit the re-stimulation of FVIII-specific murine memory B cells. Currently, we are investigating whether the same modulators operate in patients with hemophilia A and FVIII inhibitors.

3.1.2. Introduction

The development of inhibitory antibodies against factor VIII (FVIII) is the major complication in patients with hemophilia A who are treated with FVIII products. Long-term application of high doses of FVIII has evolved as an

effective therapy to eradicate the antibodies and induce long-lasting immune tolerance [1-4]. Although this therapeutic approach was introduced by Dr Brackmann and co-workers more than 30 years ago [1], little is known about the immunological mechanisms that cause the down-modulation of FVIII-specific immune responses and the induction of long-lasting immune tolerance against FVIII.

Memory B cells play an essential role in maintaining established antibody responses. Upon re-exposure to the same antigen, they are rapidly re-stimulated to proliferate and differentiate into antibody-secreting plasma cells (ASC) that secrete high-affinity antibodies [5-7]. Furthermore, memory B cells have the potential to act as very efficient antigen-presenting cells and stimulators of $CD4^+$ T cells because of the expression of high-affinity antigen receptors, MHC class II and co-stimulatory molecules [8]. It is, therefore, reasonable to believe that memory B cells have to be eradicated or inactivated for immune tolerance induction therapy to be successful in patients with hemophilia A and FVIII inhibitors. Over the past years, we have established technologies that have enabled us to study the regulation of FVIII-specific memory B cells and potential approaches to interfere with the re-stimulation of these cells *in vitro.* We have used a murine model of hemophilia A that is characterized by complete deficiency of biologically active FVIII because of a targeted disruption of exon 17 of the *FVIII* gene [9, 10]. Intravenous injection of human FVIII into these mice results in high titers of anti-FVIII antibodies that have similar characteristics to those of FVIII inhibitors in patients [11-14]. This article summarizes our most important findings in the hemophilic mouse model. Furthermore, it describes our first attempt to analyze FVIII-specific memory B cells in patients with hemophilia A and FVIII inhibitors.

3.1.3. Materials and methods

Animals

The animals used in the study were hemophilic E-17 mice. Our colony of fully inbred hemophilic E-17 mice (characterized by a targeted disruption of exon 17 of the *FVIII* gene) was established with a breeding pair from the original colony [9, 10] and crossed into the C57BL/6J background as described [15].
All mice were male and aged 8–10 weeks at the beginning of the experiments. All studies were done in accordance with the Austrian federal law (Act BG 501, 1989) regulating animal experimentation.

Treatment of mice with human FVIII

Mice received four intravenous doses of 200 ng recombinant FVIII (approximately 80 U/kg FVIII), diluted in 200 µL of Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, Irvine, UK), at weekly intervals. The recombinant human FVIII used throughout the studies was albumin-free bulk material obtained from Baxter AG (Thousand Oaks, CA).

Preparation of spleen cells from mice

Spleens were collected 7 days after the last dose of FVIII. All invasive procedures were done under anesthesia with pentobarbital (Nembutal, Richter Pharm, Wels, Austria). Spleen cells were prepared as described [16, 17].

Re-stimulation of murine memory B cells in vitro

FVIII-specific memory B cells were re-stimulated as described [17, 18]. Briefly, spleen cells were depleted of $CD138^+$ antibody-secreting cells (ASC) using a monoclonal rat anti-mouse CD138 antibody (BD Pharmingen, San Diego, CA) coupled to M-450 sheep anti-rat IgG Dynabeads (Invitrogen Dynal, Lofer, Austria). $CD138^-$ spleen cells were cultured at 1.5×10^6 cells/mL. Different concentrations of FVIII were added to the cells on day 0 as indicated. Antibodies and proteins with potential modulating activities, isotype-matched negative control antibodies or ligands for toll-like receptors (TLR) were added together with FVIII on day 0 or at later time points as

indicated. After 6 days of culture, newly formed ASC were detected by enzyme-linked immunospot (ELISPOT) assays as described [16-18]. The purity of $CD138^-$ spleen cells was analyzed by flow-cytometry [17, 18].

Antibodies and proteins for the blockade of co-stimulators

Blocking antibodies against the co-stimulatory molecules CD80 (B7.1, clone 16-10A1, hamster IgG), CD86 (B7.2, clone P03.1, rat IgG2b), CD40 ligand (CD40L, clone MR1, hamster IgG) and ICOS ligand (ICOSL, clone HK5.3, rat IgG2a) as well as the respective isotype controls were of functional grade and obtained from eBioscience (San Diego, CA). Each antibody was added at 10 µg/mL to the *in vitro* cultures on day 0. Additionally, the importance of ICOS-ICOSL and B7-CD28 interactions were evaluated by using the recombinant competitor proteins murine ICOS/Fc and murine CTLA4/Fc (both are fusion proteins of the murine protein with the Fc-part of human IgG1 and were obtained from R&D Systems; Minneapolis, MN). These proteins were used at a concentration of 10 µg/mL. Murine ICOS/Fc blocks interactions between ICOS and ICOSL, murine CTLA4/Fc blocks interactions between CD80/CD86 and CD28.

Ligands for toll-like receptors

The following ligands for toll-like receptors (TLR) were tested: zymosan for TLR2, poly I:C for TLR3, LPS for TLR4, Flagellin for TLR5, Loxoribine for TLR7 and CpG oligonucleotides for TLR9. All TLR ligands were received from InvivoGen (San Diego, CA).

Depletion of T cells

T cells were depleted from $CD138^-$ spleen cells using mouse pan-T (Thy 1.2) Dynabeads (Invitrogen Dynal) as described [17].

Cytokine analysis and proliferation assays

Cytokine analysis and proliferation assays were done as described [18].

Patients with hemophilia A

12 patients with severe hemophilia A (8-43 years old) were investigated. 6 of the patients had FVIII inhibitors (Table 1). All patients signed a form of consent.

The study was approved by the Ethics Committee of the Institute of Hematology and Transfusion Medicine, Warsaw, Poland.

Analysis of neutralizing anti-FVIII antibodies (FVIII inhibitors) in patients

FVIII inhibitors were analyzed at the central laboratory of the Medical University of Vienna, Vienna, Austria. The Bethesda assay was used as described [19].

Blood sampling and cell preparation from patients

Blood was collected and peripheral blood mononuclear cells (PBMC) were prepared using Vacutainer cell preparation tubes (CPT) with sodium citrate (Becton Dickinson, Schwechat, Austria). Cell isolation was done following the manufacturer's instructions. Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, St Louis, MO) supplemented with 2% pre-selected fetal calf serum (FCS, Hyclone, Logan, UT) was used as a washing solution.

Freshly prepared cells were frozen in RPMI-1640 (Life Technologies, Paisley, Scotland) supplemented with 40% FCS and 10% DMSO (Sigma-Aldrich, St Louis, MO) and stored in liquid nitrogen until further analysis.

In vitro re-stimulation and analysis of human circulating memory B cells

Memory B cells contained in PBMCs were re-stimulated to differentiate into antibody-producing plasma cells *in vitro* as described [20]. After 6 days of culture, newly differentiated antibody-producing plasma cells were analyzed by ELISPOT technology [20]. The frequency of antigen-specific, antibody-producing cells was calculated as a percentage of total IgG-producing cells.

The limit of detection (LD) was found to be three spots per well. These three spots were used to calculated the LD as a percentage of total spots obtained for IgG-producing cells for each individual patient.

3.1.4. Results

Re-stimulation of FVIII-specific memory B cell in vitro

We set up an *in vitro* culture system that is suitable to study the regulation of FVIII-specific memory B cells [17, 18].. For this purpose we obtained spleen cells from hemophilic mice treated with human FVIII and depleted these spleen cells of $CD138^+$ ASC. Thereby we generated a $CD138^-$ spleen cell population that did not contain any anti-FVIII ASC (Fig. 1) but contained FVIII-specific memory B, T cells and other cells. When we stimulated this $CD138^-$ cell mixture with human FVIII, FVIII-specific memory B cells were re-stimulated and differentiated into anti-FVIII ASC that could be detected as soon as 3 days after re-stimulation (Fig. 1) [17]. The maximum of newly formed anti-FVIII ASC was observed 6 days after re-stimulation (Fig. 1) [17]. In further experiments we found that the re-stimulation of FVIII-specific memory B cells in our *in vitro* culture system strictly depended on the presence of activated T cells [17]. Furthermore, a direct cell-cell contact between FVIII-specific memory B cells and activated T cells was required [17].

Re-stimulation of FVIII-specific memory B cells involves CD40-CD40L and B7-CD28 interactions but does not require ICOS-ICOSL interactions

Based on our finding that activated T cells are required to re-stimulate FVIII-specific memory B cells in our *in vitro* culture system, we wanted to know which co-stimulatory interactions would be necessary for this process. Furthermore, we were interested to find out whether blocking essential co-stimulatory interactions would prevent the re-stimulation of FVIII-specific memory B cells. We added blocking antibodies against CD40L, CD80 (B7-1), CD86 (B7-2), ICOSL or recombinant competitor proteins (mICOS/Fc, mCTLA-4/Fc) to the $CD138^-$ spleen cell cultures immediately before re-stimulation with FVIII to study the importance of the relevant ligand receptor pairs. The blockade of B7-CD28 or CD40-CD40L interactions significantly inhibited the re-stimulation of FVIII-specific memory B cells (Fig. 2) [17]. Both CD80 (B7-1) and CD86 (B7-2) contributed to the required co-stimulatory interactions with CD28. Blockade of both molecules prevented the re-

stimulation of memory cells almost completely, whereas the blockade of only one of the two molecules resulted in a partial blockade (Fig. 2) [17]. The negative control antibodies and human IgG1 (negative control for mCTLA-4/Fc) did not show any effect. In contrast to CD40-CD40L and B7-CD28 interactions, ICOS-ICOSL interactions did not contribute to the re-stimulation of FVIII-specific memory cells. Neither the addition of a blocking antibody against ICOSL nor the use of a recombinant competitor protein (mICOS/Fc) resulted in a significant alteration in the re-stimulation of memory B cells (Fig. 2) [17]. In further experiments we confirmed the specific requirements of co-stimulatory interactions for the re-stimulation of FVIII-specific memory B cells in *in vivo* studies using hemophilic mice [17].

Re-stimulation of FVIII-specific memory B cells is inhibited by high concentrations of FVIII

After specifying important co-stimulatory interactions required for the re-stimulation of FVIII-specific memory B cells, we were interested to study the potential impact of different concentrations of FVIII on this process. We tested a range of concentrations between 1 pg/mL and 100 µg/mL of FVIII (Fig. 3A) [18]. Re-stimulation of memory B cells could be detected at concentrations of FVIII that were as small as 100 pg/mL (Fig. 3A) [18]. Optimal re-stimulation was achieved at concentrations of 3-10 ng/mL, which correspond to about 3-10% of the physiological plasma concentration (Fig. 3A) . [18]. When we further increased the concentration of FVIII, inhibition of memory-B-cell re-stimulation was observed. The inhibition started at a concentration of FVIII of 100-300 ng/mL with an almost complete inhibition at 1 µg/mL FVIII (Fig. 3A) [18].

The dose-response relation for T-cell re-stimulation was very different from the dose-response relation for memory-B-cell re-stimulation. Optimal stimulation of FVIII-specific T cells was observed at concentrations of 10-30 µg/mL FVIII (Fig. 3B, 3C). Inhibition of T-cell stimulation was seen at concentrations of 100 µg/mL FVIII. Based on these results we conclude that the concentration of FVIII required for inhibition of memory-B-cell re-stimulation and the concentration required for inhibition of T-cell re-stimulation are very different (Fig. 3A, 3B, 3C), which makes it unlikely that

the inhibition of memory-B-cell re-stimulation is due to an inhibition of T-cell stimulation.

The major T-cell cytokines found in culture supernatants after stimulation of spleen cells with FVIII were IL-10 and IFN-γ (Fig. 3C), which is consistent with findings we reported previously [13, 21]. To further support these results, we analyzed the frequency of FVIII-specific T cells by intracellular cytokine staining 3 days after re-stimulation of spleen cells. We compared concentrations of 10 ng/mL, which re-stimulate, and 20 µg/mL FVIII which inhibit memory B-cell differentiation and observed a correlation between the frequency of FVIII-specific T cells producing IL-2, IL-10 or IFN-γ and the concentration of FVIII used for the re-stimulation (data not shown). We did not observe any inhibitory effects of 20 µg/mL of FVIII on T-cell stimulation despite the fact that this concentration of FVIII completely blocks the re-stimulation of FVIII-specific memory B cells [18].

Both re-stimulation and inhibition of FVIII-specific memory B cells are modulated by ligands for toll-like receptors (TLR)

Infections, particularly infections from the central venous catheter inserted in patients with hemophilia A and FVIII inhibitors during immune tolerance induction therapy (ITI), commonly cause a rise in anti-FVIII antibody titers [22]. Based on this observation, we asked whether components derived from pathogens such as viruses or bacteria modulate the re-stimulation of FVIII-specific immune memory and disturb the inhibition of memory-B-cell re-stimulation by high doses of FVIII. Microbial components are recognized by specific TLR that serve as an important link between innate and adaptive immunity. We studied the modulation of FVIII-specific memory B cells by a range of different ligands for TLR (zymosan for TLR2, poly I:C for TLR3, LPS for TLR4, Flagellin for TLR5, Loxoribine for TLR7 and CpG oligonucleotides for TLR9) [23, 24]. The most dramatic effects were seen with Loxoribine, a ligand for TLR7 (Fig. 4A) [23]. Loxoribine at 10,000 ng/mL amplified the re-stimulation of FVIII-specific memory B cells at 10 ng/mL FVIII and completely abolished the inhibition of memory-B-cell re-stimulation at 1,000 ng/mL FVIII (Fig. 4A) [23]. Furthermore, Loxoribine facilitated a re-stimulation of FVIII-specific memory B cells in the complete absence of T cells (Fig. 4B) and

even induced some re-stimulation in the complete absence of FVIII (Fig. 4A, 4B).

Next, we wanted to know whether to induce modulation of memory-B-cell re-stimulation the triggering of TLR7 by Loxoribine needed to be simultaneous with the re-stimulation by FVIII. To address this question, we started our *in vitro* culture in the presence of FVIII on day 0 and added Loxoribine at different time points during a 6-day culture. Our results indicated that triggering TLR7 by Loxoribine can be induced up to 2 days after re-stimulation with FVIII to achieve an amplification of memory-B-cell re-stimulation and a prevention of memory-B-cell inhibition in our 6-day *in vitro* culture (Fig. 5A).

Detection of FVIII-specific memory B cells in patients with hemophilia A

In the preceding sections we described several mechanisms by which FVIII-specific memory responses in hemophilic mice can be modulated. The question arises whether these mechanisms also operate in patients with hemophilia A and FVIII inhibitors. In particular, it would be important to know whether any of these mechanisms could be targeted to develop new therapeutic approaches for either the eradication of FVIII-specific immune memory or the prevention of anamnestic immune responses against FVIII in patients. To address this question it is important to develop technologies that are suitable for analyzing FVIII-specific memory B cells in patients.

We adapted a method established by Crotty et al. [24] to track FVIII-specific memory B cells in PBMC of patients with hemophilia A and FVIII inhibitors. For this purpose, PBMC were polyclonally stimulated to allow all memory B cells to differentiate into ASC. ASC specific for FVIII and HSA and the total number of IgG-secreting cells were then analyzed by ELISPOT technology (Fig. 6). The number of specific ASC directly correlates with the initial number of specific memory B cells [24].

We analyzed PBMC of twelve patients with severe hemophilia A (Table 1) for the presence of memory B cells specific for human FVIII and HSA (negative control). 6 patients had FVIII inhibitors with Bethesda titers between 1 BU/mL and 1,000 BU/mL (Table 1). 5 of the patients with inhibitors were not being treated with FVIII products at the time but with bypassing products, all

patients without inhibitors were being treated with FVIII products (Table 1). None of the patients showed detectable levels of memory B cells specific for HSA (negative control, Fig. 6A). FVIII-specific memory B cells were detected in the peripheral blood cells of one of the patients with inhibitors but not in any of the patients without inhibitors (Fig. 6A, 6B). The frequency of FVIII-specific memory B cells in the positive patient was 0.24% of total IgG memory B cells (Fig. 6A). The limit of detection for antigen-specific memory B cells was in the range between 0.02% and 0.28% of the total IgG memory B cells and varied considerably between individual patients (Fig. 6A, 6B).

3.1.5. Discussion

We studied the re-stimulation and differentiation of FVIII-specific memory B cells using an *in vitro* culture system that is based on $CD138^-$ spleen cells obtained from hemophilic mice treated with FVIII. $CD138^-$ spleen cells contain all spleen cells except $CD138^+$ ASC. Due to the nature of this mixed cell population as a source for FVIIII-specific memory B cells, it is difficult to exactly define the cell-cell interactions that are required for the re-stimulation or inhibition of FVIII-specific memory B cells. Furthermore, it is not possible to specify signal transduction pathways that are involved in the re-stimulation or inhibition of these cells. Therefore, we have further developed this method and established an *in vitro* culture system that operates with highly purified memory B cells and highly purified $CD4^+$ T cells [25, 26]. Currently, we use this improved system to study the mechanisms that are responsible for the re-stimulation and inhibition of FVIII-specific memory B cells under the conditions described in this article.

Based on our findings that the re-stimulation of FVIII-specific memory B cells requires direct cell-cell contact with activated T cells [17], we initiated experiments that focused on the modulation of FVIII-specific memory-B-cell responses by interfering with essential co-stimulatory interactions. Our results indicate that B7-1/B7-2-CD28 and CD40-CD40L interactions are essential for the re-stimulation of these cells. On the other hand, ICOS-

ICOSL interactions are not important. The B7-1/B7-2-CD28/CTLA-4 pathway is one of the best-characterized co-stimulatory pathways for T-cell activation and is also essential for T-cell tolerance [27, 28]. Qian et al. [29] were able to show that B7-2, but not B7-1, was involved in the primary immune response against FVIII in hemophilic mice. Furthermore, injecting murine CTLA-4-Ig into hemophilic mice prevented a further increase in anti-FVIII antibody titers in hemophilic mice with an established anti-FVIII immune response, indicating that CTLA-4-Ig blocks the re-stimulation of FVIII-specific memory B cells. Comparing our results [17] with those published by Qian et al. [29], we conclude that B7-2, but not B7-1, is involved in primary anti-FVIII antibody responses in hemophilic mice and that both molecules are important for the memory-driven antibody response.

CD40-CD40L interactions are a key event in T-cell-dependent humoral immune responses [30]. The results from studies on the significance of these interactions for the differentiation of memory B cells into ASC, however, conflict. Several reports suggest that CD40 signaling is important for the terminal differentiation of B cells and for antibody secretion [31-34]. Other reports show that CD40 signaling prevents the terminal differentiation of B cells [35-39]. Our results indicate that the re-stimulation of FVIII-specific memory B cells and their subsequent differentiation into anti-FVIII ASC requires CD40-CD40L interaction. The blockade of these interactions prevented the formation of anti-FVIII ASC *in vitro* and reduced it significantly *in vivo* [17]. We believe that the blockade of CD40-CD40L interactions in our system down-regulates T-cell activation and, more importantly, blocks the interaction between activated T cells and memory B cells.

Based on the successful use of high-dose FVIII for the induction of immune tolerance in patients with hemophiia A [1], we wondered whether the re-stimulation of FVIII-specific memory B cells was affected by high concentrations of FVIII. Our results demonstrate that concentrations of FVIII that are below the physiological plasma concentration of 100 ng/mL (1 U/mL) re-stimulate FVIII-specific memory B cells and induce their differentiation into ASC *in vitro* whereas concentrations that are above the physiological plasma concentration inhibit this process.

These results support the idea that the inhibition or eradication of FVIII-specific memory B cells might be an early event in the down-regulation of established anti-FVIII antibody responses in patients. The eradication of memory B cells would prevent their differentiation into ASC and, moreover, may lead to a deficiency of effective antigen-presenting cells required for the re-stimulation of FVIII-specific T cells. The induction of regulatory T cells rather than effector T cells could be the consequence of this deficiency. Currently it is not clear, however, whether high-dose FVIII ITI therapy in patients would lead to local FVIII concentrations that are comparable to the concentrations that we used in our *in vitro* experiments. Further studies are necessary to investigate this hypothesis.

Toll-like receptors (TLR) recognize invading pathogens such as viruses and bacteria and serve as an important link between innate and adaptive immunity [40, 41]. Given the importance of TLR for the regulation of adaptive immune responses, we asked how triggering TLR would influence the regulation of FVIII-specific memory B cells. In particular, we were interested to know whether the triggering of TLR would prevent the inhibition of memory-B-cell re-stimulation by high concentrations of FVIII. Our results clearly indicate that both stimulation of memory responses by low doses of FVIII as well as inhibition of memory responses by high doses of FVIII are modulated by TLR triggering. Furthermore, the triggering of TLR re-stimulates memory responses in the complete absence of T cells and to a certain degree even in the absence of FVIII. The natural ligands of TLR7 were identified as single-stranded RNA (ssRNA) [42-44]. Mouse TLR7, human TLR8 and human TLR7 recognize ssRNA viruses such as the influenza [43, 44], Sendai [45] and Coxsackie B [46] viruses. This recognition requires the internalization of the virus and its replication to release the viral RNA into endosomes, where TLR7 and TLR8 reside. The interaction between the ssRNA and TLR7/8 triggers the recruitment of the adapter molecule MyD88 leading to the activation of NF-κB and other transcription factors and the production of proinflammatory cytokines and chemokines. Based on our results, it can therefore be expected that any infection with the

indicated viruses could potentially modulate FVIII-specific immune memmory in patients with FVIII inhibitors.

In the last part of this article we present the first results of our attempts to identify FVIII-specific memory B cells in the peripheral blood of patients with hemophilia A. For this purpose, we adapted a technology that was recently described by Crotty et al [24] to human FVIII. We studied 12 patients with hemophilia A, 6 of them had detectable titers of neutralizing anti-FVIII antibodies. We could detect FVIII-specific memory B cells in one of the patients with FVIII inhibitors. This was the patient who showed the highest titers of neutralizing anti-FVIII antibodies. The frequency of FVIII-specific memory B cells in this patient was 0.24% of the total pool of IgG memory B cells. The detection limit for FVIII-specific memory B cells was in the range between 0.02% and 0.28% of the total IgG memory B cells and showed considerable variations between individual patients. The lack of detectable FVIII-specific memory B cells in 5 of the 6 patients with FVIII inhibitors might be due to one or a combination of the following reasons. 4 of the 5 patients had last received FVIII treatment between 4 and 14 years beforehand. Bypassing agents that had been given recently might not have provided sufficient stimuli to keep the pool of FVIII-specific memory B cells in the circulation large enough to be detectable. Alternatively, the remaining FVIII-specific memory B cells might have been located in secondary lymphoid organs and might have only re-circulated after re-stimulation with FVIII. Another reason for the lack of detectable FVIII-specific memory B cells in 5 of the 6 patients with FVIII inhibitors might have been the sensitivity of the assay. In its current state of development, this assay cannot detect FVIII-specific memory B cells with frequencies below 0.02% of the total IgG memory B cells. Therefore, a further improvement in the detection limit of the method might be necessary.

Summarizing our data, we conclude that FVIII-specific memory B cells are an important target for the development of new strategies to induce FVIII-specific immune tolerance in patients with hemophilia A and FVIII inhibitors. Therefore, future efforts should focus on studying the regulation of these cells

both in preclinical animal models and in patients. However, the eradication of memory B cells can only be a first step in the induction of immune tolerance in patients with FVIII inhibitors. A second step will most likely be necessary to keep a stable immune tolerance and prevent the re-induction of anti-FVIII antibodies.

3.1.6. Acknowledgements

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3.1.7. Figures

Table 1

Patients	Age (in years)	Titer of FVIII inhibitors (BU/mL) in 2006	Last treatment with FVIII	Current treatment	Historical peak inhibitor titer (BU/mL)	First detection of FVIII inhibitors
WA01	24	125	1993	bypassing agents	189	1985
WA02	27	6	2003	bypassing agents	150	1994
WA04	33	1.2	2003	no treatment	12	1993
WA05	34	1,000	1995	bypassing agents	4,340	1984
WA11	8	1	2006	high dose FVIII and rFVIIa	68	1999
WA12	16	8	1992	FEIBA	59	1993
WA03	30	0	2006	FVIII	0	no inhibitors
WA06	11	0	2006	FVIII	0	no inhibitors
WA07	33	0	2006	FVIII	0	no inhibitors
WA08	43	0	2006	FVIII	0	no inhibitors
WA09	40	0	2006	FVIII	0	no inhibitors
WA13	12	0	2006	FVIII	0	no inhibitors

Table 1: Characteristics of patients enrolled in the study in 2006.

This table was originally published in reference [20]

Figure 1

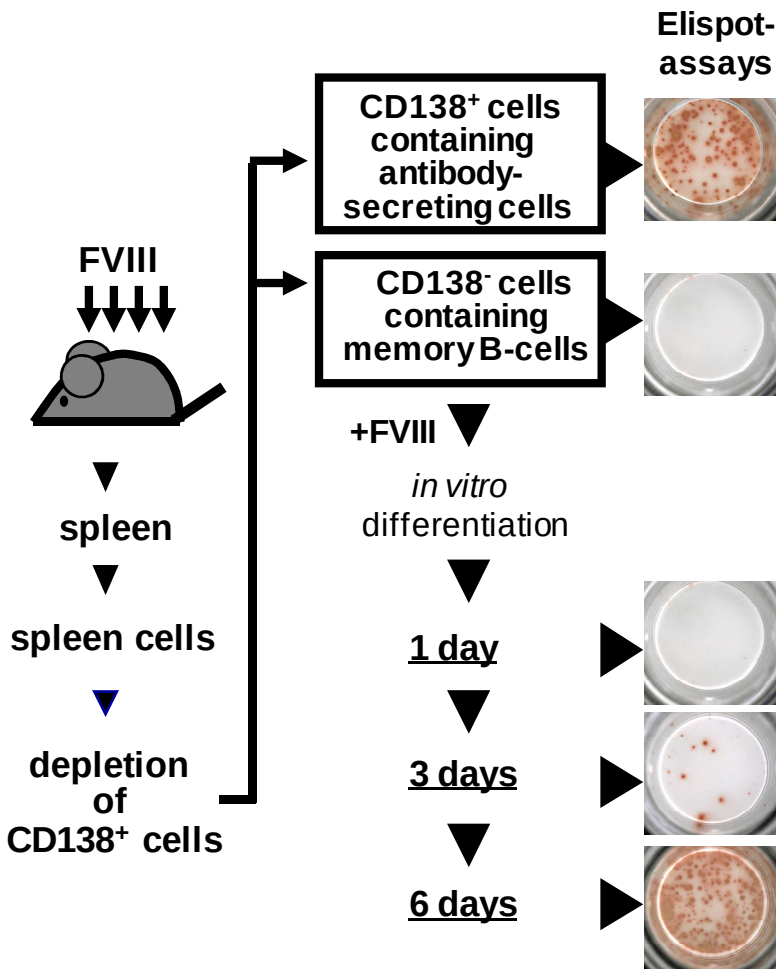

Figure 1. Re-stimulation of murine FVIII-specific memory B cells in vitro. Spleen cells were obtained from hemophilic mice treated with four intravenous doses of 200 ng (80 U/kg) FVIII and depleted of $CD138^+$ ASC. The remaining $CD138^-$ cells were re-stimulated with 10 ng/mL FVIII and analyzed for newly formed ASC after 1, 3 and 6 days of culture. ASC were analyzed by ELISPOT assays as described (Hausl 2002).

This research was originally published in *Blood*. [17]

Figure 2

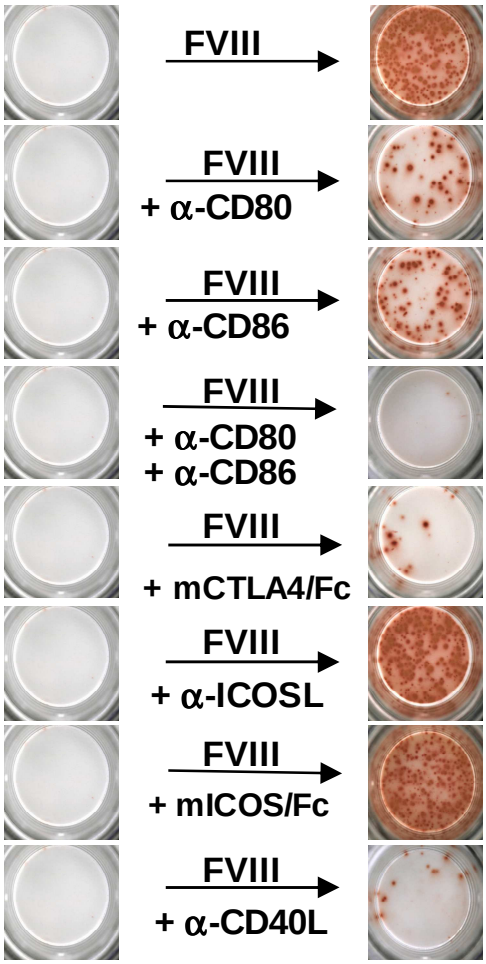

Figure 2. The antigen-specific re-stimulation of FVIII-specific murine memory B cells in vitro involves CD40-CD40L and B7-CD28 interactions but does not require ICOS-ICOSL interactions. CD138- spleen cells obtained from hemophilic mice treated with human FVIII were re-stimulated with 10 ng/mL FVIII. Anti-FVIII ASC were analyzed after 6 days of culture using ELISPOT assays. To interfere with co-stimulatory interactions, blocking antibodies (α-CD80, α-CD86, α-ICOSL, α-CD40L as indicated) or competitor proteins (mCTLA-4/Fc, mICOS/Fc as indicated) were added to the cultures at a concentration of 10 µg/mL together with FVIII. Presented are ELISPOT data obtained in a representative experiment.

This research was originally published in *Blood*. [17]

Figure 3

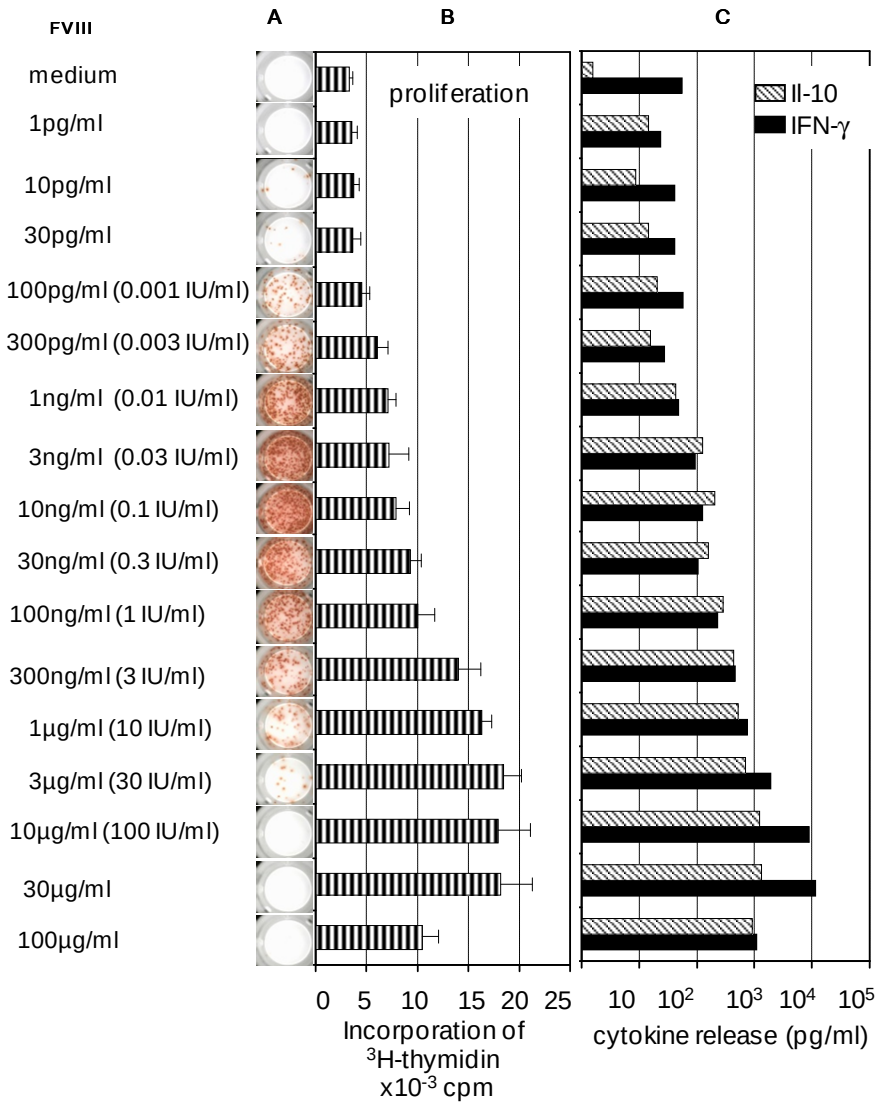

Figure 3. Concentration of FVIII determines the response of FVIII-specific murine memory B cells and FVIII-specific T cells.

CD138- spleen cells were obtained from hemophilic mice treated with human FVIII. Cells were re-stimulated in vitro with human FVIII as indicated for 3 days (B) or 6 days (A, C). Newly formed anti-FVIII ASC were detected by ELISPOT assay (A). ELISPOTs represent the results obtained in a typical experiment. Cell proliferation (B) and cytokine secretion into cell culture supernatants (C) were analyzed as described in Methods. Presented are the means and standard deviations of triplicate cultures (B) or the medians (C) obtained in a typical experiment.

This research was originally published in *Blood* [18]

Figure 4

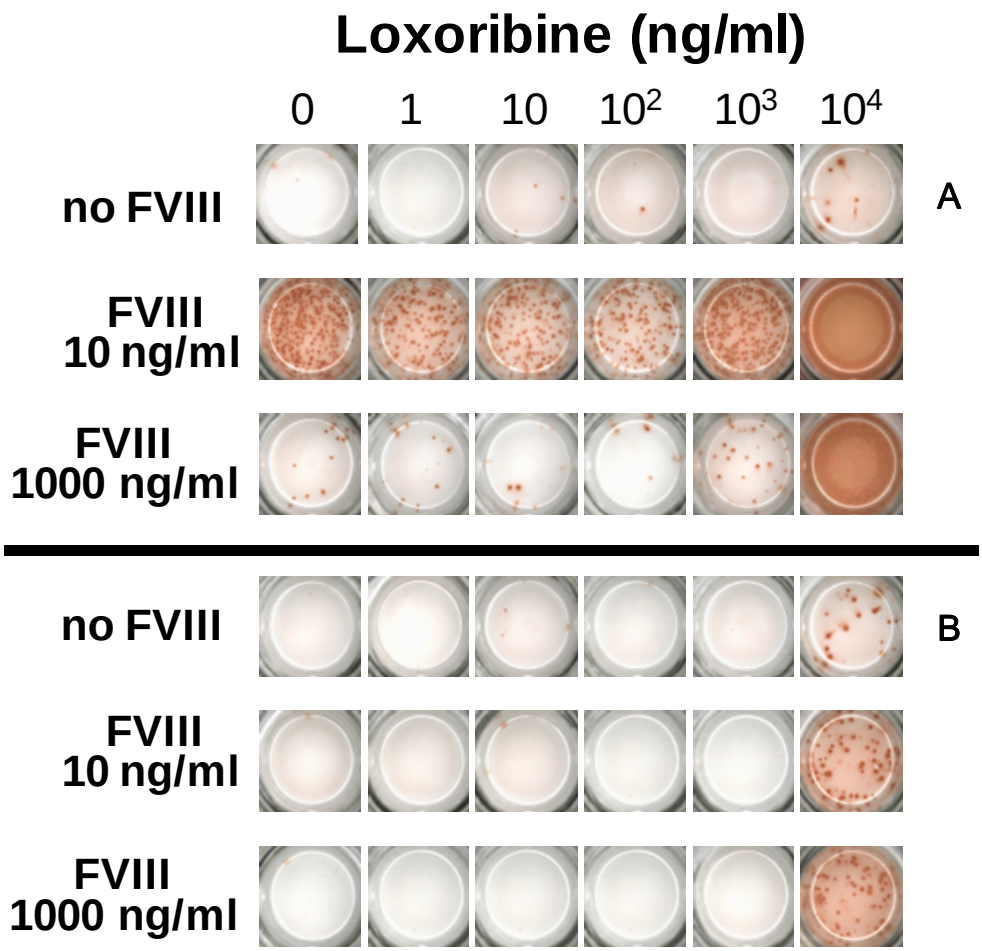

Figure 4. Loxoribine modulates both re-stimulation and inhibition of FVIII-specific murine memory B cells.

CD138- spleen cells were obtained from hemophilic mice treated with human FVIII. Cells were re-stimulated for 6 days with different concentrations of human FVIII and Loxoribine as indicated. Newly formed ASC were detected by ELISPOT assay. ELISPOTs represent the results obtained in a typical experiment.

A) total $CD138^-$ spleen cells

B) $CD138^-$ spleen cells depleted of T cells

Figure 5

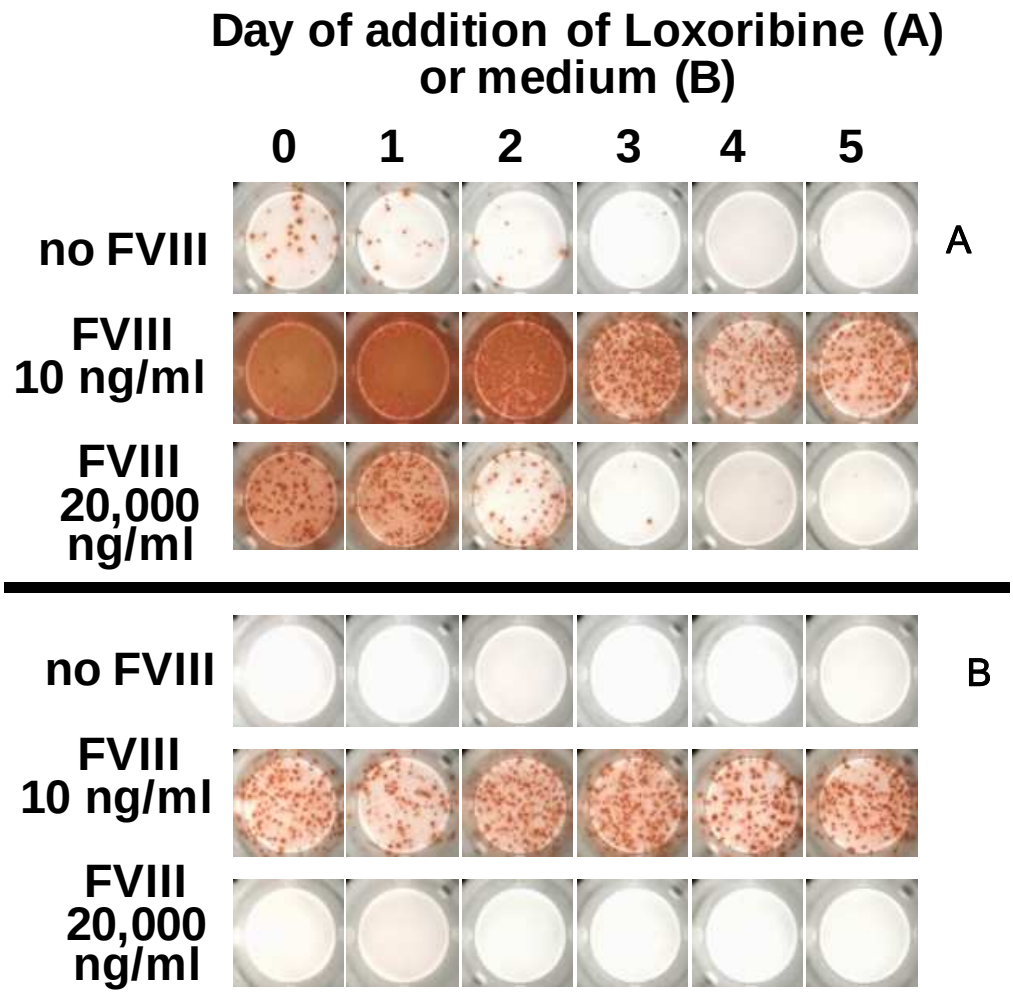

Figure 5. Time-dependent modulation of FVIII-specific memory B cells by Loxoribine.

$CD138^-$ spleen cells were obtained from hemophilic mice treated with human FVIII. Cells were re-stimulated for 6 days with different concentrations of human FVIII and with 10,000 ng/mL Loxoribine. FVIII was added on day 0. Loxoribine was added on different days as indicated. Newly formed ASC were detected by ELISPOT assay. ELISPOTs represent the results obtained in a typical experiment.

A) addition of Loxoribine at different time points as indicated

B) addition of buffer (negative control) at different time points

Figure 6

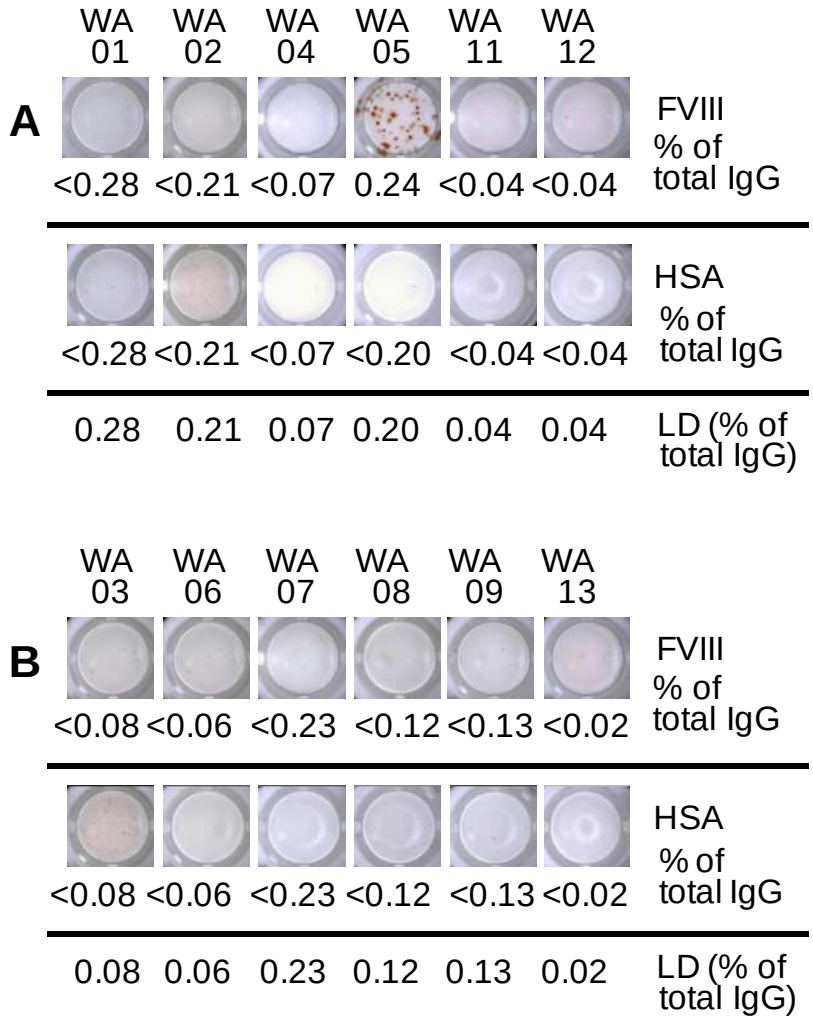

Figure 6. Tracking FVIII-specific memory B cells in patients with hemophilia A

The results of the detection of FVIII-specific memory B cells in the peripheral blood of 6 patients with severe hemophilia A and FVIII inhibitors (A) and 6 patients with severe hemophilia A without FVIII inhibitors (B) are shown. Human Serum Albumin (HSA) was used as negative control. Each spot represents a single newly differentiated anti-FVIII antibody-producing plasma cell. The percentage of antigen-specific cells related to total IgG-producing cells (% of total IgG) as well as the limit of detection (LD) were calculated for each patient.

This research was originally published at the 35th Haemophilia Symposium Hamburg 2005 [20]

3.1.8. References for Chapter 3.1

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3.2. Stimulation and inhibition of FVIII-specific memory-B-cell responses by CpG-B (ODN 1826), a ligand for toll-like receptor 9

3.2.1. Abstract

Factor VIII (FVIII)-specific memory B cells are essential components for regulating anamnestic antibody responses against FVIII in hemophilia A with FVIII inhibitors. We asked how stimulation and inhibition of FVIII-specific memory B cells by low and high concentrations of FVIII, respectively, are affected by concurrent activation of the innate immune system. Using $CD138^-$ spleen cells from hemophilic mice treated with FVIII to study restimulation and differentiation of memory B cells in vitro, we tested modulating activities of agonists for toll-like receptors (TLR) 2, 3, 4, 5, 7 and 9. Ligands for TLR7 and 9 were most effective. They not only amplified FVIII-specific memory responses in the presence of stimulating concentrations of FVIII but also countered inhibition in the presence of inhibitory concentrations of FVIII. Notably, CpG-ODN, a ligand for TLR 9, expressed biphasic effects. It amplified memory responses at low concentrations and inhibited memory responses at high concentrations, both in vitro and in vivo. Both stimulatory and inhibitory activities of CpG-ODN resulted from specific interactions with TLR9. Despite their strong immunomodulatory effects in the presence of FVIII, ligands for TLR induced negligible restimulation in the absence of FVIII in vitro and no restimulation in the absence of FVIII in vivo.

3.2.2. Introduction

Memory B cells are fundamentally important for maintaining immunological memory to ensure long-lasting protection against invading pathogens such as viruses and bacteria (1). They are also involved in long-term maintenance of immunopathologic conditions such as chronic antibody-dependent immunological disorders (2). Memory B cells have the unique capacity to rapidly differentiate into antibody-secreting plasma cells (ASC) upon re-exposure to their specific antigen, thereby replenishing the pool of plasma

cells (1). They also act as efficient antigen-presenting cells for the restimulation of $CD4^+$ T cells because they express high-affinity antigen receptors, MHC class II and co-stimulatory molecules (3).

We and others have demonstrated the presence of factor VIII (FVIII)-specific memory B cells in the circulation of patients with hemophilia A and FVIII inhibitors (4, 5). van Helden et al (5) reported the disappearance of memory B cells from the circulation of patients during successful immune tolerance induction therapy. These findings are in line with the assumption that FVIII-specific memory B cells are important for the stimulation of anamnestic antibody responses against FVIII. Little information is available on the regulation of these cells in patients with hemophilia A. Their low frequency in the circulation (0.07-0.35% of total IgG memory B cells (4, 5) and the inaccessibility of their major residencies (peripheral lymphoid organs such as the spleen (6)) are important obstacles in studying these cells in patients with hemophilia A and FVIII inhibitors. Therefore, we used the E17 hemophilic mouse model to obtain a better understanding of the regulation of these cells. Previously, we established a spleen cell culture system that enables us to study the function and regulation of FVIII-specific memory B cells (7, 8). We demonstrated that the differentiation of FVIII-specific memory B cells into ASC depends on the presence of activated T cells and certain co-stimulatory interactions (7). Furthermore, we showed that the differentiation of memory B cells is sensitive to increasing doses of FVIII. Concentrations of FVIII in the range of 0.1-100ng/mL (0.001-1U/mL) restimulate memory B cells and induce their differentiation into ASC. Higher doses of FVIII, however, inhibit FVIII-specific memory B-cell responses (8). Based on these previous results, we now asked how stimulation and inhibition of FVIII-specific memory B cells by low and high concentrations of FVIII, respectively, are influenced by concurrent activation of the innate immune system. The innate immune system becomes activated as a first line of defence against invading pathogens during natural microbial infections and primes the adaptive immune system to generate antigen-specific immune responses (9, 10). Cells of the innate immune system express various pattern-recognition receptors (PRR) that recognize conserved structures of pathogens, so-called

pathogen-associated molecular patterns (PAMP) (11). Several classes of PRR have been identified, e.g. toll-like receptors (TLR), retinoic acid-inducible gene (RIG)-I-like receptors (RLR), C-type lectin receptors or nucleotide oligomerization domain (NOD) protein-like receptors (NLR) (11, 12). We were particularly interested in the activities of agonists for TLR which represent the best characterized group of innate immune receptors with respect to known ligands, downstream signaling pathways and functional relevance (13-16).

So far, 12 different TLRs have been identified in mammals (15). They are differentially expressed on many cell types of hematopoietic and non-hematopoietic origin (13-15), either at the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) or in endolysosomal compartments (TLR3, TLR7, TLR8, and TLR9). Upon activation, TLR expressed at the cell surface can enter the endocytic pathway. The distinct localization of TLR is associated with the specific nature of their stimulatory ligands. TLR expressed on the cell surface primarily sense microbial membrane molecules such as lipopeptides, peptidoglycans, LPS and flagellin (TLR1, TLR2, TLR4, TLR5, and TLR6). TLR expressed intracellularly recognize microbial nucleic acids such as CpG-containing DNA sequences (TLR9), viral ssRNA (TLR7 and TLR8) or dsRNA (TLR3). A growing body of evidence suggests that TLR agonists modify the restimulation of memory B cells and their differentiation into ASC (17-19). Moreover, it was postulated that memory B cells can be activated by TLR agonists to differentiate into ASC in the complete absence of antigen (17, 20). Based on these findings, it is important to understand how stimulation and inhibition of FVIII-specific memory responses by different concentrations of FVIII are affected by agonists for TLR. Furthermore, one could speculate that FVIII-specific memory B cells in patients might become activated during infections or vaccinations in the complete absence of any replacement therapy with FVIII-containing products. A better understanding of how TLR agonists affect FVIII-specific memory responses is therefore of utmost importance in designing new treatments for patients with hemophilia and FVIII inhibitors.

3.2.3. Material and Methods

3.2.3.1. Hemophilic mice

Our colony of hemophilic E-17 mice (characterized by a targeted disruption of exon 17 of the *FVIII* gene) was established with a breeding pair from the original colony (21, 22) and crossed into the C57BL/6J background (23). All 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.

3.2.3.2. Treatment with human FVIII and TLR ligands

If not stated otherwise, hemophilic E-17 mice received 4 intravenous doses of 200ng recombinant FVIII (approximately 80 U/kg FVIII), diluted in 200µL of Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, Irvine, UK), at weekly intervals. The recombinant human FVIII used throughout the studies was albumin-free bulk material obtained from Baxter BioScience (Orth an der Donau, Austria).
TLR ligands were reconstituted according to the manufacturer’s instructions and administered either together with FVIII or prior to FVIII in a volume of 200µL, diluted in DPBS as indicated.

3.2.3.3. Sampling of tissue and blood

Tissue and blood samples were collected 7 days after the last dose of FVIII if not otherwise indicated. All invasive procedures were carried out under anesthesia with pentobarbital (Nembutal, Richter Pharm, Wels, Austria). Blood samples were obtained by cardiac puncture or tail snipping. The samples obtained from individual mice were added to 0.1mol/L sodium citrate at a 4:1 (vol/vol) ratio. Plasma was separated by centrifugation and stored at -20℃ until further analysis.

3.2.3.4. Preparation of spleen cells

Spleen cells were prepared as described (24).

3.2.3.5. Restimulation of memory B cells in vitro

Restimulation of memory B cells in vitro was studied as described (7, 8). Briefly, spleen cells obtained from E17 hemophilic mice treated with 4 i.v. doses of FVIII were isolated and depleted of $CD138^+$ ASC. Remaining $CD138^-$ spleen cells were cultured for 6 days (if not otherwise stated). Different concentrations of FVIII were added to the cultures at day 0. TLR ligands were added together with FVIII at day 0 or at later time points as indicated. After 6 days of culture, newly formed ASC were detected by enzyme-linked immunospot (ELISPOT) assays as described (7, 8).

3.2.3.6. Restimulation of FVIII-specific memory responses in vivo

Restimulation after priming with a single dose of FVIII

Hemophilic mice received an initial dose of 200ng i.v. FVIII on day 1. On day 9, mice received a booster injection with any of the following: PBS buffer (negative control), FVIII only, FVIII in combination with TLR ligands, TLR ligands only. All mice treated with TLR ligands on day 9 received an additional dose of TLR ligands on day 8.

Restimulation after transfer of FVIII-specific memory cells into naïve mice, as described (7, 8):

Spleen cells, isolated from hemophilic mice treated with four doses of human FVIII, were depleted of $CD138^+$ ASC. A total of 10^7 $CD138^-$ spleen cells were i.v. injected into naïve hemophilic mice. One day after cell transfer, mice were injected with a single i.v. dose of PBS buffer (negative control), with a combination of i.v. FVIII and i.p. ligands for TLR (or PBS for negative control) or with i.p. TLR ligands only.

3.2.3.7. TLR ligands

The following ligands were used: Zymosan for TLR2 (0.1 to 10,000 ng/mL); Poly I:C for TLR3 (1 to 50,000 ng/mL), LPS for TLR4 (0.1 to 10,000 ng/mL), Flagellin for TLR5 (0.01 to 1000 ng/mL), Loxoribine and Imiquimod for TLR7 (1 to 50,000 ng/mL), CpG-ODN (CpG-B, ODN1826, sequence: 5'- tcc atg acg

ttc ctg acg tt -3') for TLR9 (0.1 to 10,000 ng/mL). All TLR ligands were obtained from InvivoGen (San Diego, CA).
The following ligands served as controls: ODN2088 (CpG-DNA, sequence: 5'-tcc tga gct tga agt-3') as inhibitor for TLR9 (InvivoGen, 100 to 10.000 ng/mL), ODN1826-control (GpC-DNA, sequence: 5'- tcc atg agc ttc ctg agc tt -3') as negative control for ODN1826 (InvivoGen, 0.1 to 10,000 ng/mL) for TLR9.

3.2.3.8. Detection of anti-FVIII antibodies in blood plasma

Titers of total anti-FVIII antibodies in blood plasma were measured by ELISA as described (24).

3.2.3.9. Analysis of cytokines in cell culture supernatants

Murine interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10) and interferon γ (IFN-γ) were detected in cell culture supernatants using a Bio-Plex Mouse Cytokine 9-Plex Assay (Bio-Rad Laboratories, Hercules, CA).

3.2.3.10. Statistics

Arithmetic means were calculated using results obtained in ELISPOT assays on different days. Spots were normalized to the daily control as indicated to allow comparison of results obtained in ELISPOT assays, which were run on different days.
Statistical analyses of antibody titers were performed with SAS Version 9.1.3 of the SAS System for Linux (SAS Institute Inc., Cary, NC, USA). The null hypotheses of no differences were tested against their 2-sided alternatives at a level of 5% statistical significance. Log2-transformed titers were assumed to follow a negative binomial distribution (25). As the data were collected longitudinal (i.e. over time) a repeated measures analysis was performed using the SAS procedure GENMOD.

3.2.4. Results

3.2.4.1. Agonists for TLR7 and 9 are most effective in modulating FVIII-specific memory responses in vitro

We identified TLR agonists that modulate stimulation or inhibition of FVIII-specific memory responses by screening ligands for TLR 2, 3, 4, 5, 7 and 9 over a range of different concentrations using the previously described spleen cell culture system (7, 8). $CD138^-$ spleen cells obtained from E17 hemophilic mice treated with 4 doses of FVIII were restimulated in vitro and the impact of TLR stimulation on the differentiation of memory B cells into anti-FVIII ASC was analyzed by ELISPOT. TLR ligands were added to the cultures on day 0 in the complete absence of FVIII as well as in the presence of stimulatory (10ng/mL) or inhibitory (1µg/mL and 20µg/mL) concentrations of FVIII. Our results indicated that most TLR agonists tested modulated both restimulation and inhibition of FVIII-specific memory B cells to some degree. However, agonists for TLR7 (Loxoribine and Imiquimod) and TLR9 (CpG-ODN) were most effective (Table 1). At optimal concentrations, agonists for TLR7 and TLR9 amplified the restimulation of FVIII-specific memory B cells induced by low concentrations of FVIII considerably and countered the inhibition of memory responses induced by high concentrations of FVIII (Table 1).

In conclusion, all TLR ligands affected FVIII-specific memory responses, but TLR7 and TLR9 ligands were most effective.

3.2.4.2. CpG-ODN induces both stimulatory and inhibitory effects on FVIII-specific memory responses

When we studied the modulatory effects of CpG-ODN at different concentrations, it appeared that CpG-ODN expressed a biphasic effect. At 100ng/mL, CpG-ODN amplified the memory response at stimulatory concentrations of FVIII and countered the inhibition at high concentrations of FVIII (Figures 1 and 2). Analysis of antibody isotypes and IgG subclasses of antibodies secreted by FVIII-specific ASC showed that CpG-ODN at 100ng/mL amplified the differentiation of memory B cells into ASC of all IgG

subclasses but did not stimulate or even downregulate the differentiation into ASC of IgM and IgA isotypes (Figure 2). The analysis of T-cell cytokines secreted into cell culture supernatants during differentiation of memory B cells into anti-FVIII ASC revealed a downregulation of IL-4 and an upregulation of IFN-γ (Figure 3), which supports the idea that CpG-ODN polarizes FVIII-specific memory responses towards Th1-driven responses. Furthermore, IL-6 and IL-10 were upregulated.

CpG-ODN lost its stimulatory potential at high concentrations (1,000ng/mL and 10,000ng/mL). It inhibited FVIII-specific memory responses at low concentrations of FVIII and was no longer able to counter the inhibition of memory responses at high concentrations of FVIII (Figure 1). Analysis of cytokines secreted into cell culture supernatants during differentiation into ASC revealed a downregulation of IL-4, a downregulation of IFN-γ, no change in IL-10 and an upregulation of IL-6 when effects of 1,000ng/mL CpG-ODN were compared with those of 100ng/mL CpG-ODN (Figure 3).

Thus, CpG-ODN seemed to exert a dual function, either stimulatory or inhibitory, depending on the concentration applied.

3.2.4.3. Both positive and negative immunomodulatory effects of CpG-ODN are because of specific interactions with TLR9

The question arose whether inhibitory effects of high concentrations of CpG-ODN were because of specific interactions with TLR9 or caused by unspecific, eg toxic, effects. To address this question, we used a TLR9 blocking agent that prevented binding of CpG-ODN to TLR9. If inhibitory effects of high-dose CpG-ODN were because of specific interactions with TLR9, the addition of a TLR9 blocking agent should prevent these effects. Our results indicated that this was indeed the case. The addition of the TLR9 blocking agent prevented both the amplifying effect of low-dose and the inhibitory effect of high-dose CpG-ODN. Furthermore, a negative control DNA (GpC-DNA) did not show any modulating activities (Figures 4A and 4B). The control DNA had a similar sequence to CpG-ODN but the CpG motifs were replaced by GpC sequences, which do not have agonistic activity for TLR9.

Thus, the biphasic immunomodulatory effect of CpG-ODN was because of specific interactions with TLR9.

3.2.4.4. CpG-ODN rescues FVIII-specific memory responses suppressed by high concentrations of FVIII even when added 24 hours after FVIII

We showed that CpG-ODN at concentrations of 100ng/mL countered the inhibition of FVIII-specific memory responses by high concentrations of FVIII. The question arose if CpG-ODN could rescue FVIII-specific memory responses only when added together with FVIII or also when added at later time points. Results presented in Figure 5 demonstrate that CpG-ODN could counter the inhibition of FVIII-specific memory responses both when added together with FVIII and when added 24 hours after FVIII. CpG-ODN could rescue FVIII-specific memory responses to some degree even when added 48 hours after FVIII. However, no rescue was possible at later time points. For comparison, we tested the ability of CpG-ODN to amplify FVIII-specific memory responses at stimulating concentrations of FVIII. Our results indicate that CpG-ODN amplified the response at all time points investigated. However, the absolute number of FVIII-specific ASC decreased between days 6 and 11 after initiation of memory-B-cell restimulation, which was probably because of the limited viability of ASC differentiated from FVIII-specific memory B cells in vitro and reflects the limit of the in vitro system (Figure 5). In conclusion, CpG-ODN at 100ng/mL abrogated the inhibitory effect of high concentrations of FVIII even when added up to 2 days after addition of FVIII and amplified the stimulatory effect of low concentrations of FVIII at all time points investigated.

3.2.4.5. Modulation of FVIII-specific immune responses by CpG-ODN in vivo

Based on the data obtained in vitro, we asked whether the positive and negative regulatory effects of CpG-ODN on FVIII-specific immune responses would also be observed in vivo. Hemophilic mice were treated i.v. with 1 dose of 200ng FVIII, which does not induce detectable levels of circulating anti-

FVIII antibodies but primes the immune system for further exposure to FVIII (24, 26). 1 week after the first dose of FVIII, mice were treated with either of the following: PBS buffer (negative control), FVIII, FVIII together with different doses of CpG-ODN. Both i.p. and i.v. applications of CpG-ODN were tested. Mice treated with 200ng FVIII only developed detectable levels of circulating anti-FVIII antibodies. The antibody response was amplified when mice were treated with 1000ng FVIII (Figure 6). No modulation of anti-FVIII immune responses was observed when CpG-ODN was given i.p. (data not shown). However, a dose-dependent immunomodulatory effect became apparent when CpG-ODN was given i.v. 50 µg of CpG-ODN inhibited FVIII-specific immune responses in 50-80% of hemophilic mice treated. 5µg CpG-ODN still inhibited FVIII-specific immune responses but they were less pronounced than after 50µg. Lower doses of CpG-ODN either slightly amplified the FVIII-specific immune response (0.5µg, Figure 6) or did not show any effect (0.05µg and 0.005µg, data not shown). These results demonstrate that CpG-ODN given in vivo induces similar biphasic effects on anti-FVIII immune responses to those of CpG-ODN given in vitro. However, the stimulatory effect of low-dose CpG-ODN seemed less pronounced in vivo than that observed in vitro (compare Figures 1 and 6), which could be because of a rapid degradation of CpG-ODN after i.v. application (27).

Thus, CpG-ODN expresses biphasic immunomodulatory effects on anti-FVIII immune responses both in vitro and in vivo.

3.2.4.6. CpG-ODN in the absence of FVIII does not induce differentiation of FVIII-specific memory B cell

Previous reports suggested that memory B cells become activated to differentiate into ASC by TLR agonists in the absence of antigen (17, 20). Based on these results, one could speculate that FVIII-specific memory B cells in patients might become activated during viral or bacterial infections in the absence of any replacement therapy with FVIII-containing products. Therefore, we asked whether FVIII-specific memory B cells could be restimulated by CpG-ODN in the absence of FVIII. We tested a range of concentrations of CpG-ODN using the $CD138^-$ spleen cell culture system. We did not see an induction of memory-B-cell differentiation at any of the

concentrations tested (Figure 7A). In some experiments we observed a few spots in the ELISPOT assay. However, this was also occasionally seen in the medium controls. Therefore, we conclude that CpG-ODN either does not stimulate the differentiation of FVIII-specific memory B cells in the absence of FVIII or only stimulates the differentiation to a limited extent that cannot be clearly differentiated from the background signals.

We next asked whether other TLR ligands could restimulate FVIII-specific memory B cells in the absence of FVIII. Our results indicate that only ligands for TLR4 and TLR7 induced weak positive signals and this was only occasionally (Figure 7B). We then asked whether we would observe any restimulation of FVIII-specific memory B cells by TLR agonists in the absence of FVIII in vivo. We used 2 different methods for this purpose. In the first set of experiments, we treated hemophilic mice with 1 dose of FVIII to prime FVIII-specific immune responses and treated them with different doses of TLR agonists 1 week after dosing with FVIII. We did not see any detectable levels of anti-FVIII antibodies in the circulation (data not shown). In the second set of experiments, we treated hemophilic mice with 4 doses of FVIII and isolated $CD138^-$ spleen cells containing FVIII-specific memory cells as described (7, 8). We transferred the $CD138^-$ spleen cells into naïve hemophilic mice and treated mice 1 day after transfer with either of the following: PBS buffer (negative control), FVIII only, Loxoribine (TLR7 agonists that had induced weak effects in vitro) only, FVIII plus Loxoribine. We measured anti-FVIII antibodies in the circulation 3, 7 and 21 days after treatment. The presence of anti-FVIII antibodies in the circulation indicated the restimulation and differentiation of transferred FVIII-specific memory B cells into anti-FVIII ASC. Loxoribine amplified the restimulation of FVIII-specific memory responses in the presence of FVIII but did not stimulate any memory responses in the absence of FVIII (Figure 7C).

In conclusion, in vivo FVIII-specific memory B cells were not restimulated either by CpG-ODN or by any other TLR ligand tested in the absence of FVIII.

3.2.5. Discussion

Restimulation of FVIII-specific memory B cells is sensitive to increasing doses of FVIII. Low doses restimulate memory B cells and induce their differentiation into anti-FVIII ASC, but high doses inhibit restimulation (8). Here, we asked how stimulation and inhibition of FVIII-specific memory-B-cell responses by FVIII are modulated by concurrent activation of the innate immune system through TLR agonists. Initial screening of agonists for TLR 2, 3, 4, 5, 7 and 9 indicated that all agonists induced some degree of amplification of FVIII-specific memory responses in the presence of stimulating concentrations of FVIII. Amplification could be because of either direct effects by stimulating TLR expressed on memory B cells or indirect effects by stimulating TLR expressed on other immune cells such as macrophages, dendritic cells or lymphocytes. TLR activation initiates intracellular signaling pathways (16, 28) that induce the expression of genes encoding proinflammatory cytokines (IL-1, IL-6, TNF-alpha, IL-12) or type I interferons. Both were shown to either directly or indirectly affect B-cell responses (29-31). Moreover, TLR signaling induces the upregulation of maturation markers and co-stimulatory molecules such as CD80, CD83 and CD86 on dendritic cells (32), which could amplify the stimulation of $CD4^+$ T cells required forthe induction of memory-B-cell differentiation. In view of these findings, it is not surprising that all TLR agonists tested modulate FVIII-specific memory responses to some degree.

Agonists for TLR7 and TLR9 induced the strongest modulation. They amplified the restimulation of FVIII-specific memory B cells in the presence of stimulatory concentrations of FVIII and countered the inhibition in the presence of inhibitory concentrations of FVIII. TLR7 and TLR9 are expressed in a range of different murine immune cells, for example dendritic cells, macrophages, naïve B cells and memory B cells (33, 34). Therefore, agonists for TLR7 and TLR9 could act on FVIII-specific memory B cells directly and indirectly. Their indirect action could be via triggering TLR7 and TLR9 expressed in dendritic cells or macrophages, thereby inducing the release of pro-inflammatory cytokines and type I interferon.

When we tested different concentrations of CpG-ODN, we observed biphasic and concentration-dependent effects. Whereas 100ng/mL amplified the memory response, 1000ng/mL and 10,000ng/mL inhibited the response. Both stimulation and inhibition of memory responses were because of specific interactions with TLR9 and were seen both in vitro and in vivo. The stimulatory effect of CpG-ODN in vivo was rather weak compared with the effect seen in vitro. This might be because of rapid degradation of CpG-ODN or ineffective delivery into intracellular compartments of TLR9-expressing cells in vivo (27). Previous studies have shown that CpG-ODN and antigen (FVIII in our study) need to be delivered to the same antigen-presenting cell to express the full stimulatory activity. Different strategies for in vivo co-delivery of antigen and CpG-ODN were developed (27, 35) but it would have been beyond the scope of this study to develop such co-delivery systems for FVIII and CpG-ODN.

The amplification of memory-B-cell restimulation in vitro was associated with a downregulation of IL-4 and an upregulation of IFN-γ in cell culture supernatants, which suggests a predominant amplification of a Th1-type immune response by CpG-ODN and confirms data published by other groups using different systems (35).

The inhibitory effect of high-dose CpG-ODN raises a question about the mechanism responsible for this effect. Recent data suggested that CpG-ODN given systemically at high doses induces the enzyme indoleamine 2,3-dioxygenase (IDO) in immune cells, particularly in dendritic cells (36-38). IDO expresses immunosuppressive activities caused by both tryptophan deprivation and the production of kynurenines, which act on IDO^- dendritic cells and render an otherwise stimulatory dendritic cell capable of regulatory effects (39). IDO^+ dendritic cells could induce regulatory T cells that would prevent the activation of $CD4^+$ T cells required for restimulation of FVIII-specific memory B cells. Our in vitro data show that high-dose CpG-ODN induced a reduction of IFN-γ release into culture supernatants, which indicates a decreased activation of Th1 cells and hints at an inhibition of memory-B-cell differentiation because of an inhibition of $CD4^+$ T-cell

activation. In addition, negative regulators of TLR signaling could contribute to the inhibitory effects observed with high-dose CpG-ODN. A range of intracellular signaling molecules have been described as negative regulators of TLR signaling (40, 41) and could, therefore, contribute to the negative regulatory effects of high doses of CpG-ODN. However, our in vitro cytokine release data revealed an amplification of IL-6 release in cultures supplemented with inhibitory concentrations of CpG-ODN. IL-6 is one of the major indicators of the stimulation of TLR-triggered signal transduction pathways, which would argue against an inhibition of these pathways by negative regulators.

Alternatively, inhibition of immune responses could be mediated by the induction of IL-10, an immunoregulatory cytokine that has been shown to limit CpG responses (42). However, our in vitro cytokine release data show an increase in IL-10 release at stimulatory concentrations of CpG-ODN but no further increase at inhibitory concentrations of CpG-ODN. Therefore, at least in vitro it seems unlikely that IL-10 is involved in the inhibitory effect of high-dose CpG-ODN.

The exact mechanisms responsible for the inhibition of FVIII-specific memory responses by high-dose CpG-ODN are currently being investigated.

CpG-ODN at stimulatory concentration of 100ng/mL not only amplified the restimulation of FVIII-specific memory B cells in the presence of stimulatory concentrations of FVIII but also countered the inhibition caused by high concentrations of FVIII. The exact mechanisms responsible for the inhibitory effect of high concentrations of FVIII have not been comprehensively studied. However, we previously reported that a pan-caspase inhibitor prevented the inhibitory effects, which indicates that inhibition involves the induction of apoptosis (8). The question arises whether triggering of TLR9 by CpG-ODN might generate survival signals for FVIII-specific memory B cells that prevent the induction of apoptosis by high concentrations of FVIII. CpG-ODN has been shown to protect B cells, macrophages and plasmacytoid dendritic cells against apoptosis (43, 44). Recently, Kuo et al demonstrated that CpG-ODN upregulates Hsp90β in a TLR9/MyD88/PI3K-dependent pathway. Furthermore, they provided evidence that CpG-ODN induces its anti-

apoptotic effect by stimulating the binding of Hsp90β to bcl-2, thereby increasing the antiapoptotic activity of bcl-2, namely the inhibition of cytochrome c release and the prevention of caspase-3 activation (45). These findings could explain why CpG-ODN counters the inhibition of FVIII-specific memory-B-cell differentiation by high concentrations of FVIII. In conclusion, one would expect that an inhibition of memory-B-cell differentiation by high doses of FVIII in vivo could be countered by microbial infections that would trigger TLR9 in these cells. However, the outcome of such a scenario would probably depend on the strength of signals that are induced by high-dose FVIII on the one hand and TLR9 agonists on the other hand.

Despite its strong immunostimulatory activity in the presence of FVIII, CpG-ODN induced little restimulation in the absence of FVIII. The number of anti-FVIII ASC after restimulation of spleen cell cultures with stimulatory concentrations of CpG-ODN in the absence of FVIII in vitro was similar to or slightly above the background of negative control cultures. Furthermore, we never observed any restimulation of FVIII-specific memory responses in the absence of FVIII in vivo, either after i.v. or after i.p. application of CpG-ODN. For comparison, we tested agonists for TLRs 2, 3, 4, 5 and 7 in vitro and agonists for TLR 7 in vivo and came to similar conclusions. Although we observed weak effects in the absence of FVIII, when we tested agonists for TLR4 and 7 in vitro, we never observed any in vivo effects in the absence of FVIII. These results contrast with the findings of Bernasconi et al in the human system (17). The authors demonstrated that human memory B cells differentiate into plasma cells in response to polyclonal stimuli such as bystander T-cell help or CpG-ODN in vitro. Furthermore, they showed that antibodies to recall antigens are produced in vivo, even years after antigenic stimulation. Based on their results, the authors hypothesized that quiescent memory B cells are periodically activated by TLR agonists or bystander T-cell help to undergo self-renewal and differentiate into ASC in the absence of antigen. However, our results agree with recent results reported by Benson et al, who demonstrated that murine memory B cells neither clonally expand nor differentiate into ASC in response to inflammatory stimuli such as TLR agonists, polyclonal T-cell activation, protein vaccination or even acute

vaccinia virus infection in the absence of specific antigen in vivo (46). A number of additional studies in mice and humans provided data that could be interpreted either in favor of (47) or against (48-50) the idea that memory B cells respond to bystander inflammatory signals in the absence of the specific antigen. Clearly, our data do not support this theory. However, we cannot exclude the possibility of other inflammatory signals which were not included in our study that would be able to restimulate FVIII-specific memory B cells in the absence of FVIII.

Summarizing our data, we conclude that TLR agonists, in particular agonists for TLR7 and 9, can modulate the outcome of an anamnestic antibody response against FVIII. Depending on the actual conditions, this modulation can cause amplification or inhibition of the antibody response. However, it is difficult to predict whether natural infections would induce local concentrations of TLR agonists sufficient to induce inhibition of antibody responses.

Furthermore, we conclude that at least in the murine system it is unlikely that FVIII-specific memory B cells are restimulated by TLR agonists in the absence of FVIII. Future studies will show if this conclusion can be extended to the human immune system in patients where it would be relevant for both natural infections and vaccinations.

3.2.6. Acknowledgements

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3.2.7. Figures

Table 1

TLR-Ligand:	Control	Zymosan - TLR 2	Poly (I:C) - TLR 3	LPS - TLR 4	Flagellin - TLR 5	Loxoribine - TLR 7	Imiquimod - TLR 7	CpG-DNA - TLR 9
Conc. tested:		0.1 - 10,000 ng/ml	1 - 50,000 ng/ml	0.1 - 10,000 ng/ml	0.01 - 1,000 ng/ml	1 - 50,000 ng/ml	1 - 50,000 ng/ml	0.1 - 10,000 ng/ml
Conc. at max. stimulation:		10,000 ng/ml	1,000 - 10,000 ng/ml	1 - 100 ng/ml	1-1000 ng/ml	10,000-50,000 ng/ml	100-1,000 ng/ml	100 ng/ml
FVIII [ng/ml]								
0	0 (0-1)	1 (1)	1 (1)	2 (1-3)	1 (1)	9 (2-12)	2 (1-3)	3 (0-6)
10	100	71 (21-121)	263 (227-298)	199 (144-292)	91 (35-146)	478 (196-1023)	794 (205-1118)	676 (132-1884)
1,000	7 (3-10)	9 (5-13)	31 (28-34)	87 (42-131)	53 (11-94)	212 (187-260)	174 (126-220)	198 (44-320)
20,000	0 (0-2)	3 (1-5)	2 (1-2)	2 (1-3)	2 (1-3)	57 (12-112)	38 (19-59)	135 (24-194)
	n=10	n=2	n=2	n=2-3	n=2	n=3-5	n=3-4	n=4-5

Table 1. Screening of TLR ligands

CD138$^-$ spleen cells were obtained from hemophilic mice treated with 4 weekly doses of 200ng FVIII and restimulated in vitro with FVIII at the concentrations indicated in the presence of TLR ligands in the range of concentrations shown. Newly formed anti-FVIII ASC were detected by ELISPOT assay after 6 days of culture.

All results were normalized to compare experiments done on different days. Results obtained in differentiation cultures containing 10ng/mL FVIII only were always set as 100%. The results shown represent the highest response obtained (mean value and range) for each ligand tested.

Conc.: concentration; Max.: maximum

Figure 1

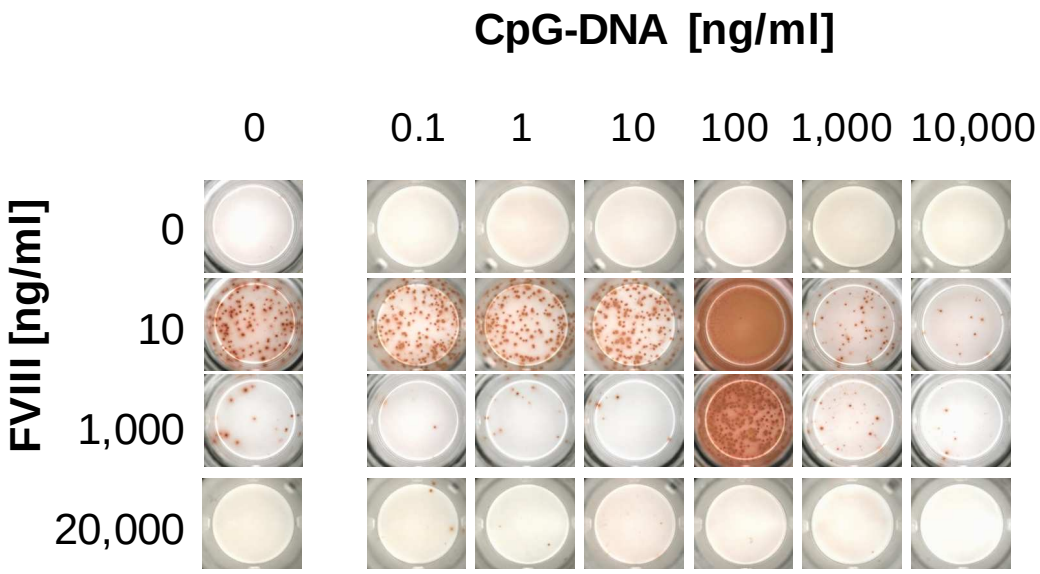


Figure1. Screening panel for in vitro restimulation of FVIII-specific memory B cells in the presence of FVIII and CpG. $CD138^-$ spleen cells were obtained from hemophilic mice treated with 4 weekly doses of 200ng FVIII and restimulated with FVIII in the presence of CpG-ODN. Newly formed anti-FVIII ASC were detected by ELISPOT assay after 6 days of culture. Each spot represents one anti-FVIII ASC. Concentrations of FVIII and CpG-ODN are indicated. A representative ELISPOT is presented.

Figure 2

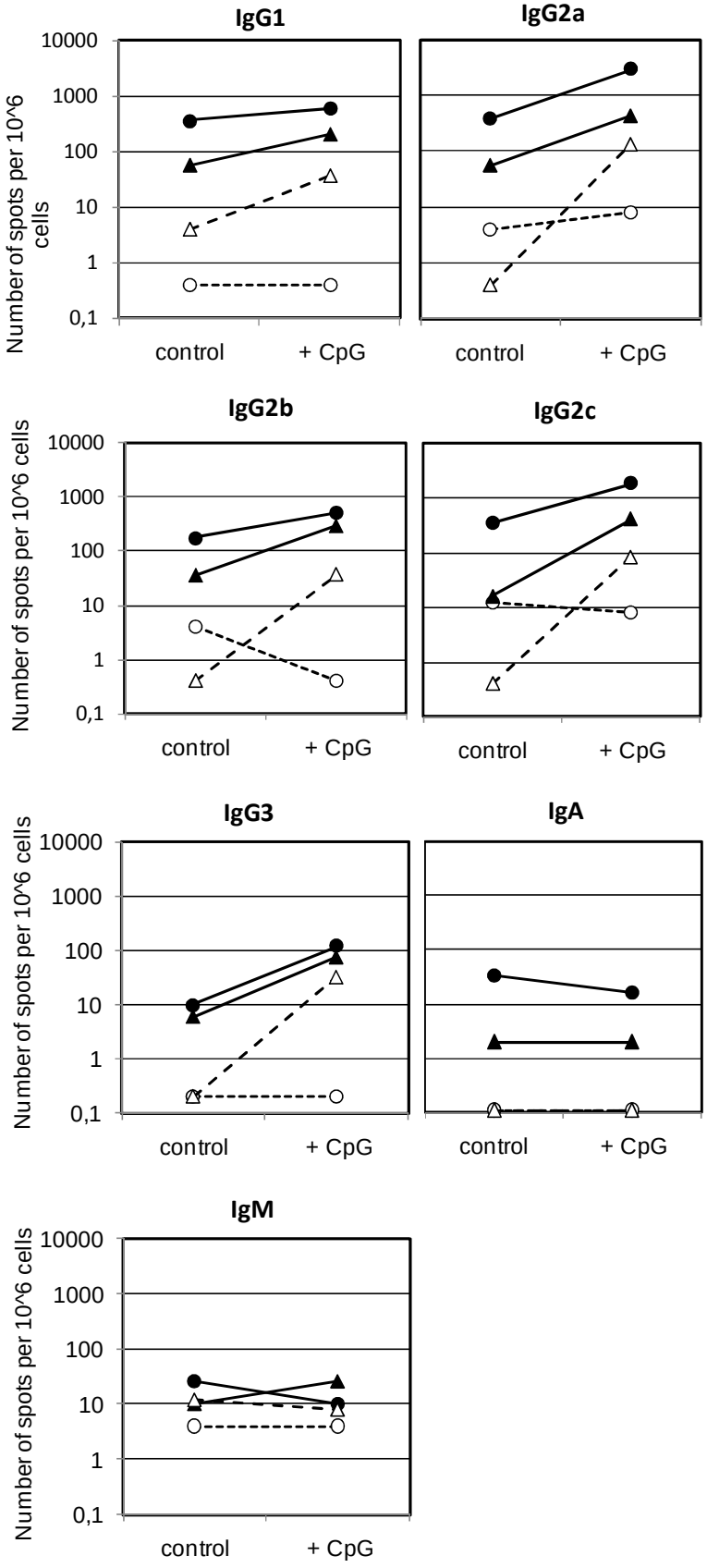

IgG1
IgG2a
IgG2b
IgG2c
IgG3
IgA
IgM
Number of spots per 10^6 cells
10000
1000
100
10
1
0,1
control
+ CpG

Figure 2. Ig isotypes and IgG subclasses of anti-FVIII ASC in vitro differentiated in the presence of FVIII and 100ng/mL CpG-ODN. $CD138^-$ spleen cells were obtained from hemophilic mice treated with 4 weekly doses of 200ng FVIII and restimulated with FVIII in the presence of medium (control) or 100ng/mL CpG-ODN (CpG). Newly formed anti-FVIII ASC were detected by ELISPOT assay after 6 days' culture. Arithmetic means of a representative experiment are presented.
medium control without FVIII (○); 10ng/mL FVIII (●); 1µg/mL FVIII (▲); 20µg/mL FVIII (△)

Figure 3

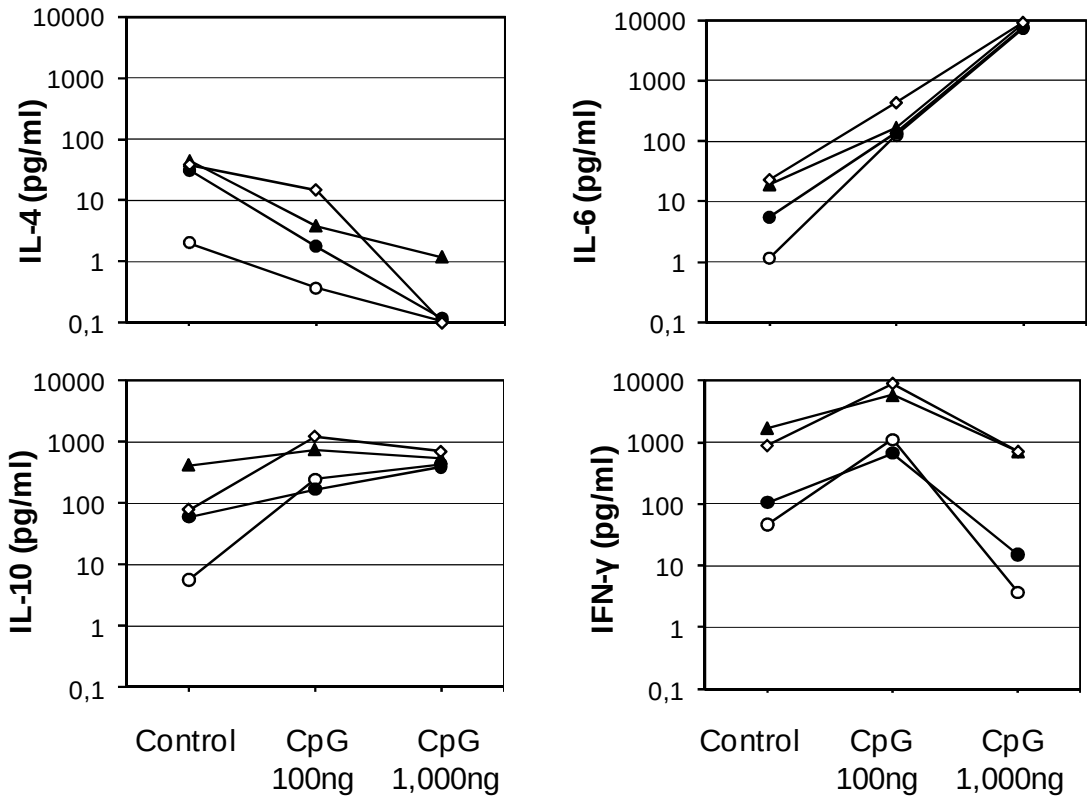


Figure 3. Cytokine release into culture supernatants during in vitro differentiation of FVIII-specific memory B cells into anti-FVIII ASC.

$CD138^-$ spleen cells were obtained from hemophilic mice treated with 4 weekly doses of 200ng FVIII and restimulated with FVIII in the presence of medium (control), 100ng/mL or 1,000ng/mL CpG-ODN. Culture supernatants were taken after 6 days' culture and analyzed for cytokines. Arithmetic means of a 2-5 experiments are presented.

medium control without FVIII (○); 10ng/mL FVIII (●); 1µg/mL FVIII (▲); 20µg/mL FVIII (◇)

Figure 4

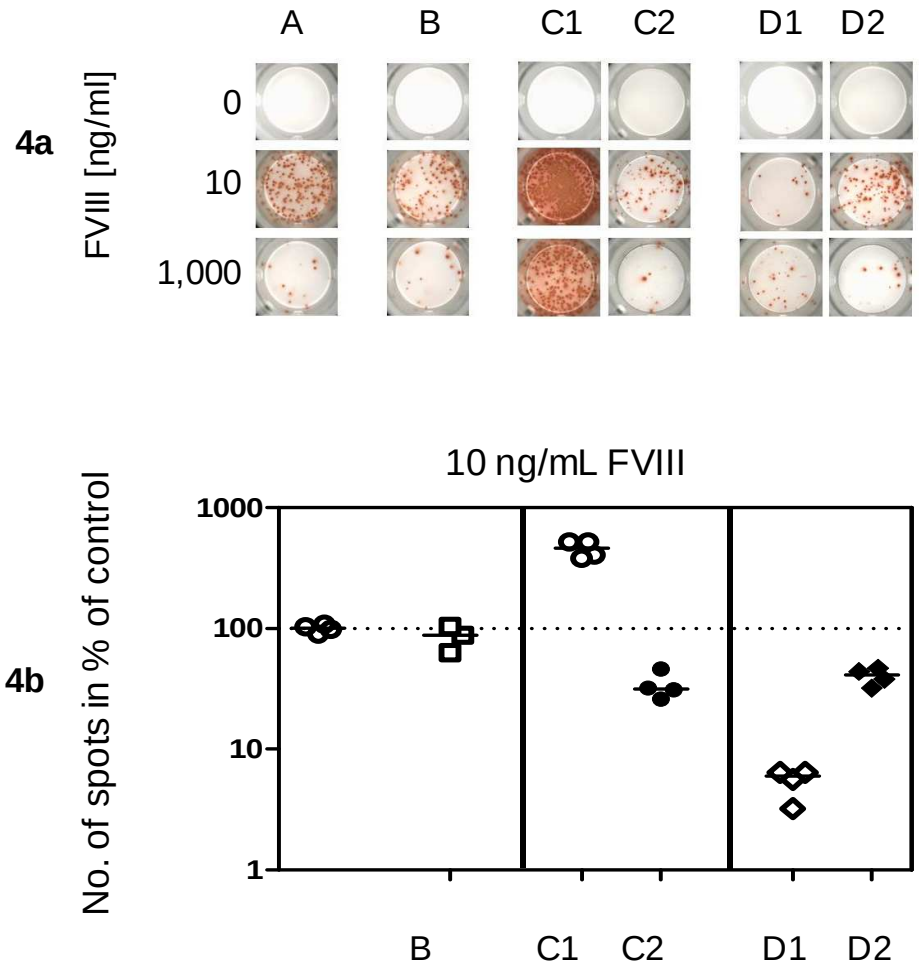


Figure 4. Both stimulatory and inhibitory activities of CpG-ODN are caused by specific interactions with TLR9. $CD138^-$ spleen cells were obtained from hemophilic mice treated with 4 weekly doses of 200ng FVIII and restimulated in vitro with FVIII in the presence of CpG-ODN or controls. Newly formed anti-FVIII ASC were detected by ELISPOT assay after 6 days' culture.

4a: Representative ELISPOT assay. Each spot represents one anti-FVIII ASC.

Cells were differentiated in the presence of A) FVIII only; B) 100ng/mL GpC-ODN (negative control of CpG-ODN); C1) 100ng/mL CpG-ODN only; C2) 100ng/mL CpG-ODN together with TLR9 blocking agent; D1) 1,000ng/mL

CpG-ODN only; D2) 1,000ng/mL CpG-ODN together with TLR9 blocking agent.

4b: Quantitative evaluation of results presented in 4A for 10ng/mL FVIII. Results of cultures differentiated in the presence of FVIII only (A in 4A) were set to 100% and presented as a dotted line. B) 100ng/mL GpC-ODN (negative control of CpG-ODN); C1) 100ng/mL CpG-ODN only; C2) 100ng/mL CpG-ODN together with TLR9 blocking agent; D1) 1,000ng/mL CpG-ODN only (D1); D2) 1,000ng/mL CpG-ODN together with TLR9 blocking agent.

Results of individual ELISPOT-analyses and the median of all individual results for each group are presented.

Figure 5

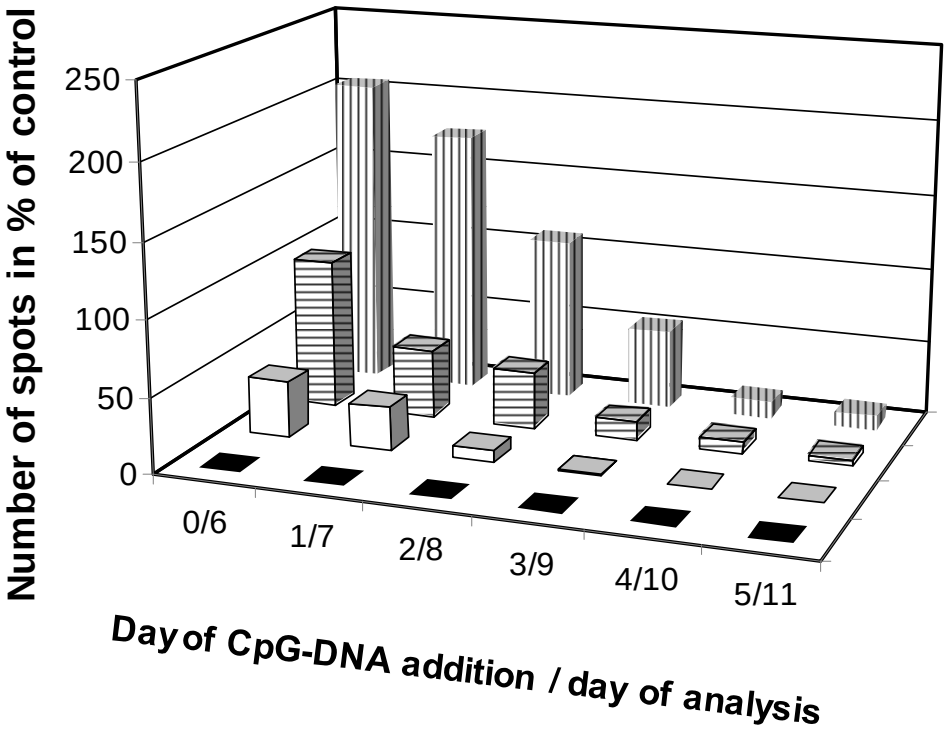


Figure 5. Immunomodulatory activity of CpG-ODN in vitro when added with a time-delay. $CD138^-$ spleen cells were obtained from hemophilic mice treated with 4 weekly doses of 200ng FVIII and restimulated with either 20µg/mL FVIII (A and B) or 10ng/mL FVIII (C and D). 100ng/mL CpG-ODN or medium were added either together with FVIII on day 0 or at different times (days 1-5) after FVIII. Newly differentiated anti-FVIII ASC were analyzed by ELISPOT assay 6 days after addition of CpG-ODN. All results were normalized in relation to results obtained with 10ng/mL FVIII without CpG-ODN (0/6), which was set to 100%. Arithmetic means of a representative experiment are presented.

20µg/mL FVIII without CpG-ODN (A); 20µg/mL FVIII + 100ng/mL CpG-ODN (B); 10ng/mL FVIII without CpG-ODN (C); 10ng/mL FVIII + 100ng/mL CpG-ODN (D)

Figure 6

6A: Treatment schedule for figure 6B (all i.v.)

label	day 1	day 8	day 9
A	200ng FVIII	PBS buffer	PBS buffer
B	200ng FVIII	PBS buffer	200ng FVIII
C	200ng FVIII	PBS buffer	1,000ng FVIII
D	200ng FVIII	0.5µg CPG-ODN	200ng FVIII + 0.5µg CpG-ODN
E	200ng FVIII	5µg CPG-ODN	200ng FVIII + 5µg CpG-ODN
F	200ng FVIII	50µg CPG-ODN	200ng FVIII + 50µg CpG-ODN

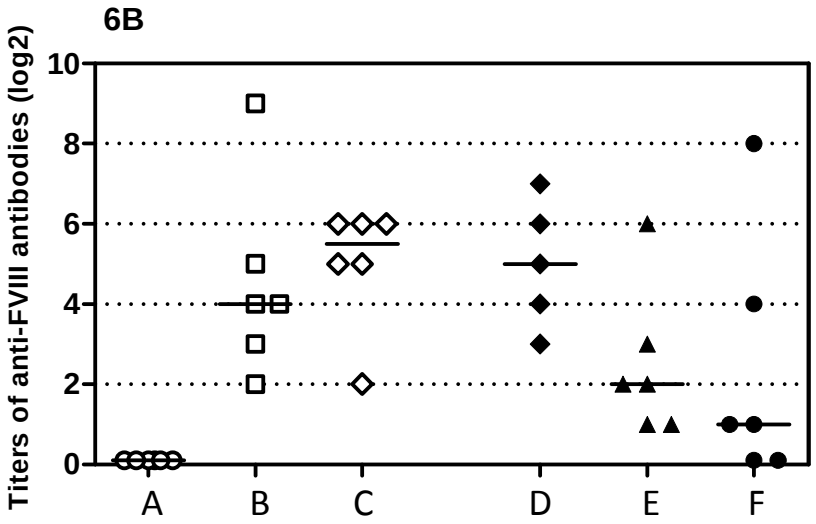


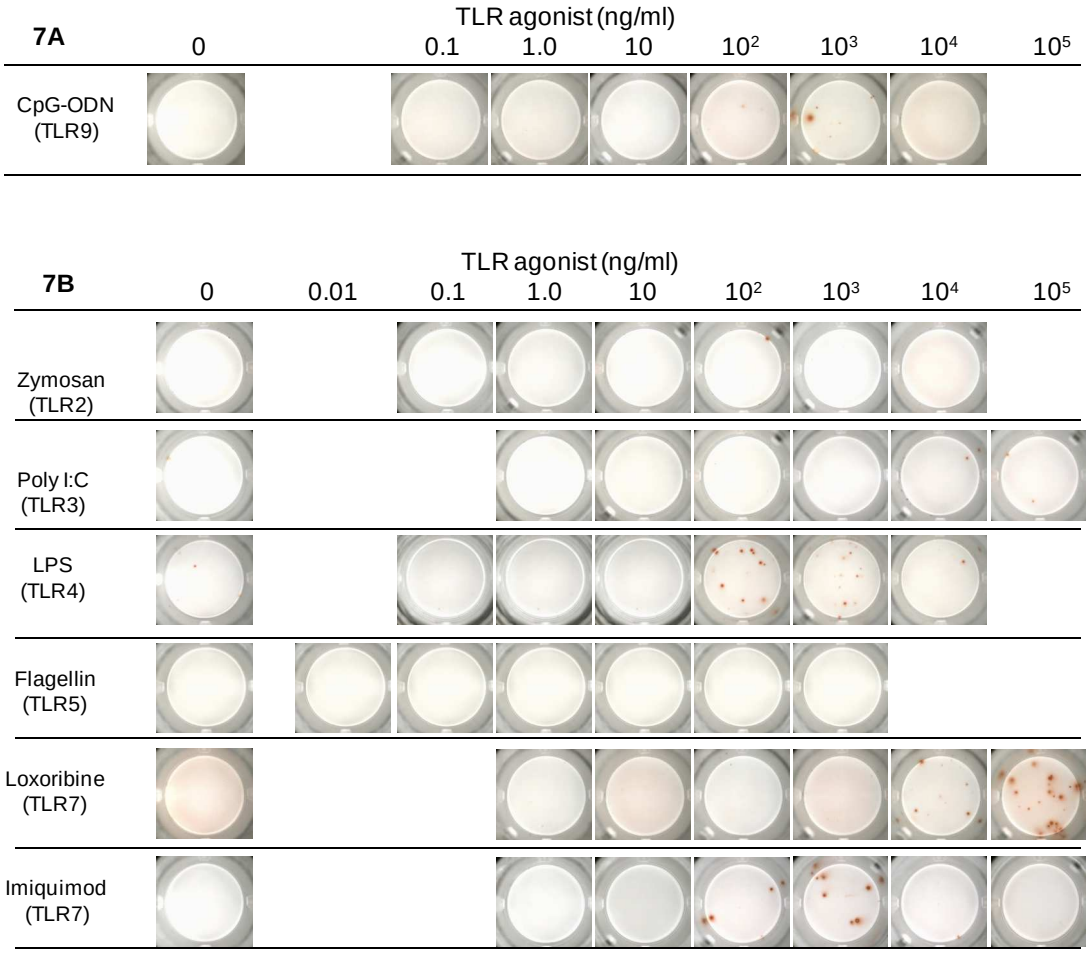
Figure 6. Modulation of anti-FVIII immune response by CpG-ODN in vivo. Hemophilic mice were treated i.v. with one dose of 200ng FVIII on day 1 to prime the immune system for further exposure to FVIII**.** On day 8, mice received either buffer or CpG-ODN to stimulate the innate immune system. On day 9, mice received either PBS buffer, FVIII or FVIII together with different doses of CpG-ODN. On day 16, blood samples were taken for the analysis of circulating anti-FVIII antibodies.

6A: Treatment schedule.

6B: Titers of anti-FVIII antibodies together with medians for each group. Each point represents the results of an individual mouse.

Results presented were confirmed in 2 independent experiments.

Figure 7


7A
TLR agonist (ng/ml)
0
0.1
1.0
10
10²
10³
10⁴
10⁵
CpG-ODN (TLR9)
7B
TLR agonist (ng/ml)
0
0.01
0.1
1.0
10
10²
10³
10⁴
10⁵
Zymosan (TLR2)
Poly I:C (TLR3)
LPS (TLR4)
Flagellin (TLR5)
Loxoribine (TLR7)
Imiquimod (TLR7)

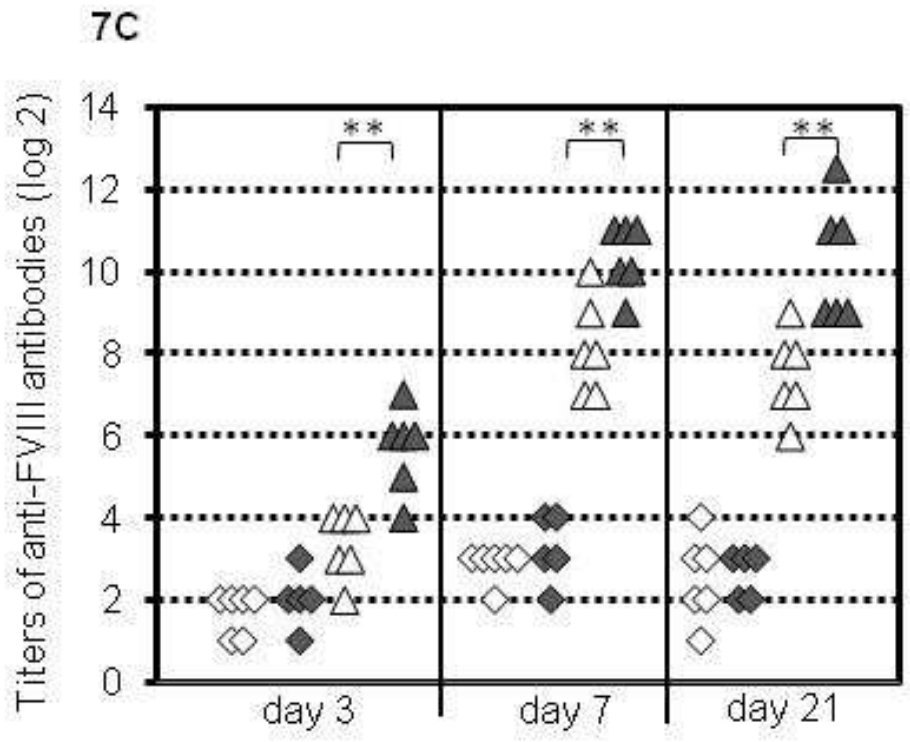

7C
Titers of anti-FVIII antibodies (log 2)
14
12
10
8
6
4
2
0
**
**
**
day 3
day 7
day 21

Figure 7. Restimulation of FVIII-specific memory responses by TLR agonists in the absence of FVIII in vitro and in vivo. 7A and 7B: $CD138^-$ spleen cells were obtained from hemophilic mice treated with 4 weekly doses of 200ng FVIII and restimulated in vitro with CpG-ODN (7A) or ligands for TLR2, 3, 4, 5, and 7 (7B) in the absence of FVIII (Loxo.=Loxoribine). Newly formed anti-FVIII ASC were detected by ELISPOT assay after 6 days' culture. Each spot represents one anti-FVIII ASC. Concentrations of CpG-ODN (7A) and ligands for TLR2, 3, 4, 5, and 7 (7B) are indicated. Representative ELISPOTs are presented.

7C: $CD138^-$ spleen cells were obtained from hemophilic mice treated with 4 weekly doses of 200ng FVIII and i.v. injected into naïve hemophilic mice. One day after cell transfer, mice were injected with a single dose of PBS buffer (◇), PBS + 1,000 µg Loxoribine (◆), 200ng FVIII (△) or 200ng FVIII + 1,000 µg Loxoribine (▲). TLR ligands were given i.p., PBS buffer and FVIII were given i.v. 3, 7 and 21 days after application, blood samples were taken for the analysis of circulating anti-FVIII antibodies. Each point represents the results of an individual mouse. ** $P<0.05$

3.2.8. References for Chapter 3.2

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3.3. Modulation of FVIII-specific memory Responses by Toll-like Receptor Ligand – A Screening Platform

3.3.1. Abstract

A major complication in the treatment of patients suffering from Hemophilia A is the development of neutralizing antibodies against factor VIII (FVIII). Memory B cells are key players in an established antibody response such as the presence of neutralizing antibodies against FVIII. Memory B cells accomplish rapid formation of antibodies upon re-exposure to the antigen. The signals controlling the re-stimulation of memory B cells have not been fully explained. The ob jective of our study was the elucidation of the role that Toll-like receptors (TLR) play in the modulation of the re-stimulation of FVIII-specific memory B cells. For this purpose we established an *in vitro* screening platform using FVIII-specific memory B cells obtained from a murine hemophilia A model. This screening platform was found to be suitable to study qualitative and quantitative changes in the re-stimulation of factor VIII-specific memory B cells induced by TLR ligands in the presence or absence of FVIII in vitro.

3.3.2. Introduction

Hemophilia A is a severe hemorrhagic bleeding disorder caused by mutations in the factor VIII (FVIII) gene. As a standard therapy, patients are substituted with FVIII concentrates intravenously. A major complication in this therapy is the formation of neutralizing antibodies against FVIII (FVIII inhibitors), which occurs in approximately 25 to 30% of patients with severe disease.[1 2]

Once an immune response to FVIII is established, memory B cells play a key role in the formation of FVIII inhibitors: upon re-exposure to the specific antigen FVIII. They differentiate into plasma cells which produce anti-FVIII antibodies at high levels. This re-stimulation of memory B cells is a fundamentally important mechanism of the adaptive immune system in antibody-dependent immunological reactions. Understanding the

mechanisms steering the processes behind is crucial for developing strategies to effective treatment of patients with inhibitor formation.

In the last few years there have been tremendous advances in the revealing of the interactions between the adaptive and the innate immune system, especially concerning the role of Toll-Like Receptors (TLRs) in antibody responses. TLRs are capable of recognizing evolutionary highly conserved pathogen-associated molecular patterns (PAMPs). Triggering of TLR by their ligands leads to the production of cytokines and the induction of inflammatory reactions.[3 4]

In primary antibody response, TLR co-signaling plays a crucial role in the initiation of cellular and humoral immune responses, hence in effective activation of dendritic cells [5 6] and as obligatory co-stimulation signal for B cells.[7]

With established immune response, the re-stimulation and further differentiation of memory-B-cells into antibody-producing plasma cells was shown to be strongly influenced by additional stimulus via TLR.[8 9 10]

To investigate the association between FVIII specific memory B cell re-stimulation and TLR triggering, we established an in vitro screening system using the murine model of hemophila A. Murine hemophila A E-17 mice are characterized by complete deficiency of functional FVIII because of a targeted disruption of exon 17 of the FVIII gene. [13 14 15] Intravenous injection of human FVIII into these mice results in high titers of anti-FVIII antibodies.[11 14]

Hausl et al demonstrated that the re-stimulation of FVIII-specific memory B cells in vitro is proportional to the concentrations of FVIII in ranges up to physiological FVIII levels in plasma. However, further increase of FVIII concentration leads to an inhibition of re-stimulation. [12]

Here, we describe a newly established in vitro screening platform that allows the rapid and comprehensive investigation of specific TLR ligands concerning their properties to modulate the FVIII-specific memory response in the presence and absence of FVIII in vitro.

3.3.3. Materials &Methods

3.3.3.1. Animals

Hemophilic E-17 mice: Our colony of fully inbred hemophilic E-17 mice (characterized by a targeted disruption of exon 17 of the FVIII gene) was established with a breeding pair from the original colony[13] [14] and crossed into the C57BL/6J background as described.[15] All 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.

3.3.3.2. Treatment with human FVIII

If not stated otherwise, hemophilic E-17 mice received four intravenous doses of 200 ng recombinant FVIII (approximately 80 U/kg FVIII), diluted in 200 µl of Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, Irvine, UK), at weekly intervals. The recombinant human FVIII used throughout the studies was albumin-free bulk material obtained from Baxter BioScience (Orth/Donau, Austria)).

3.3.3.3. Tissue sampling

Tissue samples were collected 7 days after the last dose of FVIII if not otherwise indicated. All invasive procedures were carried out under anesthesia with pentobarbital (Nembutal, Richter Pharm, Wels, Austria).

3.3.3.4. Preparation of spleen cells

Spleen cells were prepared as described previously.[16]

3.3.3.5. Re-stimulation of memory B cells in vitro

Spleen cells were isolated, re-suspended in depletion buffer (RPMI 1640

supplemented with 0,1% BSA, 1% glutamine and 1% sodium pyruvate) and depleted of $CD138^+$ antibody secreting cells (plasma cells). For depletion, cells were incubated with a monoclonal rat anti-mouse CD138 antibody (Pharmingen International) coupled to M-450 Sheep anti-rat IgG Dynabeads (Dynal ASA, Oslo, Norway). After 20 minutes of incubation at 4℃, $CD138^+$ cells were depleted using magnetic devices. This procedure was repeated once. Cell counting was done on a Z2 Coulter counter. $CD138^-$ cells, which we called memory cell pool, were cultured at 1,5 x 10^7 cells/ml in RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 10% preselected fetal calf serum (Hyclone, Logan, Utah), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Life Technologies, Paisley, Scotland) and $5x10^{-5}$ M ß-mercaptoethanol (both from Sigma-Aldrich, Irvine, UK)) for 6 days at 37℃ and 5% CO_2. Different concentrations of FVIII were added to the memory cell pool at day 0 as indicated. TLR ligands with potential modulating activities were added together with FVIII at day 0 or at later time points as indicated. After 6 days of culture, newly formed antibody secreting cells were detected by ELISPOT assays as previously described by Hausl et al.[17]

3.3.3.6. TLR Ligands

The following ligands were used to trigger TLRs: Zymosan for TLR 2 (InvivoGen, 0.1 to 10,000 ng/mL); Poly I:C for TLR 3 (InvivoGen, 1 to 50,000 ng/mL), LPS for TLR 4 (InvivoGen, 0.1 to 10,000 ng/mL), Flagellin for TLR 5 (InvivoGen, 0.01 to 1000 ng/mL), Loxoribine and Imiquimod for TLR 7 (InvivoGen, 1 to 50,000 ng/mL), CpG-ODN (CpG-B, ODN1826, sequence 5'-tcc atg acg ttc ctg acg tt -3') for TLR 9 (InvivoGen, 0.1 to 10,000 ng/mL). All TLR ligands were reconstituted according to manufacturers instruction. TLR ligands were added to the cell cultures on day 0 or as indicated.

3.3.3.7. Analysis of anti-FVIII antibody secreting cells in the spleen

Anti-FVIII antibody secreting cells in the spleen were detected by ELISPOT analysis as previously described by Hausl et al.[17]

3.3.3.8. Depletion of T cells

T-cells were depleted from $CD138^-$ spleen cells using mouse pan-T (Thy 1.2) Dynabeads (Dynal ASA, Oslo, Norway). Briefly, the cell pool was incubated with magnetic beads coupled to a monoclonal antibody directed against the Thy-1.2 antigen (mouse CD90.2) which is present on all murine T-cells. After 20 minutes of incubation at 4℃, T-cells were depleted using magnetic devices. This procedure was repeated once.´

3.3.4. Results

The objective of this study was the establishment of a screening platform for the modulation of the re-stimulation of FVIII-specific memory B cells by TLR ligands. Hausl et al showed previsously that FVIII induces either stimulating or inhibiting effects on FVIII-specific memory B cells depending on the concentration of FVIII used.[12] Based on these findings, we used up to four different concentrations of FVIII that included both stimulating and inhibiting concentrations: no FVIII as a control; 10 ng/mL (according to approx 0.1 Units/mL) as a highly stimulating concentraton; 1 µg/mL as a concentration at the lower end of inhibiting concentrations; 20 µg/mL as a very strongly inhibiting concentration.

These amounts of FVIII were tested with and without a range of different TLR ligand. TLR ligands were tested in a a broad range of concentrations that covered up to six orders of magnitude, with serial dilution steps of 1 to 10. The number of memory-B-cell-pool cells seeded in the culture vessel was standardized to $1.5x10^6$/ml. 10 mL cultures were used for all experiments. If not stated otherwise, FVIII and TLR ligand were added at day 0 of the culture periode. After 6 days of culture, cells were harvested and analyzed.

3.3.4.1. Screening platform for TLR2

TLR2 is expressed extracellular and has the capability to recognize PAMPs from various microbes. Important examples for TLR2 ligands are lipoproteins from Gram-negative bacteria, peptidoglycans and lipoteichoic adic from Gram-positiv bacteria and also zymosan which is a cell wall compound of fungi such as *Saccharomyces sp.* [4]

We used Zymosan as ligand of TLR2. Concentrations tested ranged from 0.1 to 10,000 ng/mL (see figure 1).

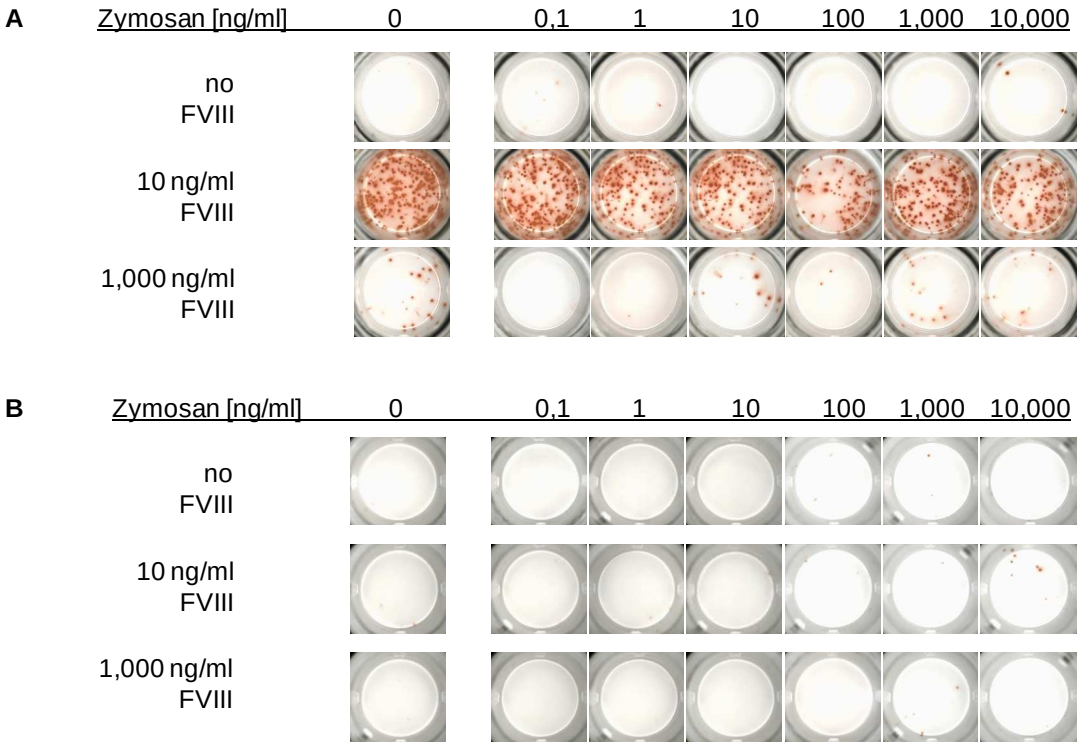

Fig.1: Screening panel for the re-stimulation of FVIII specific memory B cells in the presence and absence of ligands for TLR2.
A: Re-stimulation of FVIII specific Memory B Cells in the presence of T-cells
B: Re-stimulation of FVIII specific Memory B Cells in the absence of T-cells

Results presented in figure 1 demonstrate that the stimulation of TLR2 does not seem to influence the re-stimulation of FVIII specific memory B cells. Furthermore, no restimulation was observed in the absence of T cells.

3.3.4.2. Screening platform for TLR3

TLR3 is expressed intracellularily and recognises double-stranded RNA (dsRNA) which is produced by most viruses during their replication. [4]

In our experiments we used Poly I:C, a synthetic analogue to dsRNA, in a range of concentrations covering 1 to 50,000 ng/mL (see figure 2)

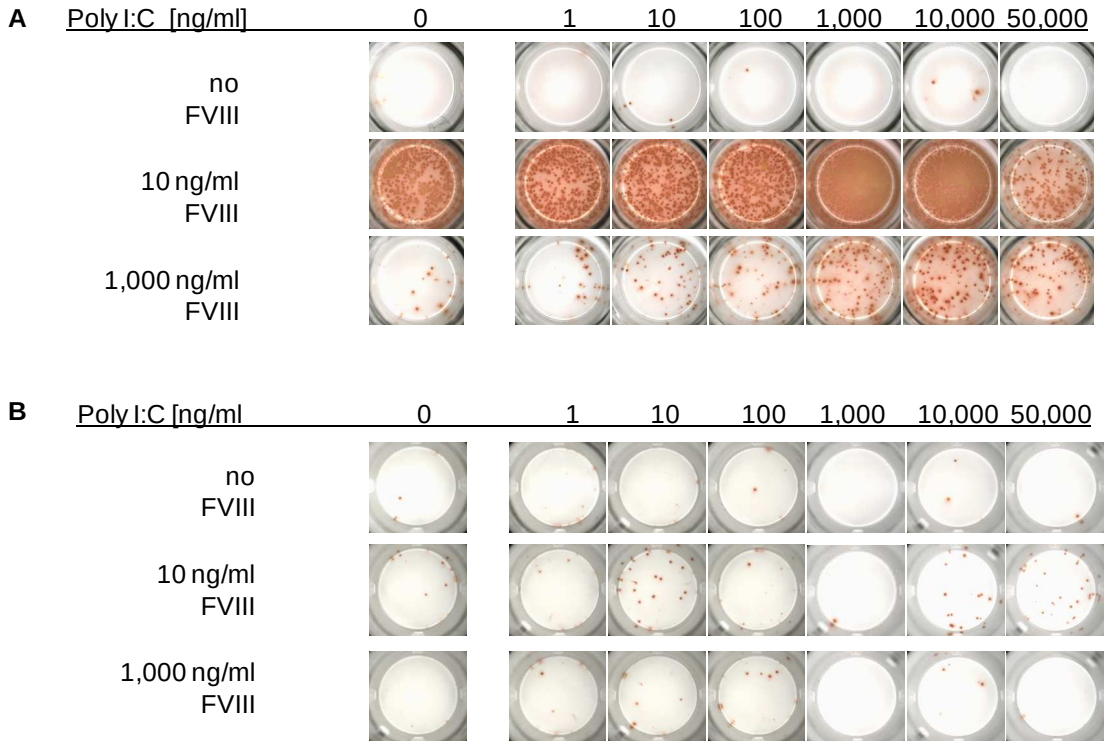

Fig. 2: Screening panel for the re-stimulation of FVIII specific memory B cells in the presence and absence of ligends for TLR3.

A: Re-stimulation of FVIII specific Memory B Cells in the presence of T-cells

B: Re-stimulation of FVIII specific Memory B Cells in the absence of T-cells

Results presented in figure 2 demonstrate that the stimulation of TLR3 with Poly I:C induces an amplification of the re-stimulation in a concentration range of 1,000 to 10,000 ng/ml.. Furthermore, the inhibiting effects of higher doses of FVIII (1000 ng/mL) can be at least partly reverted. Very little restimulation of FVIII-specific memory B cells is observed in the absence of T cells.

3.3.4.3. Screening platform for TLR4

TLR4 is an extracellular receptor and is one of the best examined and described TLRs. TLR4 has an important function in sensing lipopolysaccharide (LPS).[4]

In our experiments we used LPS in a range of concentrations covering 0.1 to 10,000 ng/mL (see figure 3).

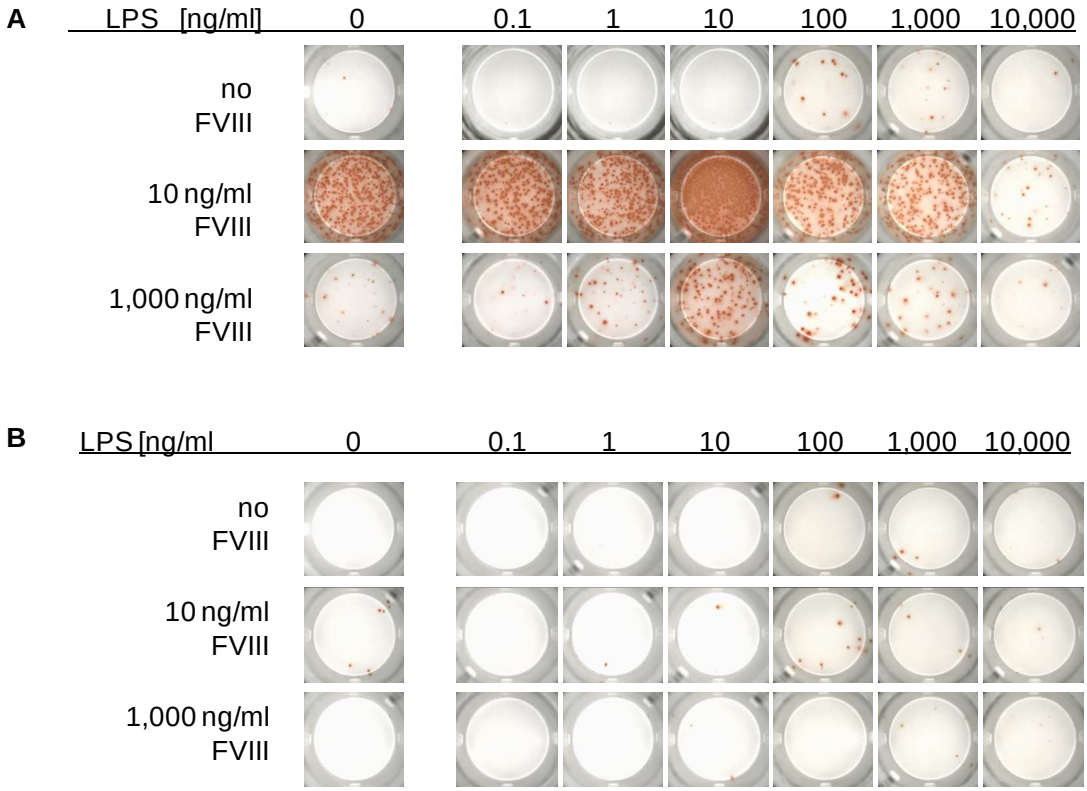

Fig. 3: Screening panel for the re-stimulation of FVIII specific memory B cells in the presence and absence of ligands for TLR4

A: Re-stimulation of FVIII specific Memory B Cells in the presence of T-cells

B: Re-stimulation of FVIII specific Memory B Cells in the absence of T-cells

Stimulation of TLR 4 with LPS shows a bi-phasic effect on the re-stimulation of FVIII-specific memory B cells At a concentration of 1ng/mL, LPS is capable of enhancing the re-stimulation and reversing the inhibiting ability of high dose FVIII. At higher concentrations of up to 1000ng/mL, LPS inhibits the re-stimulation of FVIII-specific memory B cells (see figure 3).

Furthermore, no restimulation was observed in the absence of T cells.

3.3.4.4. Screening platform for TLR5

TLR5 recognises monomeric flagellin, an evolutionary highly conserved constituent of bacterial flagella.[4]

For our experiments we used purified flagellin from S. typhimurium in a range of concentrations covering 0.01 to 1000 ng/mL (see figure 4)

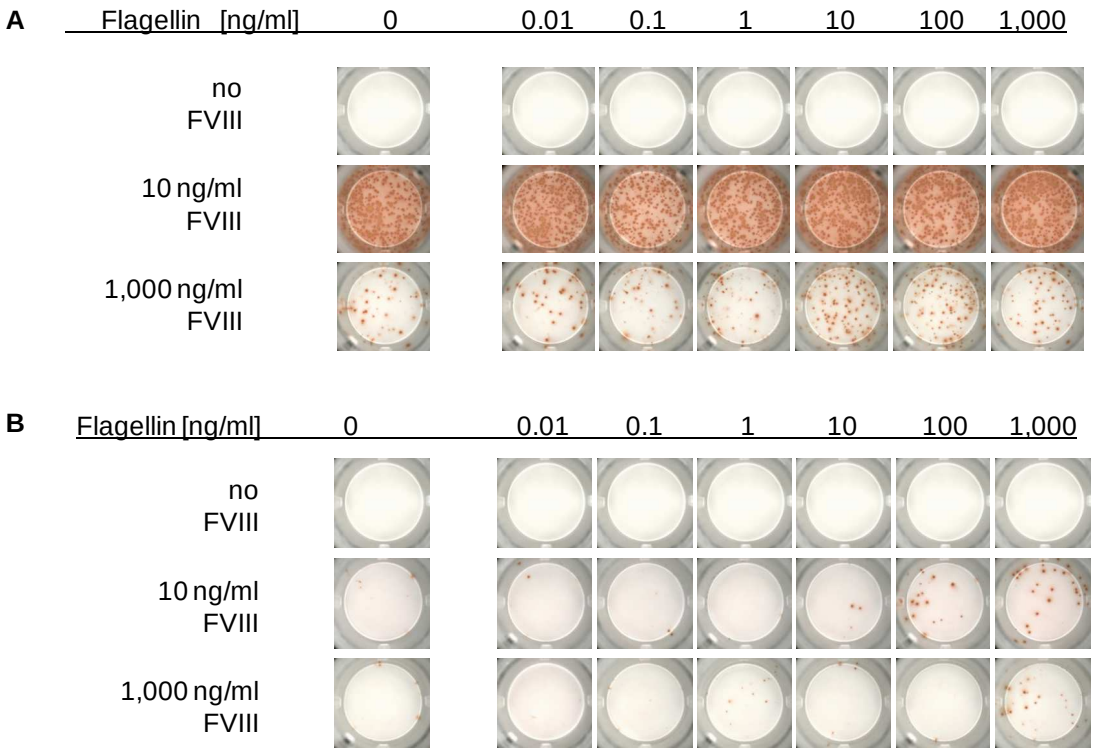

Fig. 4: Screening panel for the re-stimulation of FVIII specific memory B cells in the presence and absence of ligends for TLR 5.

A: Re-stimulation of FVIII specific Memory B Cells in the presence of T-cells

B: Re-stimulation of FVIII specific Memory B Cells in the absence of T-cells

Triggering of TLR5 in the presence of T cells does not influence the re-stimulation of FVIII-specific memory B cells (see figure 4). In the absence of T cells, triggering of TLR5 does induces little e re-stimulation of FVIII-specific memory B cells only (see figure 4).

3.3.4.5. Screening platform for TLR7

TLR7 has been shown to recognize guanosine- or uridine-rich single stranded RNA (GU-rich ssRNA) from viruses.[3]

For our experiments we used the Imidazoquinoline Imiquimod and the guanosine-analog Loxoribine in a range of concentrations covering 1 to 50,000 ng/mL (see figure 5 for the use of Imiquimod and figure 6 for the use of Loxoribine)

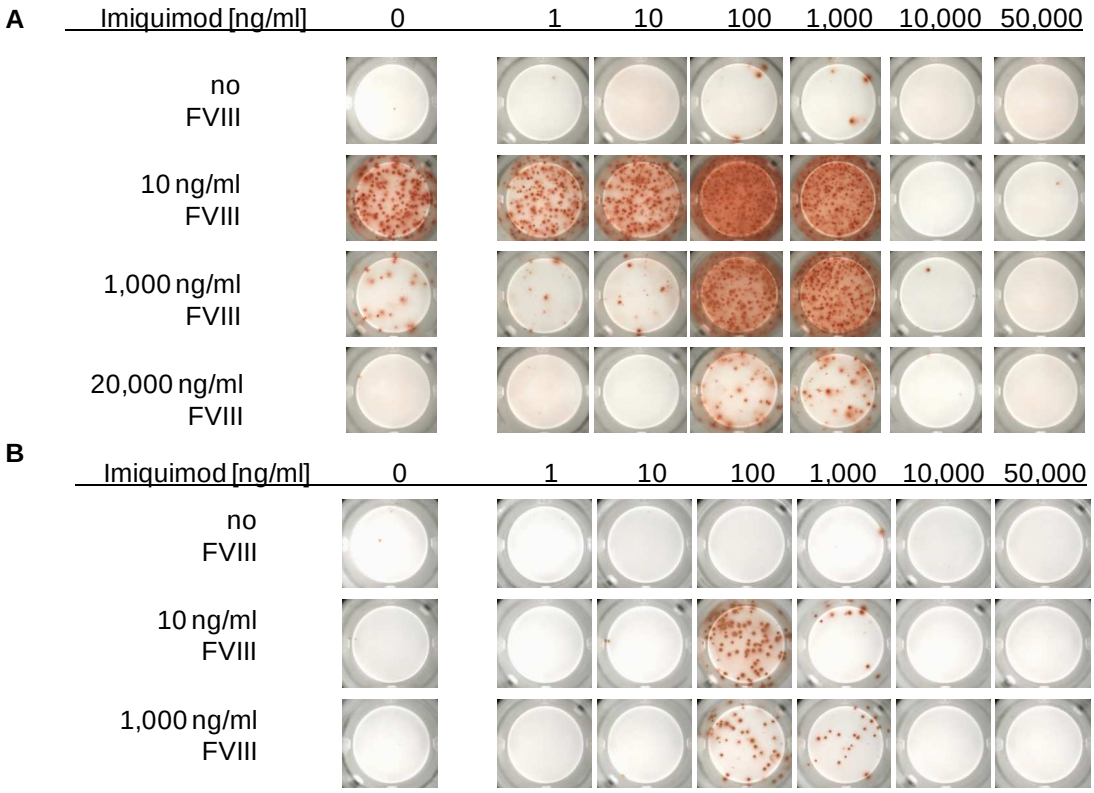

Fig. 5: Screening panel for the re-stimulation of FVIII specific memory B cells in the presence and absence of Imiquimod (ligand for TLR 7)

A: Re-stimulation of FVIII specific Memory B Cells in the presence of T-cells

B: Re-stimulation of FVIII specific Memory B Cells in the absence of T-cells

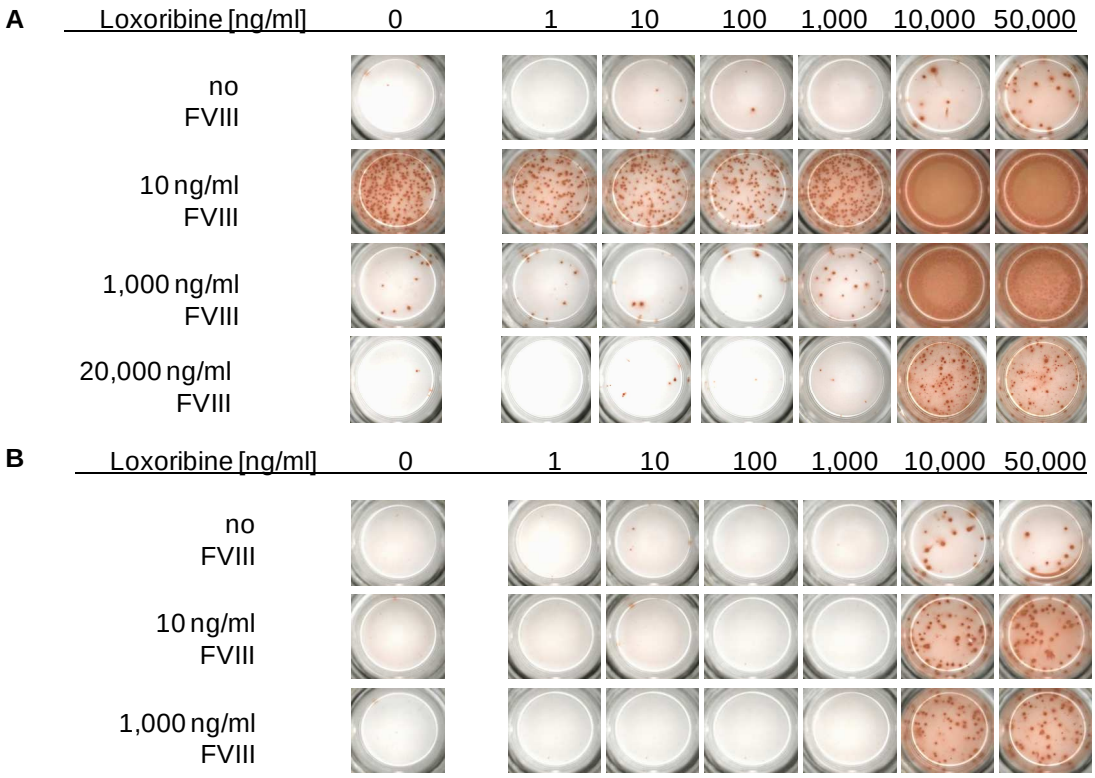

Fig. 6: Screening panel for the re-stimulation of FVIII specific memory B cells in the presence and absence of Loxoribine (ligand for TLR 7).

A: Re-stimulation of FVIII specific Memory B Cells in the presence of T-cells

B: Re-stimulation of FVIII specific Memory B Cells in the absence of T-cells

Triggering of TLR7 either with Imiquimod or with Loxoribine induces a strong modulation on the re-stimulation behavior of FVIII-specific memory B cells. Both ligands appear to possess the capability to strongly amplify the re-stimulation under stimulating doses of FVIII at concentrations of about 100ng/ml for Imiquimod and 10,000 ng/ml and above for Loxoribine (see figures 5 and 6). Furthermore, both substances are capable of completely inverting the inhibitiory function of high doses of FVIII, even if FVIII concentrations are raised to 20,000 ng/ml (figures 5 and 6).

Importantly, both Imiquimod and Loxoribine , amplify the re-stimulation of FVIII-specific memory B cells in the absence of T-cells. Some restimulation can even be seen in the complete absence of FVIII (figures 5 and 6).

Imiquimod expresses a biphasic reaction pattern Whereas concentrations between 100 and 1000 ng/ml amplify the memory response, concentrations above 1000 ng/ml cause a complete inhibition of re-stimulation, independent of FVIII concentration and presence of T-cells (figure 5).

3.3.4.6. Screening platform for TLR9

TLR9 is essential for the recognition of the CpG motif of bacterial and viral DNA.[3]

For our experiments we used CpG oligodeoxynucleotides (ODN1826), which are short single stranded synthetic DNA molecules containg CpG motifs.

ODN 1826 was used in a concentration range covering 0.1 to 10,000 ng/mL (figure 7).

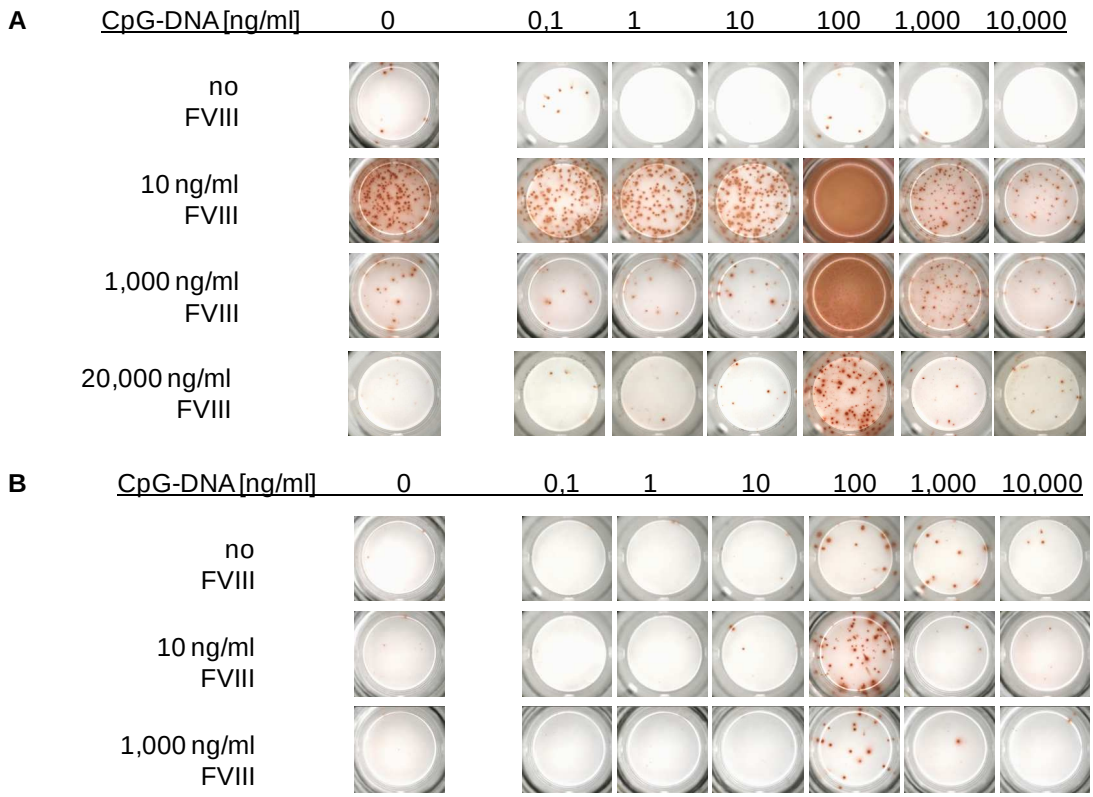

Fig. 7: Screening panel for the re-stimulation of FVIII specific memory B cells in the presence and absence of ligands for TLR 9.

A: Re-stimulation of FVIII specific Memory B Cells in the presence of T-cells

B: Re-stimulation of FVIII specific Memory B Cells in the absence of T-cells

Stimulation of TLR9 shows similar effects as described for TLR7 ligands (see Fig. 5 and 6). TLR9 triggering by CpG at 100ng/ml causes an amplification of the re-stimulation and a reversal of the inhibition of memory B cells, induced

by low and high concentrations of FVIII, respectively (figure 7). Furthermore, CpG at a concentration of 100 ng/ml allows the re-stimulation of FVIII-specific memory B cells in the absence of T-cells and even in the absence of FVIII (figure 7). However, CpG at higher concentrations of 1,000 and 10,000 ng/ml causes an inhibition of the re-stimulation even in the presence of stimulating concentrations of FVIII (figure 7).

3.3.5. Discussion

In the present study we established a rapid and comprehensive screening platform to investigate the capability of TLR ligands to modulate FVIII-specific memory responses. The readout was based on the detection of FVIII-specific antibody producing cells by ELISPOT technique as established by Hausl et al.[17] The culture periode of 6 days was chosen based on data published by Hausl et al who could demonstrate that 6 days culture is optimal for in vitro re-stimulation of memory B cells and further differentiation into antibody producing plasma cells.[16] The screening platform allows to study the influence of TLR ligands over a broad range of concentrations (six orders of magnitude) in combination with stimulating or inhibiting concentrations of the specific antigen, FVIII. Using this screening panel we were able to identify TLR ligands that showed virtually no influence on the re-stimulation pattern (eg ligands for TLR2, TLR5) and ligands which changed the re-stimulation pattern substantially (e.g. ligands for TLR7 and TLR9).

An explanation for non-responsiveness to certain TLR ligands could be the biological expression pattern of TLR. For example, TLR5 has been described to be expressed on murine T-cells and dendritic cells. However it seems not to be present on murine B cells.[18] Furthermore, due to its biological function of detecting Flagellin as a compound of bacterial flagellae, TLR5 is abundantly expressed on mucosal surfaces, the main entrance sites of bacteria,[19] [20] whereas it might be downregulated on spleen cells. Specific investigations on the cell-type-specific expression patterns of TLRs are currently ongoing to further elucidate this question.

The majority of the TLR ligands tested expressed the capability of amplifying the re-stimulation of memory B cells together with stimulating doses of FVIII (e.g. ligands for TLR3, TLR4, TLR7 and TLR9) at certain concentrations. These findings further support the important role of TLRs as stimulators of the immune system in response to pathogens. Activating TLR results mainly in the expression of genes encoding pro-inflammatory cytokines or Type I Interferon.[21] Both were shown to have direct and indirect influence on memory B cells [22] [23] [24] thus enhancing antibody production. Furthermore activation of TLR has also been described to up-regaluate co-stimulatory molecules, eg CD80, CD83 or CD86 [25] which in case of APC can increase T-cell proliferation and hence also memory B cell bystander help. It is therefore not surprising that many TLR agonists show amplifying effects concerning FVIII specific memory response.

However, increasing concentrations of ligands for TLR 4, 7 and 9, inhibit the re-stimulation of FVIII-specific memory B cells. In the case of ligand for TLR9 we could previously show that this inhibition is due to a specific interaction with TLR9 and not the result of a toxic effect.[26.] The mechanisms behind this phenomenon remain to be investigated. A recently discussed possibility could be the TLR-mediated induction of IDO (Indoleamine 2,3-dioxygenase), an immunomodulatory enzyme.[27] [28] [29] [30] IDO shows the ability to inhibit T cell proliferation in two ways. On the one hand, IDO can locally down-regulate the tryptophan catalbolism and thereby suppress T cells by depriving them of tryptophan.[29] On the other hand, IDO induces the production of kynurenines, which act on IDO^- DCs, thereby rendering an otherwise stimulatory DC capable of regulatory effects, which results in a suppression of T cell activation and eventually in suppression of immune reactions to pathogens.[31] Seen in a biological context, one could also speculate that an amplification of the immune system in case of infections is beneficial only up to a certain level, at which the danger of an overreaction of the immune system has to be avoided by active down-regulation, e.g. in case of sepsis.

Interestingly, some TLR ligands are capable of inducing a certain re-stimulation of FVIII-specific memory B cells even in the absence of T-cells.

This is notable, considering the findings of Hausl et al that direct cell contact with T cells is mandatory for the re-stimulation of FVIII-specific memory B cells.[16] However, findings from other groups already indicated that TLR signaling could re-stimulate memory B cells without T-cell help,[18 32] which indicates that a TLR-mediated signal can replace the helper function of T-cells at least to a certain extent.

Furthermore, some TLR ligands are capable of reversing the inhibiting properties of higher doses of FVIII on FVIII-specific memory B cells. This effect is of special interest considering the potential influence of infections in patients during immune tolerance induction (ITI) therapy, e.g. infections of the central venous lines necessary for ITI therapy. Patient who experience such an infection during ITI therapy are at risk of delayed success or even failure of ITI therapy due to increase anti-FVIII antibody levels during infections.[33] Considering the findings of Hausl et al, one could speculate that these ligands must be capable of preventing memory B cells from undergoing apoptosis. Hausl et al demonstrated that the inhibiting properties of high doses of FVIII could be neutralized by pan-caspase blockers, indicating that apoptosis is involved in the process.[12] Recently, Kuo et al showed that CpG-ODN can upegulate Hsp90ß in a TLR9 dependent pathway, inducing a signaling cascade which finally results in the prevention of caspase-3 activation.[34] By this way CpG-ODN could counteract potential apoptotic properties of high dose FVIII.

In order to better understand this phenomenon, it would be of great interest to know the exact mechanism of TLR mediated modulation of anti FVIII antibody production. In our study we used a heterogeneous preparation of spleen cells and investigated the influence of TLR ligands on this complex cell system. Currently we are trying to further elucidate the observed phenomena by investigating FACS sorted purified cell populations and studying the role of these purified cells populations in some detail.

The observed effects of ligands for TLRs only appeared within a narrow range of the tested concentrations. The question whether these specific

concentrations correspond to relevant concentrations during infections in vivo can currently not be answered. In vitro systems with homogenous cell suspension can hardly be compared with the highly structured and compartmentalized in vivo situation. Furthermore, absolute numbers of viral or bacterial load during an infection can only be estimated and still do not answer the question of how much TLR ligands for different TLRs would l be presented at a certain compartment. We started with in vivo experiments that should provide further information to answer this question.

3.3.6. Summary and Conclusion

In this study we established an in vitro screening plattform which allows the rapid and comprehensive investigation of the influence of TLR ligands on the the re-stimulation of FVIII-specific memory B cells. We could demonstrate that the majority of the tested TLR ligands at certain concentrations do have a significant influence on the system . TLR ligands are capable of amplifying or inhibiting the re-stimulation of FVIII-specific memory B cells at stimulating concentrations of FVIII. These effects are dose-dependend. At amplifying doses, some TLR ligands are able to induce a certain re-stimulation of memory B cells even in the absence of T-cells. Furthermore some TLR ligands show the ability to break the inhibitory properties of high doses of FVIII. The exact mechanisms behind these findings remain unclear and are amatter of current investigations.

3.3.7. Acknowledgements

We thank Elisabeth Hopfner, Nidha Abrar and Monika Grewal for technical assistance.

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4. Discussion

The development of inhibitory antibodies in up to 30% of patients is the major drawback of FVIII replacement therapy in hemophilia A. The reasons for the development of inhibitors in a moiety of patients remain still unclear. Some risk factors like type of mutation of the FVIII gene, ethnical background, environmental circumstances (e.g. concomitant invasive clinical procedures or infections, age of first exposure to FVIII) are empirically evaluated, however the root causes remain unknown and are intensely investigated.[59] [60] [61] [62] [63]

In case of development of an inhibitory antibody to FVIII in a hemophilia A patient, the standard replacement is no longer feasible as the infused FVIII is immediately neutralized, irrespective of the applied amount. Therefore the treatment of choice in case of inhibitor formation is the induction of immune tolerance (ITI). ITI can be achieved by infusion of comparatively high doses of FVIII for a long time period.[25] However, the success rate for ITI therapy is approx. 75%, leaving a quarter of inhibitor patients in the necessity of alternative and less favourable treatment options like activated FVII or bypassing agents.[18] [19]

The key to understand the reasons for the formation of inhibitory antibodies and to possible treatment strategies lies in the elucidation of the immunological mechanisms behind. The aim of the present work was to investigate a distinct aspect of the innate immune system, namely Toll-Like Receptors, with regard to their interaction with the immunological systems that lead to the formation of inhibitory antibodies to FVIII.

Toll-Like receptors went into focus of science not before the beginning of this millennium when it became evident that they are important elements of the immune system, having influence on central processes of immune responses.[41] As highly conserved pattern recognition receptors (PRRs), they are capable or identifying molecular structures different from eukaryotic multicellular organisms and usually represent constituents from pathogens. These structures are summarized under the term pathogen-associated molecular patterns (PAMP's). Prominent examples of such PAMP's are e.g. lipopolysaccharide (LPS) from gram-negative bacteria or double-stranded

RNA which is characteristic for some virus strains. Once a PAMP is detected via TLR, a signaling cascade is started, which results mainly in inflammation and establishment of adaptive immunity.[44] TLRs are expressed on most of the cells of the immune system which are responsible for the production of antibodies, including B- and T- cells as well as antigen presenting cells such as dendritic cells.[42 64 65]

It is therefore obvious to ask the question, whether TLRs also interfere in the immune response specific to FVIII. Clinical observations during the treatment of hemophilia patients indicate clearly that viral or bacterial stimuli do have an impact on the formation of FVIII specific antibodies. An example is the dreaded infection of central venous accesses, whose application is necessary for the repeated infusion of FVIII during ITI therapy. Despite the danger of severe sepsis, such infection can also lead to a increase of FVIII inhibitor levels which is highly undesirable during ITI therapy. Furthermore, there are strong recommendations to avoid any passive immunisations of patients during ITI therapy, as such stimuli are also known to raise FVIII inhibitor levels.[59 66] The involvement of TLR stimulation during these processes could be a likely cause for these well known but so far unexplained observations.

Key player in an established adaptive immune reaction to a protein like FVIII are memory B cells. Memory B cells ensure the qualitatively and quantitatively optimized fast antibody response on re-encounter with a known antigen. On contact with the specific antigen and in the normal case with assistance of helper T lymphozytes, memory B cells differentiate into plasma cells which can produce several thousands antigen-specific antibodies per second.[67] FVIII specific memory B cells have therefore been a field of intensive research, bringing exciting new insights in the re-stimulation mechanisms. [68 69 70]

In order to study FVIII specific memory B cells in an animal model as close as possible to the human system, we used a well established hemophilia A mouse model, the E17 KO mouse. The mice were developed by Bi et al in

1995 on a C57Bl/6J mouse strain background.[71 72] They are characterized by a targeted disruption of the exon 17 of the FVIII gene with a neomycin resistance gene. This gene knock out results in translation of either a truncated or partially deleted FVIII protein, which leads to a FVIII concentration in the mouse plasma below the detection limit (<0,01 IU/ ml). The so called E-17 mice express a severe phenotype of hemophilia A, meaning that they suffer from spontaneous internal bleedings, with bleedings after tail snipping being lethal.[73] This hemophilic phenotype can be corrected by the administration of human recombinant FVIII (rFVIII).[74] Human FVIII can interact with the proteins of the murine coagulation cascade due to very high sequence homologies between human and murine FVIII.[75 76] Treatment of the E-17 mice with intravenous doses of FVIII results in the development of an immune response against the administered FVIII that is very similar to the immune response arising in patients.

Hausl et al used this powerful tool and prepared single spleen cell suspensions from haemophilic mice treated with FVIII. These spleen cell suspensions, containing a major compartment of FVIII specific memory B cells were used to establish in vitro read-out systems for the study of the re-stimulation of memory B cells. Hausl et al could show that re-stimulation of FVIII specific memory B cells is antigen specific in a dose dependent manner. Doses in the range of physiological blood levels of FVIII lead to re-stimulation in vitro. Furthermore, Hausl et al demonstrated that increasing doses of FVIII which are quite above physiological level but in ranges that are used for ITI therapy, invert the effect and actively inhibit the re-stimulation of FVIII specific memory B cells.[68 69]

Based on these results, this project initially focussed on developing a platform which allows the rapid but thorough screening of TLR ligands for their capability of interfering with the re-stimulation behaviour so far described. Using the platform, it turned out clearly that most of the tested TLR ligands had the capability of significantly changing the re-stimulation pattern of FVIII specific memory B cells in the hemophilic mouse model.

TLR agonist in presence of stimulating concentrations of FVIII *in vitro*

One major effect that could be observed in the majority of tested ligands for TLR is an amplification of the specific memory response to FVIII in the presence of stimulating concentrations of FVIII, *in vitro* as well as *in vivo*. This could either be due to direct influence via TLR on the memory B cell itself or indirectly by stimulating TLR on other leucocytes which express TLR, e.g. T cells, dendritic cells or macrophages. Considering the ability of TLR to induce formation of pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-12 and TNF-alpha) or Type I Interferones, it is not surprising that these additional stimuli enhance the memory response to FVIII. Furthermore, triggering of TLR also leads to upregulation of maturation markers like CD80, CD83 or CD86 on immune cells, for example on dendritic cells. This would positively influence the maturation of T helper cells, which subsequently provide stronger helper signals for memory B cells to start differentiation into APC, finally resulting in stronger antibody response.

The testing of TLR agonists in a screening panel with concentrations ranging over six orders of magnitude revealed that several ligands, in particular ligands for TLR7 and 9, show a bi-phasic effect, depending on their concentration. While amplifying the differentiation of memory B cells at lower doses, this effect turned into an inhibition of the memory response at higher doses of TLR ligand in the presence of stimulating doses of FVIII, *in vitro* as well as *in vivo*. For TLR9 we proved that this effect is due to specific interaction of TLR ligand with its receptor. This raises the question which different mechanisms lead to the inversion of impact on memory response. One explanation described recently is the capability of TLR ligands, in particular ligands for TLR9, to induce the enzyme indoleamine 2,3-dioxygenase (IDO) in immune cells, especially in dendritic cells. IDO catalyzes the degradation of the essential amino acid L-tryptophan to N-formylkynurenine. By this, IDO has immunesuppressive capabilities by suppressing CD4+ T cells. On the one hand, IDO deprives T helper cells of tryptophan which is essential for T cell proliferation.[77] On the other hand IDO induces production of kynurenins, which show the ability of rendering otherwise stimulatory dendritic cell capable of regulatory effects, thus inducing regulatory T cells that would subsequently prevent the activation of

T helper cells.[77] [78] Our in vitro data show that high-dose CpG-DNA as agonist for TLR9 resulted in a reduction of IFN-γ in the cell culture supernatants, which is an evidence for reduced activation of Th1 cells which finally reduces the necessary bystander help for memory B cells.

Another possible explanation for the inhibitory effects of high doses of TLR ligands would be the induction of anti-inflammatory mediators like the cytokine IL-10. IL-10 has been shown to limit CpG responses, possibly by the induction if IL-10 producing regulatory T cells. However, our in vitro cytokine release data show an increase in IL-10 release at stimulatory concentration of CpG-ODN but no further increase at inhibitory concentrations of CpG-ODN. Therefore it seems unlikely that IL-10 is involved in the inhibitory effect of high-dose CpG-ODN.

TLR agonists in the presence of inhibiting concentrations of FVIII *in vitro*

Low doses of TLR ligands not only amplified the memory response in the presence of stimulating amounts of FVIII, in particular ligands for TLR 7 and 9 at low concentrations were also capable of breaking the suppression of memory response at inhibiting high doses of FVIII. The mechanisms of memory response inhibition by high doses of FVIII are not completely revealed. There is strong evidence that high doses of FVIII induce apoptosis in memory B cells, as the application of pan-caspase blockers can neutralise the suppressing effect of high dose FVIII (Ref Hausl). For CpG-ODN it has been reported that it protects B cells, macrophages and plasmacytoid dendritic cells against apoptosis.[79] [80] Furthermore, CpG-ODN can up-regulate Hsp90ß in a TLR9 dependent pathway, resulting in the prevention of caspase-3 activation and subsequently in prevention of apoptosis.[81]

The ability of TLR agonists to overcome the inhibiting properties of high doses of FVIII could be the explanation of the above mentioned complications during ITI therapy, where infections of the patient lead to increased formation of antibodies against FVIII during treatment with high doses of FVIII. An elucidation of the exact mechanisms would hence be of

utmost importance for effective measures in case of complications due to infections during ITI therapy.

TLR agonists in the absence of FVIII *in vitro*

While TLR agonists show tremendous immunestimulatory activity in the presence of FVIII, they seem incapable of inducing FVIII-specific memory B cell re-stimulation in the complete absence of FVIII, neither *in vitro* nor *in vivo*. Although we could observe some memory response in case of ligands for TLR4 and 7 in vitro, these responses were only at the level or slightly above the background signal of our assay and therefore deemed as not significant.

Reports on memory response by TLR stimulation only are ambiguous in literature. Lancavecchia et al described successful TLR-induced re-stimulation in the absence of antigen in the human system. However, recently published data showed that murine memory B cells neither clonally expand nor differentiate in to ASC in response to inflammatory stimuli such as TLR agonists, polyclonal T-cell activation, protein vaccination or even acute vaccinia virus infection in the absence of specific antigen in vivo.[82] A number of additional studies in mice an human provided data that could be interpreted either in favour of or against the idea that memory B cells respond to bystander inflammatory signals in the absence of the specific antigen. This question clearly needs further investigation.

TLR agonists in the presence of FVIII *in vivo*

The immunomodulating effects of TLR ligands found *in vitro* could also be demonstrated in our *in vivo* experiments. Co-application of TLR ligands together with FVIII resulted in amplification or inhibition of anti-FVIII antibody formation. However, the effects were less pronounced *in vivo* compared to our findings *in vitro*. With CpG-ODN we could demonstrate inhibiting effects of high doses of CpG-ODN on the formation of FVIII specific antibodies in vivo, while amplifying properties at lower doses could not be observed. This finding is remarkable as CpG-ODNs are well-known immunostimulators and therefore used as adjuvants for successful vaccinations.[83] [84] However the route of application seems to be of great importance: while subcutaneous

donation leads to a stimulation of immune reactions, systemic application leads to a suppression of immune responses[85], which is consistent to our observations.

Other reasons for the rather weak effects of CpG-ODN observed in our in vivo studies might be due to rapid degradation of CpG-ODN or ineffective delivery into intracellular compartments of TLR9-expressing cells in vivo.[86] Several authors have shown that CpG-ODN and antigen (FVIII in our study) need to be delivered to the same antigen-presenting cell to express the full stimulatory activity. Different strategies for in vivo co-delivery of antigen and CpG-ODN were developed,[87] [79] but this question was not further elucidated during this study.

In the complete absence of FVIII, none of the tested TLR ligands could elicit an antibody response specific to FVIII *in vivo*. Published data with regard to this question is contradictory. It is reported from the human system that memory B cells can differentiate into antibody-secreting plasma cells (ASC) merely by the stimulation of T cells and CpG-ODN.[88] On the other hand, recent data from the murine system indicates that murine memory B cells do not differentiate into ASC in response to inflammatory stimuli such as TLR agonsists, polyclonal T-cell activation, protein vaccination or even acute vaccinia virus infection in the absence of specific antigen in vivo. This important question therefore remains to be investigated.

Summarising the results of this thesis, we could show that TLR ligand do influence the restimulation behaviour of FVIII specific memory B cells in vitro as well as in vivo. Co-stimulation of TLR leads to amplification or inhibition of FVIII specific antibody formation. Furthermore, the inhibition of FVIII memory response by high doses of FVIII can be broken by co-stimulation with TLR ligands. These observations could be an explanation for the so far poorly understood observations during immune tolerance induction therapy treatment, where infections or vaccinations of the patients can lead to an undesired increase of FVIII specific antibody formation. The complete understanding of the underlying mechanisms could lead to more effective treatment of hemophilia A inhibitor patients.

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6. Appendix

6.1. Publications

The data presented in the results section are

published in:

6.1.1. Modulation of factor VIII-specific memory B cells.

Reipert BM, **Allacher P**, Hausl C, Pordes AG, Ahmad RU, Lang I, Ilas J, Windyga J, Klukowska A, Muchitsch EM, Schwarz HP. Haemophilia. 2010 May;16(102):25-34.

All data concerning TLR as summarized in Figures 4 and 5 were created and contributed by P. Allacher

re-submitted to the journal “Blood” after reviewers asked for minor revisions :

6.1.2. Stimulation and inhibition of FVIII-specific memory-B-cell responses by CpG-B (ODN 1826), a ligand for toll-like receptor 9

Peter Allacher, Christina K Baumgartner, Aniko G Pordes, Rafi U Ahmad, Hans Peter Schwarz and Birgit M Reipert

All data as summarized in Table 1 and Figures 1 to 7 were created and contributed by P. Allacher

to be submitted:

6.1.3. Modulation of FVIII-specific memory Responses by Toll-like Receptor Ligand – A Screening Platform

Allacher P, Baumgartner CK, Ahmad RU, Pordes AG, Schwarz, H.P., Reipert BM

All data as summarized in Figures 1 to 7 were created and contributed by P. Allacher

6.2. Oral Presentations

6.2.1. Toll-like receptor triggering modulates factor VIII-specific immune memory in murine hemophilia A with factor VIII inhibitors

Allacher P, Hausl C, Ahmad RU, Schwarz HP, Turecek PL, Reipert BM (2005).

Oral Presentation, 47th Annual Meeting of the American Association of Hematology, Atlanta , USA, December 2005
Blood 2005, **106**: 214A

Abstract:
The development of inhibitory antibodies against factor VIII (FVIII) is the major complication in the treatment of hemophilia A patients with FVIII products. Immune Tolerance Induction (ITI) therapy using long-term application of high doses of FVIII has evolved as an effective therapy to eradicate the antibodies and induce long-lasting immune tolerance. It is a common observation that infections, particularly central venous catheter infections during ITI cause a rise in anti-FVIII antibody titers what can prolong the course of ITI of possibly even lead to failure of ITI. We asked the question whether microbial components derived from viruses or bacteria modulate the re-stimulation of factor VIII (FVIII)-specific immune memory and affect the recently described inhibition of memory-B-cell re-stimulation by high doses of FVIII (Hausl et al.: Blood 2005; in press). Microbial components are recognized by toll-like receptors (TLRs) that serve as an important link between innate and adaptive immunity. TLRs can discriminate various microbial components such as lipopeptides derived from bacteria (recognized by TLR1/2 or TLR2/6), double-stranded RNA (dsRNA) derived from viruses (recognized by TLR3), lipopolysaccharide (LPS) derived from gram-negative bacteria (recognized by TLR4), flagellin derived from bacterial flagella (recognized by TLR5),

single-stranded RNA (ssRNA) derived from viruses (recognized by TLR7/8) or bacterial DNA containing the unmethylated CpG motif (recognized by TLR9).

We analyzed the re-stimulation of FVIIII-specific memory-B-cells using a murine model of hemophilia A as described previously (Hausl et al.: Blood 2004; 104:115-22; Hausl et al.: Blood 2005, in press). The following TLR ligands were tested: Zymosan for TLR 2 (0.1-10,000 ng/ml), poly I:C for TLR3 (1.0-50,000 ng/ml), LPS for TLR4 (0.1-10,000 ng/ml); Flagellin for TLR5 (0.01-1,000 ng/ml) Loxoribine for TLR7 (1.0-50,000 ng/ml) and CpG oligonuclotides for TLR9 (0.1-10,000 ng/ml). Our results indicate that none of the TLR ligands at the concentrations tested induced a significant re-stimulation of FVIII-specific memory B cells in the complete absence of either FVIIII or T cells. However, ligands for TLR3, TLR4, TLR7 and TLR9 were able to affect the inhibition of memory-B-cell-re-stimulation by high doses of FVIII and amplified the re-stimulation induced by low doses of FVIII substantially.

We conclude that triggering of TLRs by microbial components that are present during viral or bacterial infections amplify the re-stimulation of FVIII-specific memory B-cells and affect the inhibition by high doses of FVIII

6.2.2. Both stimulation and inhibition of factor VIII-specific memory B cells in hemophilia A is affected by Toll-like receptor triggering

Allacher P, Hausl C, Ahmad RU, Schwarz HP, Turecek PL, Reipert BM (2005)

Oral Presentation, Annual Meeting of the Austrian Society for Allergology and Immunology, Graz, Austria, December 2005

Abstract:

The development of inhibitory antibodies against factor VIII (FVIII) is the major complication in the treatment of hemophilia A patients with FVIII. Immune Tolerance Induction (ITI) therapy using long-term application of high doses of VIIII has evolved as an effective therapy to eradicate the antibodies and induce long-lasting immune tolerance. It is a common observation that infections during ITI, particularly central venous catheter infections, cause a rise in anti-FVIII antibody titers that can prolong the course of ITI or possibly even lead to failure of ITI. Based on this observation, we asked the question whether triggering toll-like receptors (TLR) by microbial components modulates the re-stimulation of FVIIII-specific immune memory and disturbs the recently described inhibition of memory-B-cell-re-stimulation by high doses of FVIIII (Hausl et al.: Blood 2005; Epub Aug 9). We analyzed the re-stimulation of FVIII-specific memory-B cells using a murine model of hemophilia A as described previously (Hausl et al.: Blood 2004; 104: 115-22; Hausl et al.: Blood 2005, Epub Aug 9). The following TLR ligands were tested: Zymosan for TLR2 (0.1-10,000 ng/ml), poly I:C for TLR3 (1.0-50,000 ng/ml), LPS for TLR4 (0.1-10,000 ng/ml), Flagellin for TLR5 (0.01-1,000 ng/ml), Loxoribine for TLR7 (1.0-50,000 ng/ml) and CpG oligonucleotides for TLR9 (0.1-10,000 ng/ml).

Our results indicate that none of the TLR ligands at the concentrations tested induced a significant re-stimulation of FVIIII-

specific memory B cells in the complete absence of either FVIII or T cells. However, ligands for TLR3, TLR4, TLR7 and TLR9 were able to disturb the inhibition of memory-B-cell-re-simulation by high doses of FVIII and amplified the re-stimulation induced by low doses of FVIII.

We conclude that triggering of TLR by microbial components amplifies the re-stimulation of FVIII-specific memory B-cells induced by low doses of FVIII and disturbs the inhibition induced by high doses of FVIII.

6.2.3. Ligands for toll-like receptors 7 and 9 express both positive and negative regulatory effects on the re-stimulation of FVIII-specific memory B cells in murine hemophilia A

Allacher P, Hausl C, Ahmad RU, Baumgartner B, Schwarz HP, and Reipert BM (2007)

Oral presentation, 51st Annual Meeting of the Society for Thrombosis and Haemostasis (GTH), Dresden, Germany, February 2007

Awarded “One out of the five best abstracts submitted”

Abstract:

Recently, we demonstrated that triggering of toll-like receptors (TLR) 7 and 9 amplified the antigen-specific re-stimulation of factor VIII (FIII)-specific memory B cells by low concentrations of FVIII and prevented the inhibition of memory-B-cell re-stimulation by high concentrations of FVIII [Allacher et al (2005). Blood 106: 214A]. Based on these results we asked the question how the concentration of TLR ligand influences the effects of TLR triggering.

The re-stimulation of VIIII-specific memory B cells was studied in vitro as described previously [Hausl et al. (2005). Blood 106: 3415-3422]. Imiquimod (1-50,000 ng/ml) and CpG-DNA (0.1-10,000 ng/ml) were used as ligands for TLR 7 and 9, respectively. GpC-DNA (non-stimulating) and a blocking agent for TLR 9 (inihibitory oligonucleotide sequence) were used as controls. Our results demonstrate that Imiquimod as well as CpG-DNA induced both stimulatory and inhibitory effects on memory-B-cell re-stimulation depending on the concentration of TLR ligand used. Both stimulatory and inhibitory effects induced by CpG-DNA were prevented by an inhibitory oligonucleotide sequence that blocked TLR 9. Furthermore, non-stimulating GpC-DNA did not induce any effect. These results indicate that both the stimulatory and the

inhibitory activity of CpG-DNA were due to specific interactions with TLR9.

We conclude that triggering of TLRs 7 and 9 by microbial components that are present during infections can have both positive and negative influence on the re-stimulation of FVIII-specific memory B cells depending on the local concentration of the microbial component.

6.3. Posters

6.3.1. Differential modulation of antigen-specific memory-B-cell re-stimulation by ligands of toll-like receptors 7 and 9.

Allacher P, Hausl C, Ahmad RU, Baumgartner B, Schwarz HP, and Reipert BM (2006).

Poster Presentation, Clement von Pirquet Symposium, Vienna, Austria, December 2006

Differential Modulation of Antigen-specific Memory-B-Cell Re-Stimulation by Ligands of TOLL-Like Receptors 7 and 9

Peter ALLACHER[1], Christina HAUSL[2], Rafi U AHMAD[1], Bernhard BAUMGARTNER[1], Hans Peter SCHWARZ[1,2], Birgit M REIPERT[1,2]

[1]Baxter BioScience and [2]BMT-Research GmbH, Vienna, Austria.

INTRODUCTION

Hemophilia A is caused by a deficiency of the coagulation factor VIII (FVIII) that is due to mutations in the *FVIII* gene. Patients receive replacement therapy with FVIII products. The induction of neutralizing anti-FVIII antibodies is a major complication during the replacement therapy. Immune Tolerance Induction (ITI) using long-term application of high doses of FVIII has evolved as an effective therapy to eradicate the antibodies and induce long-lasting immune tolerance to FVIII.
We showed recently (Hausl et al.: Blood 2005) that high doses of FVIII prevented the re-stimulation of FVIII-specific memory B cell and their differentiation into anti-FVIII antibody-producing plasma cells which might be an early event in the down-regulation of established anti-FVIII antibodies during ITI therapy. Triggering of toll-like receptors (TLR) 7 and 9 amplified the re-stimulation of FVIII-specific memory B cells by low concentrations of FVIII and prevented the inhibition of memory-B-cell re-stimulation by high concentrations of FVIII (Allacher et al.: Blood 2005). Based on these results we asked the question how the concentration of the TLR ligand modulates the effects of TLR triggering.

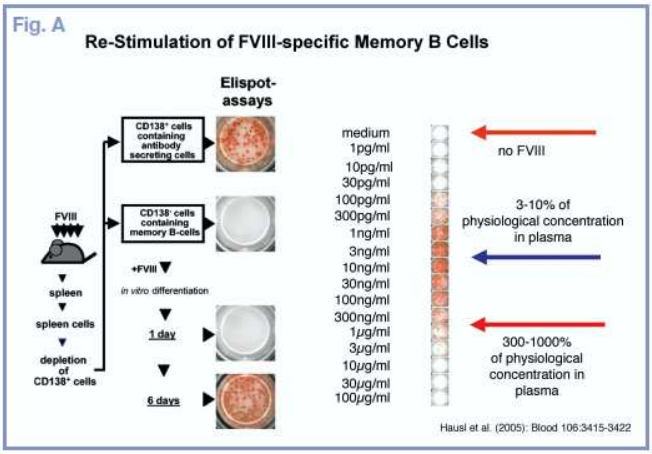
METHODS

Animals: Hemophilic mice characterized by a targeted disruption of exon 17 of the *FVIII* gene (Bi et al.: Nature Genetics 1995). Mice were male and aged 8-10 weeks.

Treatment with FVIII: Mice received four intravenous doses of 200 ng of recombinant human FVIII (Baxter BioScience) at weekly intervals.

Re-stimulation of FVIII-specific memory B cells (Fig. A): Spleen cells were obtained from hemophilic mice treated with 4 intravenous doses of FVIII. Spleen cells were depleted of $CD138^+$ cells using magnetic separation techniques. $CD138^-$ cells were re-stimulated *in vitro* for 6 days with human FVIII (10 – 20.000 ng/ml).
TLR 7 or 9 were stimulated with Imiquimod (1 - 50,000 ng/ml) or CpG-DNA (0.1 - 10,000 ng/ml), respectively. GpC-DNA (non-stimulating) and a blocking agent for TLR 9 (inhibitory oligonucleotide sequence) were used as controls. Newly formed antibody-secreting cells were detected by ELISPOT assays as described (Hausl C et al.: Thromb Haemost 2002).

Fig. A



RESULTS

Imiquimod and CpG-DNA can induce stimulatory as well as inhibitory effects on memory-B-cell re-stimulation depending on the concentration used (Fig. B and C).
Both, stimulatory and inhibitory effects induced by CpG-DNA can be prevented by an inhibitory oligonucleotide sequence that blocks TLR 9. Non-stimulating GpC-DNA does not induce any effect (Fig. D).
These results indicate that both stimulatory and inhibitory effects of CpG-DNA are due to specific interactions with TLR 9 (Fig. E).

Fig. B

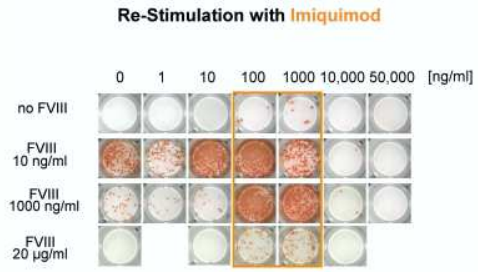


Fig. C

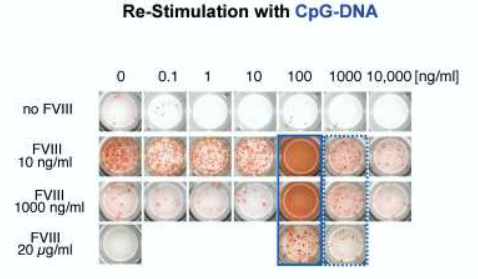


Fig. D

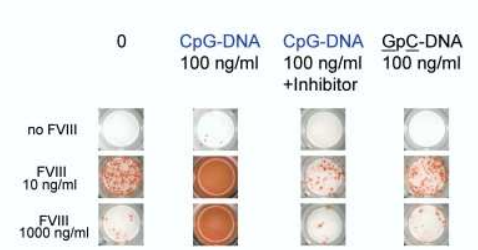
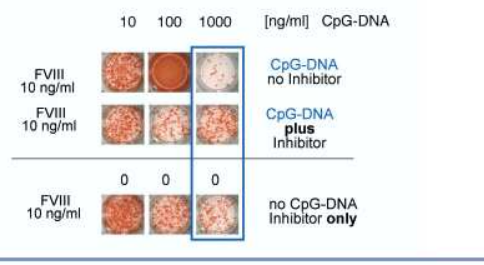


Fig. E



CONCLUSIONS

Our results indicate that triggering of TLR 7 and 9 by microbial components that are present during infections can have both positive and negative influence on the re-stimulation of FVIII-specific memory B cells depending on the local concentration of the microbial component. Both the stimulatory and the inhibitory activity of CpG-DNA are due to specific interactions with the respective receptor.

6.3.2. Agonist for toll-like receptor (TLR) 9 amplifies as well as inhibits the differentiation of FVIII-specific memory B cells into antibody-producing plasma cells in vitro and in vivo.

Allacher P, Hausl C, Pordes AG, Ahmad RU, Ehrlich HJ, Schwarz HP, Reipert BM (2008):

Poster Presentation, 50th Annual Meeting of the American Association of Hematology, San Francisco , USA, December 2008

Agonist for toll-like receptor (TLR) 9 amplifies as well as inhibits the differentiation of FVIII-specific memory B cells into antibody-producing plasma cells in vitro and in vivo

Allacher P[1], Hausl C[2], Pordes AG[2], Ahmad RU[1], Ehrlich HJ[1],Schwarz HP[1], Reipert BM[1]
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BMT RESEARCH

OBJECTIVE

Antibody responses against factor VIII (FVIII) are the major complication in patients with Hemophilia A who are treated with FVIII. Memory B cells are the key players in the maintenance of long-term antibody responses. The objective of the studies presented was the examination of the potential impact of viral and bacterial infections on the re-stimulation of FVIII-specific memory B cells.

INTRODUCTION

Memory B cells are essential for maintaining long-term antibody responses. They can persist for years even in the absence of antigen and are rapidly re-stimulated to differentiate into antibody-producing plasma cells when they encounter their specific antigen [Fig. 1].
Previously we demonstrated that ligands for TLR 7 and 9 amplify the differentiation of FVIII-specific memory B cells into anti-FVIII antibody-producing plasma cells at low concentrations of FVIII and prevent the inhibition of memory-B-cell differentiation at high concentrations of FVIII [Fig. 2].
The modulation of FVIII-specific memory-B-cell responses by agonists for TLR is highly relevant for the design of new immunotherapeutic approaches in patients with FVIII inhibitors because TLR are activated by a range of different viral and bacterial components. Specifically, TLR 7 is triggered by single-stranded RNA derived from viruses and TLR 9 is triggered by bacterial DNA containing unmethylated CpG motifs.

Fig. 1

Re-stimulation of FVIII-specific memory-B-cells *in vitro*

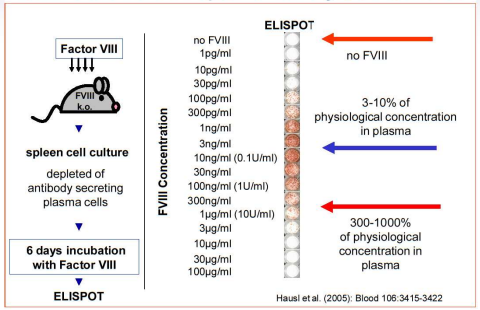
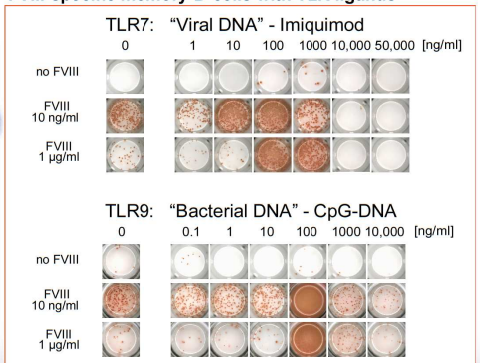


Fig. 2

Amplification and inhibition of the re-stimulation of FVIII-specific memory-B-cells with TLR ligands



METHODS

Animals: Hemophilic mice characterized by a targeted disruption of exon 17 of the *FVIII* gene (Bi et al.: Nature Genetics 1995). Mice were male and aged 8-10 weeks.

Re-stimulation of FVIII-specific memory B cells *in vitro*
Spleen cells were obtained from hemophilic mice treated with 4 intravenous doses of FVIII given in weekly intervals. Spleen cells were depleted of CD138+ cells using magnetic separation techniques. CD138- cells were re-stimulated in vitro for 6 days with human FVIII (10 – 1.000 ng/ml).
Imiquimod (1 - 50,000 ng/ml) or CpG-DNA (0.1 - 10,000 ng/ml) were added at day 0, respectively.
GpC-DNA (non-stimulating) and a blocking agent for TLR 9 (inhibitory oligonucleotide sequence) were used as controls.
Newly formed antibody-secreting cells were detected by ELISPOT assays as described (Hausl C et al.: Thromb Haemost 2002).

Re-stimulation of FVIII-specific memory B cells *in vivo*
Mice were primed with a single intravenous dose of 200 ng FVIII on day 0 to stimulate the generation of FVIII-specific memory B cells. Memory B cells were re-stimulated with FVIII on day 7. CpG-DNA was given 24 h prior to the second dose of FVIII (day 6) as well as together with FVIII (day 7).
We analyzed titers of anti-FVIII antibodies in the circulation of these mice one week after the second dose of FVIII by enzyme-linked immunosorbent assay (ELISA) as described (Hausl C et al.: Thromb Haemost 2002).

RESULTS AND CONCLUSION

Our results reveal a biphasic modulating effect of CpG DNA on the re-stimulation of FVIII-specific memory B cells and their differentiation into anti-body-producing plasma cells.

Both in vitro and in vivo studies show that CpG DNA at high doses inhibits the re-stimulation and differentiation of FVIII-specific memory B cells [Fig. 2 & 4]. However, CpG DNA at low doses amplifies these processes. [Fig. 2].
Experiments using a receptor blockade for TLR 9 indicate that both stimulatory and inhibitory effects are due to direct interaction with the TLR 9 [Fig. 3]

Our findings demonstrate the modulating effects of bacterial and viral infections on the regulation of FVIII inhibitor development.

Fig. 3

Specifity of TLR ligand modulation

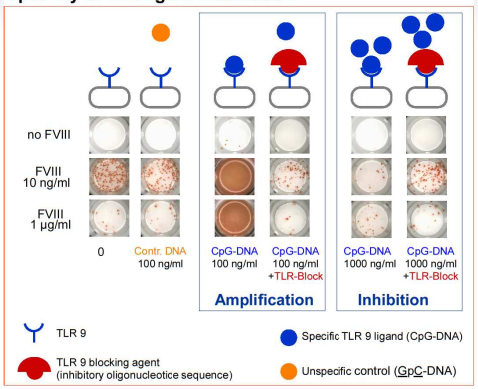
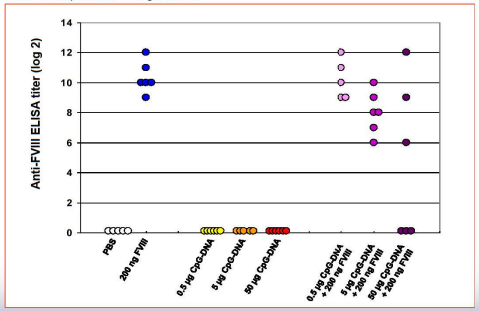


Fig. 4

Modulation of FVIII specific memory-B-cell re-stimulation *in vivo*
Each dot represents a single animal



This work was supported by Baxter BioScience and in part by a grant from the Center for Innovation and Technology City of Vienna, Austria.
Poster presented at the 50th ASH Annual Meeting, San Francisco/USA 2008

7. Curriculum vitae

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Education

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1993 – 1999	studies of biology, University of Vienna, Austria
1998 – 1999	diploma thesis at the department of pharmacognosy, group of Prof. Brigitte Kopp
2004 – 2010	PhD-thesis at the department of Immunology, Baxter Innovations GmbH, Vienna, Austria

Work experience

05/1992 – 09/1993	Employee of “Johann Laska und Söhne GesmbH”, Vienna, Austria, Department of merchandise management and IT
07/1997 – 09/1999	Employee of the Plant Breeding Unit of the FAO/IAEA Agriculture and Biotechnology Laboratories, Seibersdorf, Austria; lab assistance and IT
since 06/2000:	Employee of Baxter, Vienna, Austria
06/2000 – 05/2007	GLP Quality Assurance
since 06/2007	Research Scientist Immunology

8. Danksagung

Herrn Prof. H.P. Schwarz möchte ich für die Möglichkeit danken, diese Arbeit im Bereich Immunologie bei Baxter durchführen zu können, bei der er mich – trotz meiner ungewöhnlichen firmeninternen Position - immer unterstützt hat.

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Nicht zuletzt möchte ich meiner Familie und allen Freunden danken, die mich mit viel Rücksicht und Aufmunterung den gesamten Weg begleitet haben.

Im besonderen Andenken an meinen Vater, Josef Allacher, der die Fertigstellung dieser Arbeit leider nicht mehr erleben durfte.