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Exploration of Anti-idiotypic Antibodies for Elimination of fVIII Inhibitors in Hemophilia A Patients

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II. Zusammenfassung

Hämophilie A, ausgelöst durch eine Mutation im Gen des Gerinnungsfaktors VIII (fVIII), führt zu einer verzögerten Gerinnung und Spontanblutungen in Patienten. Je nach Ausprägung der Mutation ist fVIII funktionsgemindert oder funktionsunfähig. Therapeutisch lässt sich fVIII in Form eines plasmatischen oder rekombinanten Präparats substituieren. Aufgrund einer unzureichenden Immuntoleranz gegenüber dem substituierten fVIII entwickeln circa 30% der Patienten allogene neutralisierende Antikörper (Inhibitoren) gegen den Faktor. Als Erstlinientherapie gegen Inhibitoren wird eine Immuntoleranzinduktion (ITI) durch hochdosierte fVIII-Gabe eingesetzt. Die ITI führt bei etwa 30% der Patienten nicht zum Therapieerfolg. Wirkmechanismen der ITI sind Gegenstand der Forschung, da die unzureichende Kenntnis über Mechanismen und prognostische Faktoren der ITI die Behandlung verkomplizieren. Unter anderem wird die Bildung von anti-idiotypischen Antikörpern erforscht, welche als natürliche Regulatoren des Immunsystems vorkommen. Sie wurden unter anderem in Immunglobulinpräparaten und in Patientenplasma nach erfolgreicher ITI detektiert.

Durch Bindung funktioneller Epitope innerhalb der fVIII-Domänen beeinträchtigen Inhibitoren die Funktion von FVIII innerhalb der Gerinnungskaskade. Inhibitoren gegen die A2- und C2-Domäne kommen gehäuft vor, aber auch die C1-Domäne trägt signifikant zur Immunogenität des fVIII bei. Die Polyklonalität der existierenden Inhibitoren erschwert eine gezielte Therapie. Die vorliegende Arbeit beschäftigt sich mit der Generierung von Anti-Idiotypen zur Eliminierung von Inhibitoren. In diesem Rahmen wurde die Phage-Display-Technologie angewandt, um spezifische *single chain variable fragments* (scFv) gegen humane polyklonale anti-fVIII Antikörper aus Patientenplasma und gegen zwei monoklonale C1-Domänen-spezifische Antikörper zu selektieren.

Da in vorherigen Experimenten mit Inhibitor-haltigem Vollplasma keine erfolgreiche Selektion von Anti-Idiotypen möglich war, wurden im ersten Projekt dieser Arbeit anti-fVIII-Antikörper aus humanem Plasma aufgereinigt. Hierzu wurden Protein A-Chromatographie und fVIII-gekoppelte Affi-Gel® Affinitätschromatographie angewandt. Dabei wurden fVIII-spezifische Antikörper erfolgreich aufgereinigt. Die

isolierten anti-fVIII Antikörper wurden im Anschluss als Zielmoleküle für die Selektion von Anti-Idiotypen eingesetzt. Bei der Analyse selektierter scFv-tragender Phagen zeigte sich, dass keiner der Phagen alleinig die fVIII-spezifischen Antikörper band, sondern auch die unspezifische IgG-Kontrolle. Das Selektionsprotokoll wurde daraufhin angepasst, was zu einer Reduktion von unspezifischen Bindern führte. Jedoch konnten auch hier keine anti-idiotypischen Phagen gegen fVIII-spezifische Antikörper selektiert werden. Grund hierfür war möglicherweise die hohe Variabilität der polyklonalen Antikörper und die eingeschränkte Vielfalt der Phage-Display-Bibliotheken.

Im zweiten Teil dieser Arbeit verfolgten wir die Selektion von anti-idiotypischen scFvs, die sich gezielt gegen die C1-Domänen-spezifischen monoklonalen Antikörper GMA8011 (murin) und LE2E9 (human) richten. Aus therapeutischer Sicht könnte ein individuelles Anti-Idiotypengemisch dazu verwendet werden gezielt Inhibitoren in Patienten zu neutralisieren. Hierbei konnten ein GMA8011-spezifischer (H2C1) und zwei LE2E9-spezifische (H3G7, H3F10) scFv-tragende Phagen isoliert werden. In weiteren Experimenten zeigte nur der GMA8011-spezifische scFv ein kompetitives Bindungsverhalten in Gegenwart von fVIII, was auf eine anti-idiotypische Bindung an das Inhibitorparatop hindeutet. Die LE2E9-spezfischen scFvs konnten die Bindung von LE2E9 an fVIII nicht verhindern. Somit ließ sich hier kein anti-idiotypisches Verhalten feststellen. Für weitere Charakterisierungsschritte wurden die scFvs in den Antikörperkontext (scFv-Fc) kloniert und rekombinant hergestellt. Im Antikörperkontext zeigten alle drei scFvs eine konzentrationsabhängige Bindung an den Zielantikörper sowie die Isotypen-Kontrolle. Die Bindespezifität aus dem Phagenkontext konnte nicht reproduziert werden. Dass bei keinem der drei scFvs eine Bindung an das Paratop des Zielantikörpers vorliegt, bestätigte sich in Kompetitionsexperimenten mit fVIII.

Die Selektion fVIII-Inhibitor-neutralisierender Antikörper erweist sich als anspruchsvoll. Aufgereinigte anti-fVIII-Antikörper aus Patientenplasma als Zielmolekül im Biopanning einzusetzen führte zu keinem Erfolg, möglicherweise durch die zu große Diversität der Zielmoleküle. Die Generierung eines monoklonalen Anti-Idiotypengemischs zur gezielten Therapie einzelner Patienten erweist sich als mühsame Methode. Wir konnten Inhibitor-bindende scFvs gegen LE2E9 und GMA8011 selektieren, in Analysen jedoch kein anti-idiotypisches Verhalten nachweisen. Die hohe Spezifität der Bindung des

Inhibitorparatops an das Zielepitop, welches sich auf wenige Aminosäurereste beschränkt, fordert eine ebenso spezielle Beschaffenheit des Anti-Idiotypen, welche in den untersuchten Phagenbibliotheken nicht gefunden werden konnte. Weitere Versuche mit monoklonalen Inhibitoren und Anti-Idiotypen sollen zu einem besseren Verständnis der Paratop-Epitop-Interaktion führen.

III. Abstract

The genetic mutation of the coagulation factor VIII (fVIII) results in a defective or missing protein, leading to a malfunctioning blood coagulation. The resulting disease is called hemophilia A. Depending on the severity of the mutation, affected patients experience an increased risk of pathologic bleeding after minor trauma or even sudden bleeding events. Substitution therapies with extrinsic fVIII exist using plasmatic or recombinant fVIII products. Due to an insufficient immune tolerance towards substituted fVIII, about 30 % of patients develop allogenic neutralizing antibodies (inhibitors) against substituted fVIII products. The gold standard of treating inhibitors is the immune tolerance induction (ITI), where patients are given frequent, high doses of fVIII to induce an immune tolerance. ITI therapy fails in about 30 % of patients. Mechanisms of action of ITI are part of research, as insufficient knowledge about mechanisms and prognostic factors complicate treatment. For example, the development of anti-idiotypic antibodies, which occur naturally as a regulatory mechanism of the immune system, are being studied. Such anti-idiotypes have been detected in immunoglobulin preparations and in patients after successful ITI.

Inhibitors interfere with fVIII function in coagulation by binding functional epitopes within fVIII domains. Inhibitors against the A2 and C2 domain are predominantly found, however also the C1 domain has been shown to be highly immunogenic in some patients. The polyclonality of inhibitors aggravates the understanding and treatment of these. The present project therefore focusses on the selection of synthetic anti-idiotypic antibodies to target inhibitors in patients. The phage display method was applied to, for one, isolate anti-idiotypic *single chain variable fragments* (scFvs) specific against human polyclonal anti-fVIII antibodies and second against two C1 domain-specific inhibitory monoclonal antibodies (mAbs).

In the first project, anti-fVIII antibodies were purified from human plasma to serve as target molecules. A previous project showed that using full plasma as a target did not yield anti-idiotypic antibodies from phage display. For the purification, protein A chromatography and fVIII-coupled Affi-Gel® chromatography were applied. The isolated antibodies were next used as targets for the selection of anti-idiotypic scFvs. Analysis revealed that none of the selected phages solely bound the anti-fVIII antibody target.

Consequently, the test protocol was modified, which resulted in a reduction of unspecific binders. Yet, no target-specific binders were isolated from phage pools. Reason for this may have been the high diversity of the polyclonal antibody target and the limited diversity of the phage libraries.

The aim of the second project, was the selection and characterization of scFvs, that target the paratopes of C1 domain-specific mAbs GMA8011 and LE2E9. From a therapeutic viewpoint, the preparation of an anti-idiotypic antibody pool, tailored to each patient's inhibitor population, could help neutralize inhibitors in patients. Ultimately, one GMA8011-specific scFv-carrying phage clone (H2C1) and two specifics to LE2E9 (H3G7, H3F10) were isolated. In further experiments, only the GMA8011-specific scFv showed competitive behavior in presence of fVIII, pointing towards an anti-idiotypic binding to the inhibitor paratope. The LE2E9-specific scFvs did not prevent binding of the inhibitor to fVIII. Hence, no anti-idiotypic behavior could be determined. For further characterization, selected scFvs were genetically fused to Fc antibody fragments and recombinantly produced. In this antibody format, all three scFvs showed concentration dependent binding to the target and the isotype control. The binding specificity to the target, observed in phage context, could not be reproduced. Competition experiments with fVIII confirmed that none of the scFvs bound the paratope of their target inhibitor.

The selection of anti-idiotypic scFvs from phage display libraries proves to be effortful. Polyclonal anti-fVIII antibodies purified from hemophilic plasma appear to be unsuitable as a target for phage display, likely due to the high diversity of the target molecules. Furthermore, the preparation of an individualized anti-idiotypic pools for patients by selecting scFvs against single inhibitory mAbs proves to be difficult. The selection of scFvs against anti-C1 inhibitors GMA8011 and LE2E9 produced three promising scFv-carrying phages. However, analysis could not detect anti-idiotypic behavior. Further research with inhibitors, monoclonal and polyclonal, and anti-idiotypic antibodies should be performed to bring better insight into the highly complex paratope-epitope interaction.

1 Introduction

1.1 Blood Coagulation

Blood coagulation, or hemostasis, plays a key role in the organism´s ability of self-healing. After an injury, the coagulation cascade is activated, to prevent massive blood loss. During primary coagulation, blood vessels contract and activated thrombocytes adhere to damaged endothelial cells to form a clot [reviewed in 1]. As this primary thrombus is unstable, secondary coagulation takes place to form a mesh of fibrin over the primary clot. The secondary coagulation cascade is responsible for the lasting occlusion of the wound. It embodies a series of interactions of proteins, called coagulation factors and cofactors, which catalyze several coagulant mechanisms. These coagulation factors are mostly serine proteases, which circulate through the blood as inactive forms. Blood clotting can be initiated in two ways. The extrinsic system is triggered, when a blood vessel is injured and tissue factor (TF), which is expressed by extravascular cells, is exposed to coagulation factor VII (fVII) in the blood[2]. Activated fVII (fVIIa) and TF form an extrinsic tenase complex, so called because it is able to activate factor X (fX)[3]. The intrinsic pathway is catalyzed when blood interacts with exposed collagen fibers in the blood stream. Factor XII (fXII) is activated and initiates the activation cascade of factor XI (fXI) and factor IX (fIX). FIX partners up with activated cofactor VIII (fVIIIa), forming the "intrinsic" tenase complex[4,5]. Overall, the intrinsic pathway is slower but more efficient than the extrinsic pathway [reviewed in 6].

At the end of both pathways stands the formation of the prothrombinase complex, composed of activated fX and cofactor V, which then converts prothrombin to thrombin[7]. Thrombin is the central operator of the cascade, primarily responsible for the splicing of fibrinogen into fibrin monomers, which form a dense network over the primary thrombus[8]. This secondary clot is also called a red thrombus, because erythrocytes are caught in it.

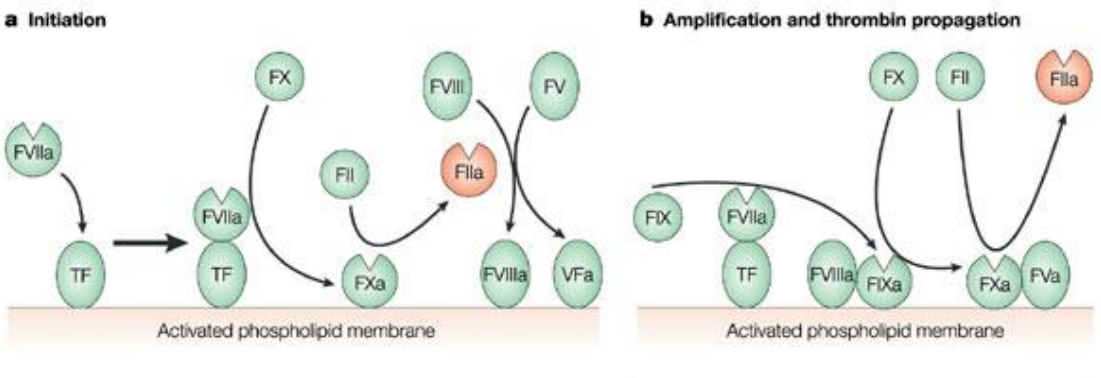

Figure 1 Blood coagulation cascade (adapted from Bishop *et al.*)[9]. a) Coagulation is initiated by activated fVII in complex with TF, which functions as a fX activator. FXa launches the activation of thrombin (fII). Cofactors fVIII and fV, activated by thrombin, function in a positive feedback to enhance thrombin activation. b) Activated fIX (fIXa) and fVIIIa form the tenase complex, which provokes amplification of coagulation by boosting fX activation.

Several feedback mechanisms regulate the extent of blood coagulation. For instance, coagulation is restricted to the site of injury, because coagulation factors only function as membrane bound enzymes on exposed, negatively charged phospholipid membranes of endothelial cells and activated thrombocytes. Additionally, thrombin works in two directions. Besides being the essential activator of fibrin, it also enhances further activation of fV, fVIII and fIX, fXI, fXIII and platelets, in a positive feedback loop. On the other hand, thrombin induces an anticoagulant mechanism by activating protein C, which then inactivates cofactors fVa and fVIIIa. Protein C is located on intact endothelial membranes, which limits the extend of coagulation to the site of injury. Circulating protein S functions as a cofactor for protein C, improving its anticoagulant activity[10]. Despite all regulatory mechanisms to either improve or limit coagulation for efficient clotting, defective clotting factors disrupt the system and may result in severe bleeding disorders. Among others, mutations in the fVIII gene can lead to a defective fVIII molecule or complete deficiency, both resulting in the bleeding disorder hemophilia A.

1.2 Structure and Function of Factor VIII

FVIII plays a center role in hemostasis, as fVIIIa and fIXa form an enzymatic complex, which activates fX. FVIII is produced in liver sinusoidal endothelial cells and extrahepatic endothelial cells[11,12,13]. It is a glycoprotein consisting of 2332 amino acids forming six domains in the order A1-A2-B-A3-C1-C2. Domains A1-A2-B make up the heavy chain (HC)

and domains A3-C1-C2 form the light chain (LC). The domains are connected via acidic linkers ha1, a2 and a3 [reviewed in 14,15]. Within the Golgi apparatus, fVIII undergoes several post-translational modifications. It is cleaved at the B domain to form the LC and the HC. They then form a heterodimeric complex connected via non-covalent bonding, which will then be secreted into the blood[16,17]. The protein travels through the circulatory system, bound non-covalently to vWF, which prevents premature activation, proteolysis and endocytosis. This improves the life span of fVIII. On its own, fVIII has a life-span of only two hours, while in complex with vWF it can last up to twelve hours [reviewed in 18]. The LC, or more precisely the C2 domain, is mainly responsible for binding to vWF. Additionally, domains C1-C2 and A3 of fVIII hold the major binding sites for phospholipid membranes of endothelial cells and platelets[19–21]. Thrombin and fXa activate the cofactor by cleavage at certain residues within the heavy chain[17,22]. Upon activation, fVIIIa is released from the fVIII/vWF-complex and forms the membrane-bound tenase-complex with fIXa and calcium on the surface of activated thrombocytes and endothelial cells[16,23]. The A2 domain holds the binding site for fIXa[24]. As its cofactor, fVIIIa highly increases the enzymatic activity of fIXa, and takes a rate-determining position in the activation of thrombin[19,23].

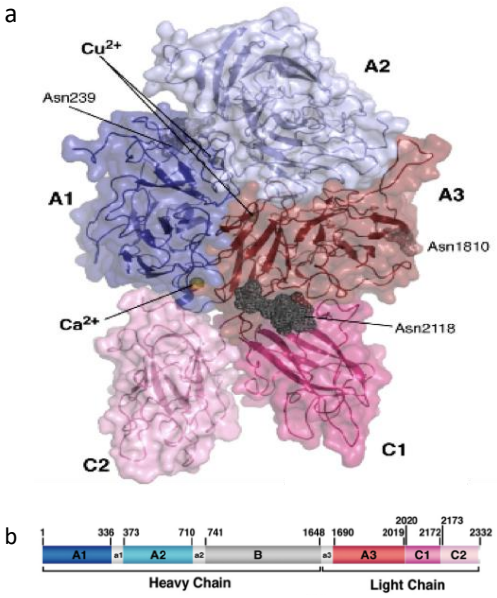

Figure 2 Structure of fVIII (adapted from Ngo *et al.*)[25]. a) Three-dimensional crystal structure of B domain-deleted fVIII. The model contains domains A1, A2, A3, C1, and C2. The model also includes two Cu^{2+} ions, one Ca^{2+} ion, and three carbohydrate fractions linked to asparagines (Asn). b) The model shows full length fVIII composed of the heavy chain (A1-a1-A2-a2-B) and the light chain (A3-C1-C2)

1.3 Hemophilia A

Several genetic mutations result in the defective expression or total lack of cofactor VIII, causing the disease hemophilia A (HA). Due to the X-linked heritage, almost all patients are men. Affected females are usually conductors with a residual fVIII activity, that causes little or no bleeding events. With an incidence of 1 in 5000 newborn boys, HA is part of the group of rare bleeding disorders. Mutations like deletions, inversions, insertions and punctual mutations within the gene are responsible, of which intron-22-inversion presents the most common cause of severe HA[26].

The disease is commonly classified into severe, moderate or mild, based on the residual fVIII procoagulant activity in the blood. fVIII activity <1% causes severe symptoms, levels between 1-5% cause moderate cases, and levels between 5-15% cause mild HA. Patients with a residual activity above 15% are not at risk of sudden bleedings[27]. As explained above, fVIII plays an important role in the effectiveness of intrinsic coagulation. Especially in severe cases, patients already show first signs during birth, such as prolonged bleeding of the umbilical cord. The next age at which patients may become apparent is during toddlerhood, when the risk of mild trauma rises with increased mobility. For instance, a trivial fall on the head may cause a deadly brain bleed. Besides exaggerated bleeding after trauma, patients suffer from sudden, non-traumatic intramuscular and intraarticular bleeding. Consequent deformation of the joints and contractions of the muscles reduces the patients' mobility. By the time they reach young adulthood, patients may already be dependent on a wheelchair[28,29]. In total, the quality of life and life expectancy diminishes with the severity of the disease. Nonetheless, patients with fVIII activity above 1% already show less sudden bleeding incidents and levels above 40% do not result in such at all[27]. From a therapeutic viewpoint, even a slight restoration of fVIII activity will improve symptoms of patients.

1.4 Hemophilia A Therapy and Anti-fVIII-Inhibitors

Standard therapy of HA consists of prophylactic substitution with fVIII containing products. Modern substitution therapy for fVIII deficiency started during the 1970s, when plasma concentrates of fVIII became available. However, these products posed high risks of viral infection, such as hepatitis C or human immunodeficiency virus. Today, plasma-

derived fVIII concentrates have become much safer and are still commonly used in HA prophylaxis. Additionally, progress in genetics has enabled the production of recombinant fVIII (rfVIII) from eukaryotic cell-lines[30, reviewed in 31]. While patients with mild hemophilia usually only require substitution during surgery or post-trauma, severe hemophiliacs undergo weekly fVIII substitution. When born with a severe defect, patients start regular treatment at a very young age, usually before their first birthday, with lifelong therapy foreseeable. Despite the obvious benefits of the treatment, no medical treatment comes without side effects. In about 30% of treated patients with severe HA, allogenic inhibitory antibodies (inhibitors) against (foreign) fVIII develop and cause treatment failure[32].

1.4.1 Risk Factors for Inhibitor Development

Why neutralizing anti-fVIII antibodies emerge is still subject to research. Interestingly, the chance of development of fVIII-inhibitors in HA patients is ten times higher than in any other coagulation factor deficiency[33]. In the past, several risk factors associated with inhibitor development have been identified and will be mentioned below. Anti-fVIII antibodies may also sporadically occur in healthy individuals with normal fVIII activity. This autoimmune disease is called acquired hemophilia A (AHA)[34].

Physiologically, during the maturation of the immune system, starting prenatally, the body “learns” to tolerate its own components. In patients with defective or missing fVIII such a tolerance towards fVIII is likely not build up sufficiently or specific to crippled protein. Later, when exposed to exogenous fVIII during therapy, the immune system can recognize the human protein as “foreign” and induces an inhibitor response. In short, the first step of the immune response is antigen presenting cells (APCs) taking up exogenous fVIII via endocytosis and presenting antigenic peptides via MHC II molecules to T cell receptors (TCR) of CD4+ T cells[35]. Upon activation, these T cells induce several immune mechanisms, which lead to the proliferation of anti-fVIII-specific B cells and differentiation into antibody-secreting plasma cells. These inhibitors eventually cause substitution therapy to fail and risk new bleeding events.

The likelihood of a hemophilia A patient for developing a fVIII-specific immune response depends on several reasons, such as the MHC class II phenotype, which determines

whether fVIII peptides will be presented to TCRs and the tolerance of T cells to fVIII. In healthy humans, T cell maturation in the thymus is strictly controlled and autoreactive T cells to self-antigens are outsourced through apoptosis. In hemophiliacs, tolerance induction of premature T cells does not occur or is only specific to altered 'self'-fVIII. The immune system is more likely to recognize the substituted fVIII as 'foreign', when its molecular structure strongly differs from 'self'-fVIII [reviewed in 36]. This explains why patients with certain mutations are more prone to develop inhibitors. Such mutations are nonsense mutations, great deletions or rearrangements, like the intron-22-inversion[37,38]. Activated thrombin catalyzes the parallel activation of a systemic inflammatory response. Thus, when high-dosage fVIII triggers coagulation in HA patients, the immune system might take this as a 'danger signal'. This may explain the immunologic rejection of administered fVIII with inhibitors[39].

Unfortunately, many more factors, other than genetics, attribute to the chance of inhibitor formation. Treatment-related risk-factors, such as the product type, the exposure days during treatment, the dosage and switching between different fVIII drugs are suspected to play a key role in provoking inhibitor development[37,38]. For instance, some studies have suggested that recombinant factor products are associated with a higher risk for inhibitor development, compared to plasma-derived products. Other studies repute this observation [reviewed in 31]. Whether early prophylaxis is preferable or poses a risk is controversially being discussed, nevertheless because the total constellation of risk factors of a patient impacts the chances of developing inhibitors[40]. Due to the multifactorial nature of inhibitor formation and their diverse appearance, it is still impossible to foretell the risks of inhibitor manifestation in HA patients. This adds to inhibitors being the most feared complication of HA therapy. The development of inhibitory antibodies against fVIII is currently the most serious complication of hemophilia A patients undergoing fVIII replacement therapy.

1.4.2 Characteristics and Immunology of fVIII-Inhibitors

The immune response to fVIII results in the formation of a polyclonal immunoglobulin G (IgG) antibody population. While inhibitors of all IgG subclasses have been identified, subclasses IgG1 and IgG4 predominantly occur. The immune attack may be directed

against every domain of fVIII. Inhibitors interfere with blood coagulation by impairing fVIII function in numerous ways. While so, each inhibitor maintains a high domain specificity, binding a specific combination of amino acid residues that form the epitope. Some interfere with the interaction of fVIII and phospholipid membranes or fIX. Others shorten the life span of fVIII by interfering with the complex formation with vWF or proteolytically deactivating fVIII. These qualities depend on the domain in which the inhibitor epitope is located. Figure 3 visualizes the different mechanisms in which inhibitors interfere with the fVIII function. While inhibitory antibodies pose the greatest fear, non-neutralizing antibodies, that bind non-coagulatory epitopes of fVIII, also exist. Even though they present a much lower affinity to fVIII, they may form immune complexes and thus benefit fVIII elimination[41].

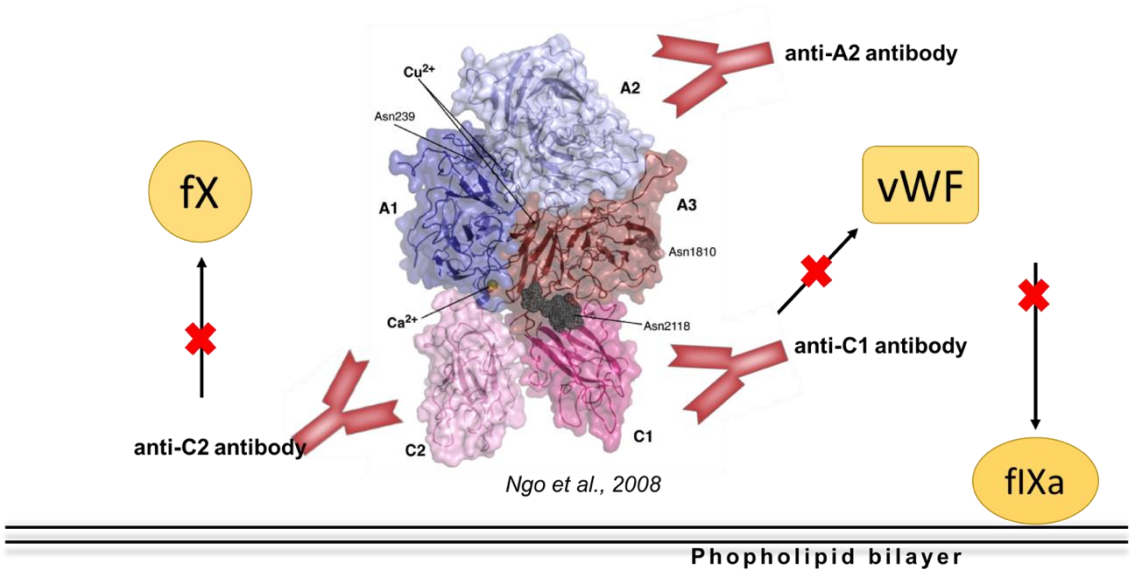

Figure 3 Mechanisms of fVIII Inhibition (adapted from Ngo *et al.*)[25]. Inhibitory antibodies directed against epitopes within the different domains of fVIII can cause interference of fVIII interaction with coagulant partners, such as coagulation factors, vWF and phospholipid bilayers.

While inhibitors against all domains have been identified, domains A2 and C2 are found to be immunodominant in HA and AHA[42,43]. Within the A2 domain, a total of eight different epitope groups have been identified via epitope mapping. For the C2 domain, five different epitope groups have been found. Recent studies demonstrated that C1 also contributes significantly to the immunogenicity of fVIII in congenital and acquired hemophilia[44]. It has been shown that the C1 domain is responsible for vWF and phospholipid binding, as well as involved in the uptake of fVIII by dendritic cells. Batsuli *et*

al. studied the binding properties of anti-C1 mAbs, that are responsible for a reduced fVIII level in the blood. They concluded that bleeding episodes in HA patients with C1 inhibitors may be caused by increased clearance of fVIII due to weakened fVIII/vWF complex formation[45]. Human anti-C1 mAb LE2E9, isolated from a mild HA patient, for example, competes with vWF and incompletely inhibits fVIII activity[46]. Overall research has demonstrated the immunogenic part-taking of C1.

Inhibitor titers are measured in Bethesda Units (BU/ml). One Bethesda Unit is defined as the amount of inhibitors needed to reduce fVIII activity in normal plasma by 50%. The assay is performed by incubating inhibitor-positive plasma in different dilutions with normal plasma. Following this, fVIII activity is measured and compared to fVIII activity in healthy plasma. The quotient of both measurements is used to determine the inhibitor titer[47]. Patients are divided into *high responders* with measurements above 5 BU/ml and *low responders* with titers between 0.6-5.0 BU/ml. Titers lower than 0.6 BU/ml are considered inhibitor negative. While *low responders* are able to receive fVIII substitutions, fVIII is ineffective in *high responders*[27]. This emphasizes the danger that inhibitors present to a hemophilic patient.

1.5 Immune Tolerance Induction Therapy

As pathogenicity of anti-fVIII antibodies is not fully understood, preventing or eradicating them successfully poses a difficult task. The only successful treatment for inducing immune tolerance towards fVIII consists of frequent administration of mostly high dosages of fVIII. This therapy is called immune tolerance induction, or ITI. Analysis of patients' plasma after successful ITI revealed, that the development of anti-idiotypic antibodies is one reason for successful eradication of fVIII inhibitors. Anti-idiotypic antibodies are a common regulatory mechanism of the immune system. They specifically bind the paratope of an antibody, preventing it from interacting with its epitope. This way, the immune system controls the impact of self-directed antibodies[48]. In the case of ITI treated patients, the immune system develops such antibodies, which are directed against the paratope of inhibitors and the membrane immunoglobulin of the B cell receptor of a memory B cell. This way, anti-idiotypic antibodies neutralize the effect of

inhibitors and prevent re-stimulation of memory B cells[49,50]. However, much research is still needed to fully understand the mechanisms of tolerance induction.

Different protocols of ITI are known in the world. The German guidelines (Deutsche Ärztekammer 2008) suggest up to 100-200 IU/kg body weight fVIII twice a day in patients with high inhibitory titers[40]. The success rate lies at about 76% [40], which also means that about 25% of ITI treatments fail and leave patients with low fVIII activity and little options to prevent bleeding risks. Moreover, ITI is not suitable for patients with acquired inhibitors, due to the autoimmune nature of the disease, compared to the rejection reaction against foreign fVIII by congenital hemophiliacs[51].

Effective alternative treatments after failed ITI therapy are scarce. Bypassing agents, like activated prothrombin complex concentrate (aPCC) and recombinant factor VII (rfVIIa), which engage in the coagulation cascade, can improve coagulation. However, even at recommended dosages the products have a small therapeutic spectrum and may cause thrombogenic events in patients[40]. Another option poses the application of the CD20-specific monoclonal antibody Rituximab, which depletes the immune system of all B cells. This drug has already been used off-label to eliminate fVIII-specific B cells in inhibitor-positive HA patients[40]. Unfortunately, this treatment poses strong negative side effects, as it depletes all CD20-positive B cells. Moreover, in some patients, fVIII-specific B cells producing inhibitors redeveloped from naïve B cells after successive fVIII substitution[52].

1.6 Experimental Research

Many experimental approaches for preventing and treating inhibitory antibodies are currently under investigation. One approach aims at the creation of less immunogenic fVIII products. The structure of the fVIII protein has been well analyzed and several inhibitor specific epitopes within the different domains have been characterized[25,45,53,54]. Inhibitors bind specific amino acid residues within the epitope, which can be replaced by site directed mutagenesis. Two major techniques for this are the alanine scanning mutagenesis and the use of human/porcine fVIII hybrids[55]. Porcine fVIII holds a slightly different set up of amino acids within its domains, resulting in a lower cross-reactivity with anti-human fVIII inhibitors. A recombinant B domain-deleted porcine fVIII (OBI-1) has shown improvement in hemostasis in patients with allo- or autoantibodies[56,57]. The

creation of human/porcine fVIII hybrids aims at altering epitopes of fVIII, creating a molecule that is less immunogenic in hemophilic patients. Parker et al. demonstrated a reduced inhibitory response to a hybrid human/porcine fVIII in mice, that were immunized with human fVIII, while maintaining the same hemostatic efficacy[58]. Another option to reduce immunogenicity of proteins is the PEGylation of such. By adding polyethylene glycol (PEG) to the structure of a molecule, such as fVIII, its molecular weight is increased and a hydrophilic cloud is created around it. This causes the molecule to be less prone to proteolysis. A PEGylated recombinant fVIII demonstrated prolonged half-life and studies suggest a decreased immunogenicity of the product[59]. A recombinant Fc-fused fVIII molecule demonstrated an increased half-life in patients with severe hemophilia, allowing a less frequent dosing. Simultaneously, none of the tested patients developed inhibitors, suggesting a better immunogenic tolerance towards the product[60,61]. The underlying mechanism argued, is the existence of regulatory T cell epitopes in the Fc chain of IgG antibodies[62]. Regulatory T cells (T_{regs}) play a center role in controlling the development of defensive immune response and inducing tolerance in humans. The recognition of T_{regs} epitopes induces T_{reg} populations and suppresses the immune response to co-present antigens. Schmidt et al. reviewed several methods to induce tolerance to fVIII in mice. For example, fVIII knock-out (fVIII-/-) mice, fed with fVIII antigen, resulted in the suppression of inhibitor populations through the development of fVIII-specific Tregs [reviewed in 63]. Yoon and Schmidt *et al.* successfully created a fVIII-specific human T_{reg} clone. They built a chimeric antigen receptor by using a fVIII-specific scFv, which was transduced into T_{regs}. These engineered T_{regs} were capable of suppressing fVIII-specific T effector cells *in vitro. In vivo* experiments with $fVIII^{-/-}$ mice showed a reduction in antibody production against fVIII[64].

1.6.1 The Role of Anti-idiotypic Antibodies in Research of Hemophilia A and Inhibitors

As mentioned above, the success of ITI is believed to partially be a result of anti-idiotypic antibodies. The role of anti-idiotypic antibodies has since played an important part in research for treating inhibitors of HA patients. Gilles *et al* discovered anti-fVIII antibodies and anti-idiotypic antibodies simultaneously existing in healthy individuals[48]. In 1984,

Sultan *et al.* described a partial restoration of fVIII activity in HA patients with inhibitors after administering high-dose intravenous immunoglobulin (IVIg). This effect was attributed to natural presence of anti-idiotypic antibodies in IVIg[65]. The success of ITI treatment is also partially attributed to the development of anti-idiotypic antibodies, as such were found in plasma of patients, who underwent successful ITI[49,50]. Gilles *et al* attempted to recreate the induction of anti-idiotypic antibodies in a mouse model. They immunized $fVIII^{-/-}$ mice with the anti-fVIII mAb BO2C11, which triggered an immunogenic reaction and the formation of anti-idiotypic antibodies to the corresponding mAb. Indeed, these anti-idiotypic antibodies were capable of neutralizing the respective mAb in test assays. Furthermore, *in vivo* experiments on hemophilic mice, treated with fVIII and the respective inhibitor, achieved restoration of fVIII activity after application of the anti-idiotypic antibody[66] .

Previously, our lab successfully selected a synthetic peptide from phage displayed peptide libraries, screened against inhibitor-positive plasma. In test assays, this peptide sequence mimicked a dominant fVIII epitope and was able to reduce inhibitory activity in tested plasma, partially restoring fVIII activity[67]. Similar results were found in other studies, using polyclonal human anti-fVIII antibodies as targets[53,54]. However, due to several reason peptides are unsuitable for effective neutralization of inhibitors. Inhibitor-specific scFvs, selected from phage libraries, pose a possible alternative to peptides. They present a longer half-life when used in *in vivo* experiments. Furthermore, scFvs can be fused into a scFv-Fc antibody format, which is necessary to be used in a therapeutic setting. Our research group successfully isolated scFvs for monoclonal inhibitors GMA8021 and GMA8006, using synthetic phage display libraries. Transformed into scFv-Fc proteins, they were capable of neutralizing their corresponding inhibitor and restore fVIII function *in vitro* and *in vivo* to an extent[68]. As the production of synthetic scFv-Fc is comparably simple, the generation of a pool of synthetic anti-idiotypes presents a possible option to neutralizing a significant number of inhibitors in patients. Additionally, inhibitor-specific scFvs could be used as the targeting moiety in immunotoxins for the selective elimination of fVIII-specific memory B cells. This approach would allow precise eradication of inhibitors.

1.7 Phage Display

Phage display describes a method of protein affinity selection that uses bacteriophages to express a molecule of interest, such as a protein, on the phage´s surface, while the protein DNA is packed within the phage's genetic material. This link between phenotype and genotype allows the selection of ligands for target molecules and their easy identification[69,70].

A virus that infects bacteria are known as a bacteriophage. It consists of a single strand DNA (ssDNA) surrounded by a tube-like protein membrane. A common bacteriophage used in phage display is phage M13, which belongs to a group of non-lytic filamentous phages that infect *Escherichia coli (E. coli)* bacteria that express an F-pilus. The phage's membrane is made out of approximately 2700 copies of the major coat protein PVIII. One tip of the phage is lined with five copies of the minor coat protein PIII, which is responsible for infection of the bacteria[71]. PIII is also most commonly used for expression of the desired molecule in phage display. Such a PIII fusion protein diminishes the infectivity of PIII with the F-pilus of bacteria[70]. To reduce this effect, phagemids were developed. They are plasmids, that only carry DNA of the PIII-fusion-protein and a sequence for secretion. An antibiotic resistance sequence is added to allow selection of infected *E. coli* during reproduction. To enable the reproduction of fully functioning bacteriophages, bacteria are also infected with a helper phage, such as KM13, which carries all genes of the structural components to assembly a fully functioning phage. A new phage will then express both wild type coat proteins and PIII fusion proteins. This reduces the interference of PIII fusion proteins during infection[69]. Figure 4 demonstrates a schematic illustration of a wild type M13 bacteriophage and one that expresses a scFv fused with a PIII protein.

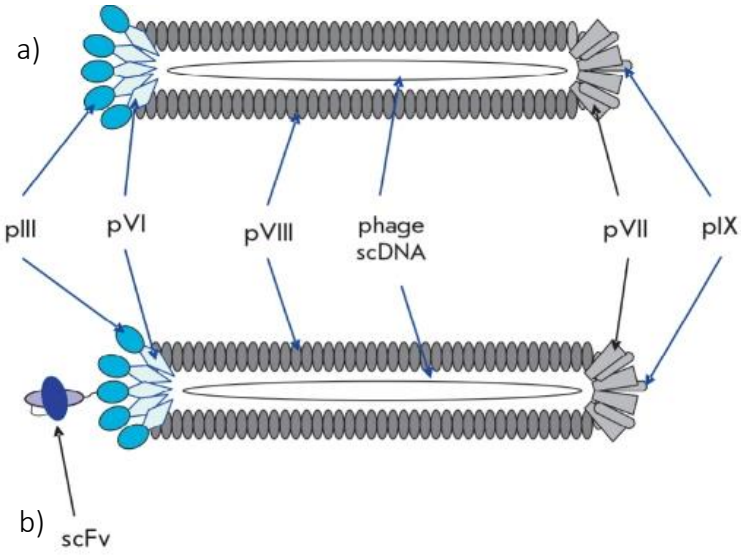

Figure 4: Schematic illustration of a) wild type and b) phage expressing a PIII fused with a scFv [from 71]. The ssDNA plasmid, carrying the genome of the phage, is surrounded by a cylindrical shell composed mainly of major coat protein PVIII. One tip is composed of minor coat protein PIII. b) PIII is fused to a scFv, as encoded in the genetically modified genome.

A phage display library is composed of numerous phage clones, expressing a vast variety of peptides, proteins or antibodies. To isolate phage clones, a selection process called biopanning, which is based on binding affinity, is used [73]. During the process, the target is immobilized and incubated with polypeptide-carrying phages. Specific binding phages are then used to infect *E. coli* bacteria. *E. coli* will then reproduce numerous copies of phage particles. The selected phages are analyzed by DNA sequencing, to recover the sequence coding for the unknown binding molecule[71].

1.8 Objective

Research on new diagnostic and treatment options for hemophilia A patients with inhibitors is of great importance, as they present the most serious complication of HA patients undergoing fVIII replacement therapy. The selection and characterization of anti-idiotypes, capable of neutralizing inhibitors and thereby restoring fVIII activity represents a possible approach to finding a treatment against fVIII inhibitors. Synthetic scFvs, that specifically bind the paratope of inhibitors can mimic anti-idiotypic behavior and thus interfere with inhibitory action.

In the first part of this work, we attempt to isolate scFvs from synthetic human phage display libraries, targeting polyclonal inhibitors from human plasma. Purified polyclonal

anti-fVIII antibodies from an inhibitor-positive human plasma pool will serve as a target. A method to purify anti-fVIII antibodies specifically, using affinity chromatography with protein A and fVIII-coupled Affi-Gel®, was developed and will be applied for this research[74]. Inhibitor-binding scFv-carrying phage clones will be enriched throughout several selection steps and later tested for their binding characteristics. ScFvs that show anti-idiotypic activity will then be fused with Fc chain fragments of murine and human origin to create scFv-Fc proteins. Their anti-idiotypic potential will be characterized in ELISA experiments. Hope is, that the selection of scFvs, using polyclonal human targets, will result in a wider range of specific scFvs and possibly have a greater chance of cross-reactivity with other fVIII-inhibitors. Also, a successful selection of anti-idiotypes for one patient, would open the possibility of applying this method as an individual treatment option against inhibitors.

In the second approach, we aim to select scFvs against two C1 domain-specific mAbs: human LE2E9 and murine GMA8011. This approach will add anti-idiotypes specific for C1 to the list of already existing scFv-Fc proteins specific for domains A2 and C2. Because it was shown that both anti-C1 mAbs recognize epitopes in human plasma, we theorize that specific-scFvs will also recognize inhibitors in these patients. Moreover, LE2E9 and GMA8011 show competitive behavior with one another, when binding fVIII and C1. The assumption was made that they may recognize identical epitopes[44,74]. Thus, selected scFvs for each mAb will be tested for their ability to cross-react with the other mAb. Confirmed cross-reactivity between both inhibitors will harden the theory of identical epitopes. This will also be a first attempt to generate anti-idiotypic scFv-Fc proteins directed against a human mAb. The clinical goal consists of preparing distinct anti-idiotypic mixtures to neutralize inhibitors in individual patients.

For both experiments, we will be using human naïve scFv libraries HuScL-2 and HuScL-3 with diversities of 1.4-1.6x10^9, supplying a great diversity of available antibodies. Separate biopannings, including three to four rounds of selection, will be performed using polyclonal anti-fVIII antibodies as well as LE2E9 and GMA8011 as target molecules.

2 Methods

2.1 Ethical Statement

We only used residual plasma samples from anonymized patients, obtained for medical examination.

2.2 fVIII-inhibitor-positive Patient Plasma

The HA patient plasma had to fulfill certain criteria to qualify for the experiment. We chose an anonymized patient's plasma, which was left-over from medical examination. The patient's inhibitory titer had to be sufficiently high, to enable a successful purification of plenty anti-fVIII antibodies. The chosen plasma samples showed inhibitory titers ranging from 93 BU/ml to >20000 BU/ml, which was analyzed by Bethesda assay (data not shown).

2.3 Protein Biochemistry

2.3.1 Purification of IgG Antibodies

Protein A Affinity Chromatography is an optimal method to clear IgG from suspension. It was therefore chosen as the first step to purify anti-fVIII-specific IgGs from human plasma. Moreover, it was used to extract scFv-Fc fusion proteins (scFv-Fc) from supernatant after production in eukaryotic cells. Working with Protein A columns took place at 4°C.

Anti-fVIII-specific antibodies were extracted from an anonymized inhibitor-positive patient's plasma pool. Plasma samples were initially heated at 56°C for 30 min to inactivate fVIII. The plasma samples were diluted in protein A binding buffer (200 nM) in a 1:20 ratio and completed to 5 ml with protein A binding buffer (20 nM), resulting in a final buffer concentration of 20 nM. Plasma samples were filtrated using a Millex-GS syringe filter unit (0.22 µm)

Cell culture supernatants containing scFv-Fcs were diluted in protein A binding buffer (200 nM), with a final buffer concentration of 20 nM, and kept on ice. Supernatants were

filtrated using a NALGENE™ Reusable Bottle Top Filter Unit with cellulose acetate filters (0.2 µm) before and after adding protein A binding buffer.

Before each purification, HiTrap Protein A HP columns (1 ml) were washed with 5 ml H_2O and primed with protein A binding buffer (20 nM) using a peristaltic pump. Then, samples were loaded onto the column at a slow rate of 1 ml/min. After they had passed through, the column was washed with 10 ml protein A binding buffer (20 nM). Antibodies were eluted from the column by connecting it to an ÄKTAprime. For ÄKTA program settings see Table 14. Eluted protein samples were neutralized with 250 µl protein A neutralization buffer. Finally, fractions containing protein were pooled and protein concentration was measured using a NanoDrop® 1000 spectrophotometer.

IgG concentrates from patient plasma were loaded onto a Zeba Spin column and rebuffered in Buffer A for Affi-Gel® HZ chromatography. ScFv-Fc solutions were rebuffered in 1xPBS using a Vivaspin 20 concentrator or SnakeSkin™ Dialysis Tubing (22 mm), dialyzing three times for three hours at 4°C. Concentrated protein samples were then stored in small aliquots at -20°C.

Protein A columns were recycled as follows: the column was cleansed using 10 ml of elution buffer and 10 ml Protein A binding buffer (20 nM). Next, the column was rinsed with 10 ml water. It could then be stored in ethanol or directly used for the next purification. If formerly stored in ethanol, it was rinsed with 10 ml water before reutilization. Before further purification, the column was equilibrated using Protein A binding buffer as described above.

2.3.2 Affi-Gel® HZ Column for Purification of Anti-fVIII-specific Antibodies

For extraction of fVIII-specific antibodies from the purified IgG pool of the human plasma, an Affi-Gel® HZ column was coupled with rfVIII (Kogenate®) and then loaded with IgG samples. In the process, rfVIII carbohydrate chains bind to the hydrazide functional group of the gel matrix. For this experiment, we followed protocol by Gilles *et al.*[41]. The binding capacity of Affi-Gel®, according to the manufacturer, is 1-5 mg IgG or fVIII per ml Affi-Gel®. Gilles *et al.* coupled 360 µg rfVIII to 1ml of Affi-Gel®.

First, 5500 IU rfVIII were dissolved in 4 ml coupling buffer and then dialyzed three times for three hours at 4°C against coupling buffer. Next, carbohydrate chains of rfVIII needed

to be oxidized using BSA, $NaIO_4$ and NaCl solutions at working concentrations according to the manufacturer's instructions. The mix was incubated for 1 hour at room temperature (RT) in the dark. Glycerin was added to a final concentration of 20 mM and incubated for 10 min to inactivate $NaIO_4$. The rfVIII solution was dialyzed again three times for three hours at 4°C against coupling buffer.

3.8 ml of Affi-Gel® HZ/isopropanol were washed three times using coupling buffer. Prior, isopropanol was removed by centrifugation. Between washing steps, the mix was centrifuged for 1 min at 100x g to remove supernatant. Next, the rfVIII dialysate was combined with the gel, at a final ratio of ~360 µg rfVIII per ml of packed gel. The mix was well combined by rotating for 24 hours at RT.

Before adding the patient's IgG, the gel mixture was centrifuged for one minute at 100x g and the supernatant was removed. Then, the gel was washed three times with washing buffer. Next, the matrix was equilibrated with buffer A three times. All centrifugation steps of Affi-Gel® were set 100x g for 1 min at low acceleration and deceleration.

The total volume of purified human IgG was added to the gel matrix and incubated for four hours at 4°C. Afterwards, the mixture was loaded onto a PolyPrep® Chromatography column. The column was washed with 16 ml washing buffer B and anti-fVIII-specific antibodies were eluted with 16 ml elution buffer C, 16 ml regeneration buffer D and 16 ml protein A elution buffer. Flow through of each buffer was collected separately and kept on ice. The protein A elution buffer eluate was neutralized 1:4 with Protein A neutralizing buffer. Eluates were dialyzed three times for three hours at 4°C against PBS and concentrated using a Vivaspin 20 concentrator. Extraction of anti-fVIII antibodies was analyzed by ELISA (for detail see section 2.3.8).

2.3.3 Preparation of Magnetic Beads for Phage Display Selection

Biopannings were performed in three or four rounds of selection. For each round, Dynabeads Myone™ Streptavidin C1 were prepared and loaded with the target antibody or the negative control antibody. 20 µl of beads for the negative selection and 15 µl for the positive selection were placed in 2 ml reaction tubes and washed five times with 500 µl washing buffer. Beads were separated from buffers using a magnetic concentrator.

For biopanning, targeting human anti-fVIII antibodies, 0.4 µg biotinylated anti-human IgG were added per µl beads in a total of 500 µl stock buffer, to achieve saturation (max. binding capacity: 0.2 µg per µl beads). For biopanning, targeting murine anti-fVIII antibodies, the same ratio of antibodies per µl beads was applied using biotinylated anti-mouse IgG. The mix was incubated for one hour at RT using a rotator. Afterwards, beads were washed five times with washing buffer and blocked with 500 µl blocking buffer for one hour under rotation at RT.

Next, beads coupled with biotinylated antibodies were coupled with the corresponding target anti-fVIII antibodies. Beads coupled with biotinylated antibody have a maximum binding capacity of 0.4 µg per µl beads for the target antibody. To secure saturation, the target antibody or respectively the negative control antibody was added at 1.5 times the required volume. Meaning, 0.6 µg target antibody were added per one µl coupled beads in a total volume of 500 µl stock buffer, diluted to working concentrations. Incubation lasted two hours at RT rotating.

2.3.4 Affinity Selection (Biopanning)

The aim of both projects in this work was to select scFvs, that bind anti-fVIII-antibody paratopes. Target antibodies were a pool of purified anti-fVIII antibodies from human plasma as well as anti-C1 domain mAbs GMA8011 and LE2E9. Biopanning was applied for affinity selection of scFv-carrying phages from phage-displayed libraries. The process of biopanning, including positive and negative selection, is visualized in Figure 5. The positive selection entailed the incubation of scFv-carrying phages with immobilized target antibodies. The negative selection exposed target-binding phages to an isotype-antibody of the same IgG isotype. Phages, not binding the paratope of the target, were eliminated this way. A total of three or four rounds was performed.

As described in section 2.3.3, antibodies were coupled via biotinylated IgG to 15 µl magnetic beads for positive selection and 20 µl of beads for negative selection. Prior to biopanning, prepared beads were washed five times using washing buffer. A magnetic concentrator was used throughout biopanning to separate beads from buffers.

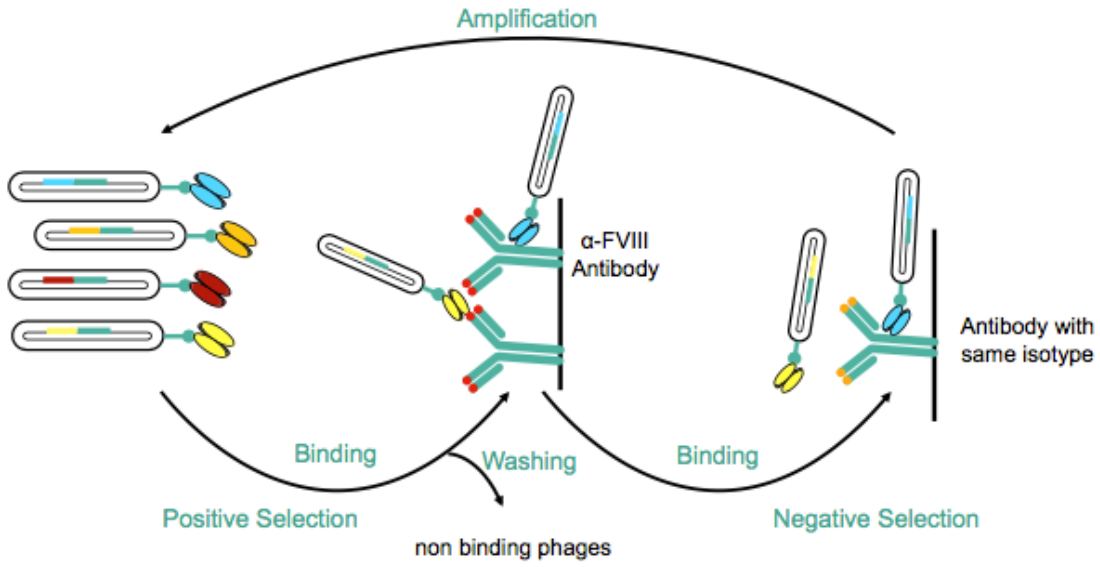

Figure 5 Scheme of Phage Display Library Screening (Biopanning)[74]. During selection rounds scFv-carrying phages from phage libraries are presented to immobilized anti-fVIII antibodies. Non-binding antibodies are washed off, while binding antibodies are eluted and transferred to a negative selection. Here, an unspecific isotype antibody binds unspecific phages. scFv-carrying phages specifically binding the paratope of the anti-fVIII antibodies are then taken and amplified for the next round of selection.

For the first round of selection, $3x10^{11}$ plaque forming units (PFU) of the phage libraries HuScL-2 and HuScL-3 were added to each 15 µl beads coupled with target in a total volume of 500 µl stock buffer, diluted to working concentrations. The mix was rotated for two hours at RT. Non-binding phages were removed by washing the beads ten times with 500 µl washing buffer. Bound phages were eluted from the beads by adding 192 IU rfVIII in 450 µl PBS+Ca^2 and incubating one hour at RT under rotation. The eluate was transferred to 20 µl of beads with coupled isotype antibodies and rotated for two hours at RT for negative selection.

Afterwards, supernatants were moved to new reaction tubes and combined with 50 µl of trypsin. The KM13 helper phage carries a trypsin cleaving site within the PIII protein. Trypsin cuts PIII from the helper phage, leaving them non-infective, while phagemids that carry a scFv fusion will not be affected. After 10 min of incubation at RT, 250 µl of the digested eluate were added to 1.75 ml of *E. coli* TG1 culture with an optical density (OD_{600}) of 0.4 for 30 min at 37°C for infection. Left-over eluate was stored at 4°C. Dilutions of infected bacteria were prepared for phage titration. Phage titration will be thoroughly described in the chapters below. The remaining bacteria were centrifuged at

2000x g for 25 min and resuspended in 100 µl supernatant medium, before being spread out onto a 25 cm 2xYT$_{amp/glu}$ agar dish. Plates were placed at 37°C for overnight growth of bacterial colonies. Amplification of selected phage clones was performed as in section 2.3.5.

In the following rounds of selection, 1x10^{12} PFU phage precipitates from the previous rounds were used. Also, washing of the beads during the positive selection was repeated 15 times with 500 µl washing buffer.

2.3.5 Amplification of Selected Phages

The bacterial colonies on the 25 cm plate were washed off using 7 ml 2xYT$_{amp/glu}$ medium. The suspension was diluted 1:1000 in 50 ml 2xYT$_{amp/glu}$ medium and incubated at 37°C, 225 *rotations per minute* (rpm) until reaching log phase at OD_{600} of 0.4. As a backup, 500 µl of the bacterial suspension was mixed with PBS/glycerol and stored at -80°C.
After reaching log phase, 10 ml of the bacterial culture were infected with 5x10^{10} PFU KM13 helper phage for 30 min at 37°C. Afterwards, the bacteria were centrifuged at 3000x g for 10 min. The pellet was resuspended in 50 ml 2xYT$_{amp/kan}$ medium. Phage amplification was proceeded overnight through incubation at 30°C and 225 rpm.
The following day, the bacterial culture was centrifuged at 10000x g for 30 min to remove bacteria. The supernatant was moved to a new reaction tube and phages were precipitated by adding 50% PEG/NaCl (2.5 M) at a 1:5 ratio and leaving the mixture on ice for one hour. Phages were separated from medium via centrifugation at 10000x g for 15 min. To clear the phage pellet of bacterial debris, it was resuspended in 1.6 ml 1xPBS and centrifuged again at 13000x g for 10 min. The pellet was discarded. For the second precipitation, the supernatant and 50%PEG/NaCl (2.5 M) were combined at a 1:5 ratio and left on ice for another hour. Thereafter, the phages were separated from the mixture by centrifugation at 10000x g for 15 min and the pellet was resuspended in 1 ml PBS/NaN_3 and stored at 4°C.

2.3.6 Determination of Phage Titers

To determine the titer of phage suspensions, the absorption of the phages' DNA was measured at 269 nm and 320 nm using a NanoDrop® spectrophotometer. The following equation, developed by Day *et al.* in 1979, to calculate titers was used[75]:

$$\frac{(A_{269}\text{-}A_{320})\times 6\times 10^{16}}{\text{bp phage genome}} \times \text{dilution factor} = \text{phage titer [phages/ml]}$$

Additionally, the titer was determined by preparing diluted plate cultures of phage stocks to determine the number of phage-infected single clones. As described above, 1.75ml of *E. coli* TG1 (OD_{600}=0.4) were combined with 250 µl of phage preparation for infection at 37°C for 30 min. Infected bacteria were diluted 10^{-2} to 10^{-4} in PBS. 20 µl of each dilution was spread out onto $2xYT_{amp/glu}$ agar dishes. After overnight growth at 37°C, bacterial colonies were counted and PFU/ml were calculated.

2.3.7 Production of Single Phage Clones

After selection rounds of the biopanning process was finished, phages were prepared for analysis. Single clones were picked from titration plates and separately transferred into wells of 96-well culture plates containing 200 µl $2xYT_{amp/glu}$ medium. Bacteria were allowed to grow overnight at 30°C and 115 rpm. 100 µl PBS/glycerol were added to each well of the original culture plates and stored at -80°C for back-up purposes. Before PBS/glycerol was added, 5 µl of pre-cultures were diluted in 200 µl fresh $2xYT_{amp/glu}$ medium and incubated for two hours at 37°C and 115 rpm. Next, bacterial cultured were infected with $1x10^9$ helper phage for 30 min under the same conditions. Plates were then centrifuged at 2000 rpm for 10 min and supernatant medium was replaced by 200 µl $2xYT_{amp/kan}$ medium. Bacterial pellets were resuspended and incubated overnight at 30°C at 115 rpm, for phage production. Supernatants, containing phage clones, were analyzed in ELISA experiments.

To amplify promising phage clones on a larger scale, the respective bacteria from the back-up plates were inoculated with 5 ml $2xYT_{amp/glu}$ medium and incubated overnight at 37°C, 225 rpm. The precipitation of phages followed the same protocol as the precipitation during the biopanning. Phage precipitates were then further studied.

2.3.8 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was used to analyze the binding characteristics of selected scFv phages and scFv-Fcs. Furthermore, it was used to test for presence of anti-fVIII antibodies in the different steps of Affi-Gel® HZ affinity purification. ELISAs were performed using 96-well microtiter plates. Coating of wells was performed over night by incubating target protein in 100 µl PBS at 4°C. Analysis was performed in duplicates or triplicates. Plates were washed three times with PBST using an ELISA-plate-washer after each incubation step. To avoid unspecific interactions, all coated wells were blocked with 200 µl 5% MPBST for two hours at RT. All other incubation steps were also performed two hours at RT.

ELISAs were developed using 100 µl o-phenylenediamine (OPD, activated with 1:4000 H_2O_2) for 5 -10 min in darkness and the reaction was stopped by adding 100 µl H_2SO_4 (0.5 M). A Tecan Sunrise™ ELISA reader was used to measure the adsorption at 492 nm and 620 nm for reference. When selecting binding phage-clones, a cut-off was set at 0.2 absorption units (AU). Phages displaying signals below this cut off where defined as non-binders.

2.3.8.1 Detecting Anti-fVIII Antibodies after Affi-Gel® HZ Affinity Purification

For detecting anti-fVIII antibodies after Affi-Gel® HZ affinity purification 0.5 IU rfVIII were immobilized on plates using PBS+Ca^{2+} as buffer. The input, flowthrough, wash and eluates from Affi-Gel® HZ affinity purification steps were incubated in a series of dilutions (undiluted; 1:10; 1:100; 1:200). Afterwards, 100 µl HRP-conjugated goat anti-human IgG, diluted 1:5000 in MPBST, was added to each well and incubated. ELISA signals were developed and detected as described above.

2.3.8.2 Binding Characteristics of Selected scFv-carrying Phage Clones

Half of the wells of microtiter plates were coated with 0.1 µg of the respective target antibody and the other half with isotype control antibody. To analyze bacterial supernatants, containing scFv-carrying phages, 30 µl of bacterial supernatant from phage amplification were mixed with 70 µl of PBS in coated wells. For advanced analysis, $1x10^9$ PFU of precipitated phages were added to coated wells in 100 µl PBS. For titration of scFv-

carrying phages, concentrates were prepared in series of dilutions in PBS and tested for binding to coated antibodies.

Competition of scFv-carrying phages with rfVIII was tested by first adding 2 IU of rfVIII in 50 µl PBS+Ca^{2+} to coated wells for 30 min at RT and then adding phage precipitates in concentrations leading to 75 % binding signal. Simultaneously, the control was performed with PBS instead of fVIII.
To detect scFv-carrying phage clones, 100 µl of HRP-conjugated anti-M13 IgG, diluted 1:4000 in MPBST, were added to each well. ELISA signals were developed as described above.

2.3.8.3 Binding Characteristics of scFv-Fc Fusion Proteins

To test scFv-Fcs for binding to their respective anti-fVIII antibody, wells of microtiter plates were coated with 0.1 µg of the respective target antibody and isotype control antibody in 100 µl PBS. A series of dilutions of the scFv-Fc in PBS was prepared. HRP-conjugated goat anti-human IgG, diluted 1:5000 in MPBST, was used for detection of scFv-Fc with a hIgG1-Fc chain. HRP-conjugated rabbit anti-mouse IgG, diluted 1:5000 in MPBST, served for detection of scFv-Fc with an mIgG2c-Fc chain.

Competition of scFv-Fcs and anti-fVIII mAb with rfVIII was examined as follows: Microtiter plates were coated with 0.5 IU rfVIII per well. First, a master mix of GMA8011 or LE2E9, with concentrations leading to 50-75 % binding to fVIII was set up. These measurements were previously taken by our lab (data not shown). Second, a series of two- or ten-fold dilutions of the fusion protein was created, starting with a 400-fold molar excess to the monoclonal antibody. At last, the master mix was combined 1:1 with each dilution and incubated for one hour at 37°C. 100 µl of each mix was added to fVIII-coated plates. In this type of ELISA, the anti-fVIII mAb was detected, using HRP-conjugated goat anti-human IgG for LE2E9 and HRP-conjugated rabbit anti-mouse IgG for GMA8011. Detection antibodies were diluted 1:5000 in MPBST. ELISAs were developed as described above.

2.3.9 Bethesda Assay: Measuring Inhibitor Activity

The inhibitory antibody titer is determined through Bethesda assay. Inhibitor positive plasma samples were prepared in a serious of dilution with imidazole buffer and

combined equally with normal fVIII plasma. Mixtures were incubated at 37°C for two hours. 5 µl of dilutions were added 1:10 to imidazole buffer and then mixed with 50 µl fVIII deficient plasma and 50 µl Dade® Actin® FLS. Coagulation was initiated by adding 50 µl $CaCl_2$. Time until clotting was measured. A fVIII activity reference was determined by using normal plasma instead of inhibitor-positive plasma in the set up. Results of residual fVIII activity were divided by the fVIII activity reference and multiplied with the dilution factor. Results were then translated to a calibration curve and matched with an inhibitory titer. One Bethesda unit (BU) describes the amount of inhibitor needed to diminish fVIII activity by 50 %.

2.4 Molecular Biology

2.4.1 Bacterial Cultivation

A single colony of *E. coli* TG1 was produced over night in 100 ml of 2YT medium at 37°C and 225 rpm. The next day, the culture was diluted 1:100 in 2YT medium and incubated under the same conditions until reaching log phase (OD_{600}=0.4).

2.4.2 DNA Preparation

Bacterial clones were cultured in 5 ml or 150 ml 2xYT medium mixed with antibiotics and grown over night. To isolate DNA from bacteria, the GeneJET Plasmid Miniprep Kit was used following the manufacturer's instructions. The NucleoBOND PC500 Kit was used for purification of larger quantities of plasmid DNA using 150 ml overnight culture. To isolate DNA from agarose gel after electrophoresis, the peqGOLD Gel Extraction Kit was chosen. DNA samples were measured with a NanoDrop® 1000 spectrophotometer and stored at -20°C.

2.4.3 Agarose Gel Electrophoresis

DNA from PCR was separated by gel electrophoresis using 1 % (w/v) agarose containing TAE gel. Roti®-GelStain, diluted 1:20, was used for visualization of DNA. DNA samples were mixed 1:5 with 6x DNA gel loading dye and loaded onto the gel. Electrophoresis was set up at 100 V for 30 min.

2.4.4 Cloning of scFv DNA into Fc Chain Vector Plasmids

Preceding ligation of scFv DNA with vector plasmids, both had to be digested using restriction enzymes *Nco*I and *Not*I. In separate digestion reactions purified scFv DNA and 50 ng of desired vector DNA were each combined with CutSmart® Buffer, following one hour of incubation at 37°C.

ScFv sequences were ligated into vector plasmids pCMX-mG2c-Fc and pCMV-hG1-Fc in front of the Fc region (see Table 13). The NEB Quick Ligation™ Kit was used according to the manufacturer instructions. 50 ng digested vector DNA and threefold molar excess of scFv DNA were combined and the volume was adjusted to 10 µl with water, before adding 1 µl of T4 DNA ligase diluted in 10 µl 2x ligation buffer. After five minutes of incubation at RT, mixtures were used for transformation of chemically competent *E. coli* Top10.

2.4.5 Production of Chemically Competent E. coli

A clone of *E. coli* Top10 was cultured overnight in LB medium. The overnight culture was diluted 1:50 in fresh LB medium and incubated at 37°C until reaching an OD_{600} of 0.4. The culture was then left on ice for 10 min and centrifuged at 3220x g at 4°C for 10 min. The pellet was resuspended in 40 ml ice-cold $CaCl_2$. After an incubation time of three hours, the mix was centrifuged again and resuspended in 4ml $CaCl_2$/glycerol. Aliquots containing 50 µl of cells were frozen in liquid nitrogen and stored at -80°C. To define the competence of bacteria, aliquots of newly prepared chemically competent cells were transformed with known amounts of DNA (0.05 - 0.5 ng). $1x10^6$ colonies/µg DNA were considered as satisfactory.

2.4.6 Transformation of Chemically Competent *E. coli*

E. coli strain Top10, made chemically competent, was used for transformation with plasmid DNA or ligation samples. For the process, all of the ligation sample was added to 50 µl of bacteria and left on ice for 30 min. Afterwards, the mix was left on a heating block at 42°C for 30 seconds for heat shock, then directly transferred on ice for another three minutes. Finally, 1 ml of LB medium was added and incubated for 45 min at 37°C under rotation. 200 µl of the bacterial mix were spread onto LB_{amp} agar plates. The rest was

centrifuged shortly and the pellet was resuspended in a smaller volume before it was plated onto a second LB_{amp} agar plate.

2.4.7 Amplification of DNA Fragments by Polymerase Chain Reaction (PCR)

Colony PCR served to identify bacterial clones, which carried properly ligated vectors (see Table 13). For each construct, ten bacterial colonies were picked from agar plates after transformation with the ligated product from 2.4.4 and mixed with 5 µl of 10x Taq buffer, 1 µl dNTP, 1 µl primer for and 1 µl primer rev and 0.2 µl Taq polymerase in a total volume of 50 µl.

PCR run-through was performed as follows:

Cycles	Temperature [°C]	Time [min]	
1	95	5	Initial denaturation
30	95	1	Denaturing
	61	0.75	Annealing
	72	1/kb	Elongation
1	72	5	Final elongation

2.4.8 DNA Mutagenesis by Overlap Extension PCR

DNA sequences of selected scFvs H2C1 and H3F10 contained a stop codon TAG, which had to be replaced by a glutamine-encoding codon through site-directed mutagenesis. An overlap extension PCR, following Heckman *et al.*'s PCR protocol using Phusion® High-Fidelity DNA Polymerase, was performed for each scFv DNA template. Figure 6 demonstrates how overlap extension PCR is used for DNA mutagenesis. Two types of primers were designed. The flanking outer primers (a and d) recognize the 5' end of the gene sequence. The inner primers (b and c) carry the mutation, that will replace the stop codon with a glutamine-encoding codon. In a first set of PCRs, the DNA sequence of the scFv was combined with the primers a and b in one PCR setting and primers c and d in another PCR setting. This produced new DNA templates AB and CD, carrying overlapping

complementary segments at the 3' end. When combined in a second PCR set up, segments AB and CD are denatured and the 3' strands will anneal. The newly created AD sequence will no longer carry the stop codon, but the mutagenic glutamine codon[76].

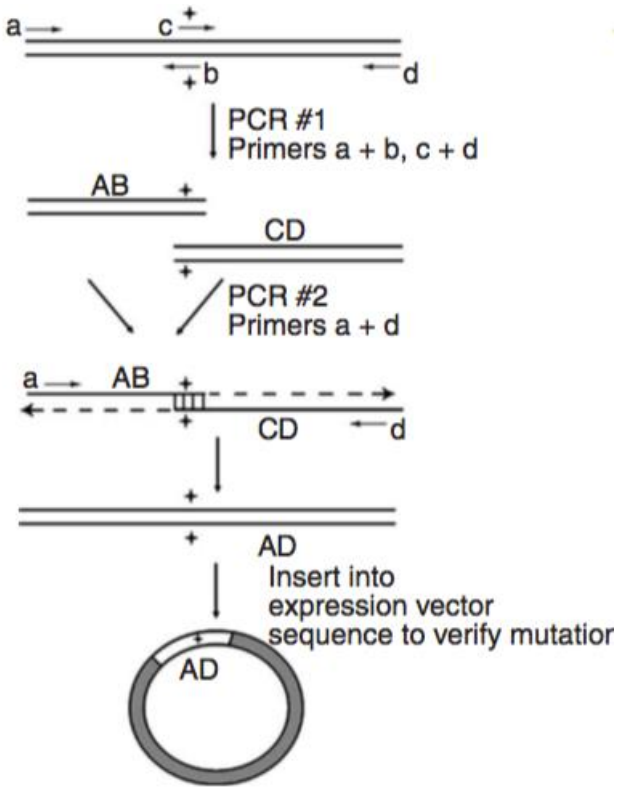

Figure 6 Schematic Principle of PCR-mediated overlap extension for site-directed mutagenesis.[76]
In PCR #1, site-directed mutagenesis is achieved by using inner mutagenic primers (b and c) and outer primers (a and d), generating PCR sequences AB and CD. PCR #2 was set up to splice products AB and CD. For this, both products were denatured and DNA strands anneal at their overlapping, complementary ends. Amplification of product AD is then started by outer primers a and d. Product AD is then ligated into chosen vector plasmids.

The mixtures for PCR were prepared as follows:

4 µl	HF buffer
0.4 µl	dNTP
2.5 µl	outer primer [20µM]
2.5 µl	inner primer [20µM]
40 ng	scFv-carrying phage DNA **
0.5 µl	Phusion® polymerase
add 20µl	H_2O

* See Table 11 for primers used

** for the third PCR, DNA material from fragments AB and CD were added in a fitting ratio, considering the base pair length and weight of each fragment

The following PCR program was installed in a Gene Amp® PCR System 9700:

Cycles	Temperature [°C]	Time [sec]	
1	98	30	Initial denaturation
30	98	5-10	Denaturing
	72	10 *	Annealing
	72	15-30/kb	Elongation
1	72	5	Final elongation

* Annealing time in the third PCR was 45 seconds.

To isolate the DNA products, we ran the PCR product through gel electrophoresis, as described in the protocol by Hackman and Pease *et al.* For detail, see section 2.4.3. The gel band with the expected base pair size of the desired DNA segment was cut out. PCR products were then purified from gel, using the peqGOLD Extraction Kit.

2.4.9 DNA Sequencing

For sequencing, DNA samples of 400-500 ng were infused with 2.5 µl of 10 µM sequencing primer in a total volume of 10 µl and sent to GATC biotech for sequencing.

See Table 10 for primers used.

2.5 Cell Biochemistry

2.5.1 Cultivation and Transient Transfection of Eukaryotic Cells

HEK 293T cell line was cultivated in DMEM-FBS at 37°C and 5% CO_2.

Eukaryotic 293T cells were transiently transfected with the desired scFv-Fc-encoding vector (see Table 13 for product information). For each vector, 16 culture plates were prepared each with $5x10^6$ HEK 293T cells in 8 ml DMEM-ITS. Cells were left to settle overnight at 37°C and 5% CO_2. Per plate, a mix of 5 µg vector, 1 ml DMEM-FBS and 50 µl polyethylenimine (PEI) was combined, vortexed and left for 10 min at RT. 1 ml of the mixture was added gently to each plate. Cells were incubated overnight for transfection. The following day, plates were washed using 8 ml PBS and filled with 8 ml DMEM-ITS. On the following three days, supernatants containing secreted proteins were collected and subsequently replaced with fresh medium. Collected media were centrifuged before being stored at -80°C since further use. ScFv-Fcs were purified from cell culture supernatants using protein A affinity chromatography (see section 2.3.1)

3 Results

The development of fVIII inhibitors proposes the most difficult complication in patients with hemophilia A. While the majority of fVIII inhibitors are directed against the A2 or C2 domain of fVIII, it has recently been shown that many patients also possess antibodies against the C1 domain[44]. Overall, the presence of anti-fVIII antibodies can so far only be treated by ITI therapy, putting patients under debilitating and money-consuming stress, with therapy success being incalculable. This shows the need for novel therapeutic approaches for hemophilia A patients with inhibitors. One focus lies in the concept of anti-idiotypic antibodies, as anti-idiotypic antibodies occur naturally in the immune system as a regulatory factor neutralizing anti-fVIII antibodies and thereby restoring fVIII activity[48].

3.1 Selection of ScFvs Specific for Human Anti-fVIII Antibodies Purified from Patient Plasma

For the first project, we pursued to select anti-idiotypic scFvs using purified anti-fVIII antibodies from a human plasma pool. In previous experiments, our lab already attempted to select scFvs against polyclonal anti-fVIII antibodies by using whole plasma samples from inhibitor positive patients. However, several different approaches did not lead to a successful selection of inhibitor-specific scFvs[74]. To improve the probability of finding anti-idiotypic scFvs for polyclonal anti-fVIII antibodies, we performed a biopanning with initially purified anti-fVIII antibodies from an inhibitor-positive human plasma. For this purpose, we chose plasma samples from an anonymized patient with high Bethesda Units ranging 5000-20000 BU/ml. Previous analysis of fVIII-specific antibodies in these plasma samples revealed dominance of the IgG4 subclass, mainly directed against HC and C2 of fVIII molecules (data not shown) *.

*Analysis was performed by Dr. Kerstin Brettschneider

3.1.1 Purification of Human Anti-fVIII Antibodies from Patient Plasma

The anti-fVIII antibodies were purified from plasma in a two-step procedure. First, IgG antibodies from plasma sample were extracted using Protein A affinity chromatography. Protein A knowingly binds IgG subclasses 1, 2 and 4. Previous IgG subclass analysis showed that our patient had an insignificant concentration of IgG3 antibodies (data not shown), which is why Protein A chromatography offered a suitable purification system.

Secondly, fVIII-binding antibodies were separated from the IgG pool using Affi-Gel® hydrazide agarose coupled with fVIII. For this, rfVIII was coupled to the Affi-Gel® matrix as described in section 2.3.2. Briefly, 5500 IU rfVIII were coupled via oxidized carbohydrate chains to hydrazide functional groups of the Affi-Gel® matrix. After loading the patient's purified IgG pool onto the matrix, non-binding IgG were washed off and fVIII-binding antibodies were eluted in three ways, using buffers of 50% ethylene glycol (pH 6), 75% ethylene glycol (pH 6) and 0.1 M citric acid (pH 3). Figure 7 schematizes the purification process.

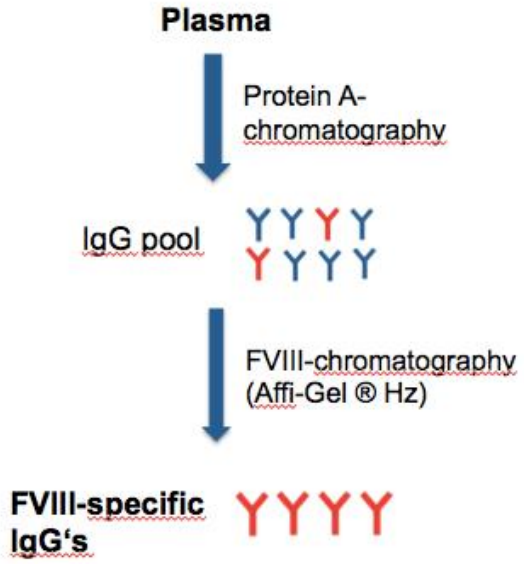

Figure 7 Purification of fVIII-specific IgG. Human IgGs were purified from plasma samples by protein A chromatography. Eluted IgGs were incubated with fVIII-coupled Affi-Gel® matrix to bind fVIII-specific IgG. Bound antibodies were eluted using different elution buffers (for further detail see 2.3.2).

The outcome of the procedure was verified by ELISA using fVIII as immobilized antigen. The IgG solution from protein A purification, was tested and is described as 'input' in Figure 8. Furthermore, the 'flow through' and 'wash' as well as all eluates of buffer 'C', buffer 'D' and citric acid were tested for IgG binding fVIII.

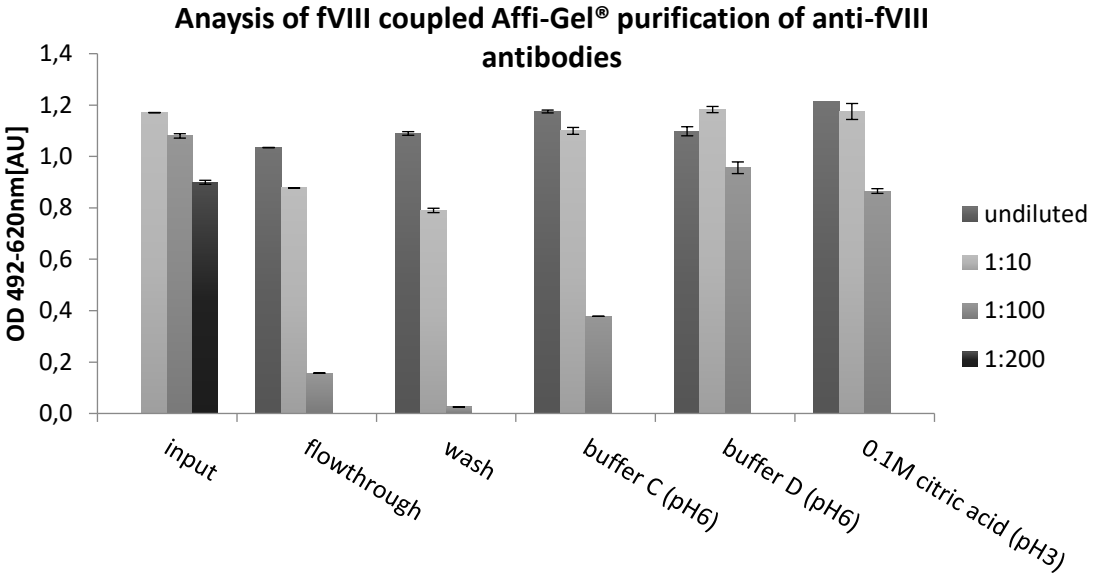

Figure 8 Analysis of Affi-Gel® purification of anti-fVIII antibodies. 0.5 IU rfVIII were immobilized on microtiter plates. Input IgG, flow through, wash and eluates were added in different dilutions (undiluted, 1:10, 1:100, 1:200). Binding antibodies were detected using HRP-conjugated goat anti-human IgG. Errors bars indicate standard deviation (SD) of duplicates.

ELISA binding signals of fVIII-specific antibodies in the input and eluates were strong, showing that anti-fVIII antibodies were present in the IgG pool obtained from Protein A purification and in antibodies eluted from Affi-Gel®. Signals of eluates of buffer D and citric acid appeared the strongest, suggesting that the antibody yield was the highest. Considerable signals of 'wash' and 'flow through' from Affi-Gel® suggest that fVIII antibodies were lost during purification.

The inhibitory activity of pooled anti-fVIII antibodies after elution was detected as 15967.63 BU/ml, suggesting the purification of fVIII inhibitors. In comparison, the original plasma probes' inhibitory titers ranged from 5000-20000 BU/ml. A total of 450 µg IgG was collected during fVIII affinity chromatography. Overall, extraction of polyclonal human anti-fVIII antibodies was successful and purified antibodies could be used as targets for the selection of scFvs.

3.1.2 Selection of ScFvs Specific for Human Anti-fVIII Antibodies

In the next step, phage display was applied to isolate scFvs specific for human anti-fVIII antibodies. For this, human naïve scFv libraries HuScL-2 and HuScL-3 were used. For negative selection, as described in section 2.3.4., an IgG-pool of 50 healthy humans was used. After three selection rounds composed of one positive and one negative selection, 48 selected phage clones from each phage library were analyzed for their binding behavior to immobilized human anti-fVIII antibodies and IgG control in ELISA.

Out of the analyzed phage clones picked from HuScL-2, 22 solely bound the IgG control and 15 bound the target as well as the IgG control. Eleven clones did not show signals above the defined cut-off and were dismissed as non-binders. From HuScL-3, we found 40 binding phage clones out of which 18 bound exclusively the IgG control. Eight phage clones were non-binders. None of the selected phage clones showed specific binding to the anti-fVIII antibody target.

Because we only selected unspecific phages, we tested whether selection and amplification during rounds led to an effective accumulation of specific binders and a depletion of non-specific binders. Using phage precipitates from each round*, E. coli* TG1 were infected and phages were produced. Single phage clones were then analyzed for binding specificity in ELISA.

All selection rounds of HuScL-2 revealed only phages binding both target and IgG control. Outcomes showed that while in the first round, 10 out of 48 tested clones showed binding signals in ELISA experiments, the number of binding clones in round two and round three was reduced down to two and one. (Table 1) Repeating the same experiment with selected phages from HuScL-3, we were unable to grow any bacterial clones infected with phages from selection rounds, even though phage titers were calculated as high in phage precipitates.

Table 1: Summary of analysis of phage clones from each selection round of biopanning with HuScL-2 against purified anti-fVIII antibodies from human plasma

Selection Rounds (R)	Number of phage-clones tested	Unspecific binding clones		IgG control only binding clones		Anti-fVIII antibody only binding clones
1	48	10	21%	3	30%	0
2	48	2	4%	1	50%	0
3	48	1	2%	0	0%	0

In a second experiment, we tested precipitated phage pools of each selection round for binding to anti-fVIII antibodies and the IgG control, to see if there is a noticeable trend of phages being accumulated or depleted. For this, an ELISA was set up, where equimolar amounts of anti-fVIII antibody target and IgG control were immobilized and phage precipitates from each selection round were added. Figure 9 visualizes the selection of binding phage clones over the three selection rounds. A rise in signal intensity over the rounds of phages, binding both target and isotype control, suggests an overall accumulation of binding clones. A reduction of IgG control binders cannot be seen. Investigated phage precipitates, selected from HuScL-3, showed no signals in ELISA for any selection round.

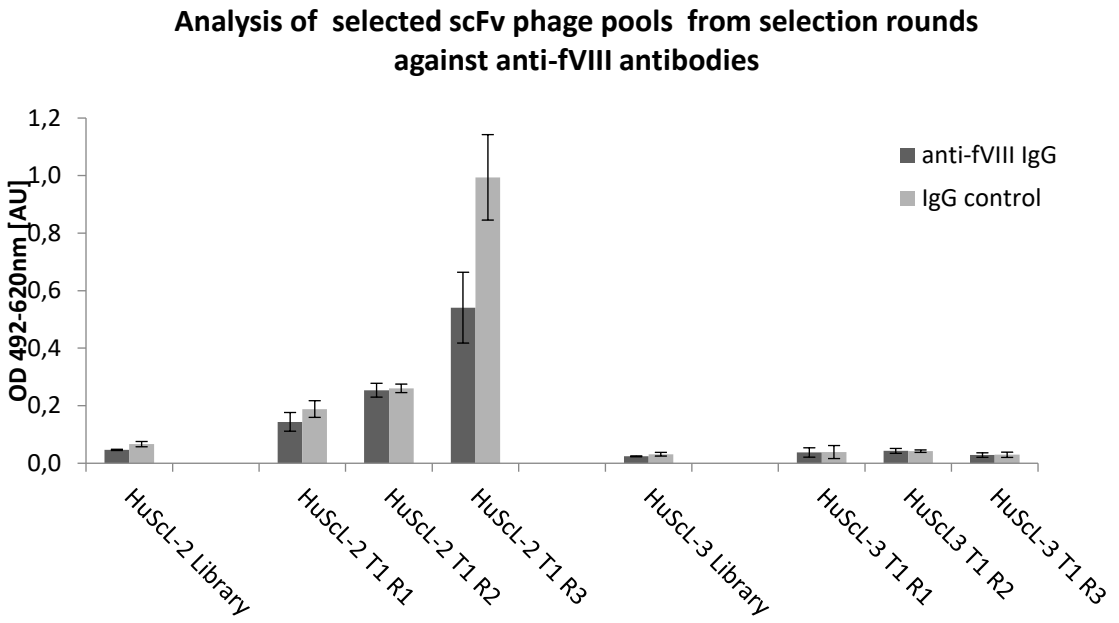

Figure 9 Analysis of pooled selected scFv-carrying phage clones against purified anti-fVIII antibodies from human plasma. 0.1 µg polyclonal anti-fVIII antibodies and control IgG were immobilized on microtiter plates. $1x10^9$ PFU from phage pools of individual selection rounds (R1, R2, R3) were added to wells and detected with HRP-conjugated anti-M13 IgG. Error bars indicate SD of mean of duplicates. HuScL-2 Library and HuScL-3 Library represent the binding signal of the native phage libraries. T1 defines the first biopanning trial. R1, R2, and R3 represent the selection rounds.

Following the first results we stopped exploring the HuScL-3 library. To improve the selection, we repeated the biopanning starting with the phage precipitate from HuScL-2 of the first selection round due to limited availability of the library. This time an additional fourth selection round was performed. In round three and round four, phage solutions underwent two negative selections to increase the chances of eliminating unspecific phages binding to control IgG. Figure 10 displays the outcomes of the second biopanning trial. Here, 63 cultivated phage clones were tested in ELISA for binding to anti-fVIII antibody target and control IgG. We documented 42 binders, out of which seven solely bound isotype antibodies (16%). No target specific binders were selected. In comparison to results from the first biopanning (Table 1), the number of IgG control binding phages was reduced by 43%. This suggests that the doubled negative selection of third and fourth round in the second biopanning successfully reduced IgG control binding phages.

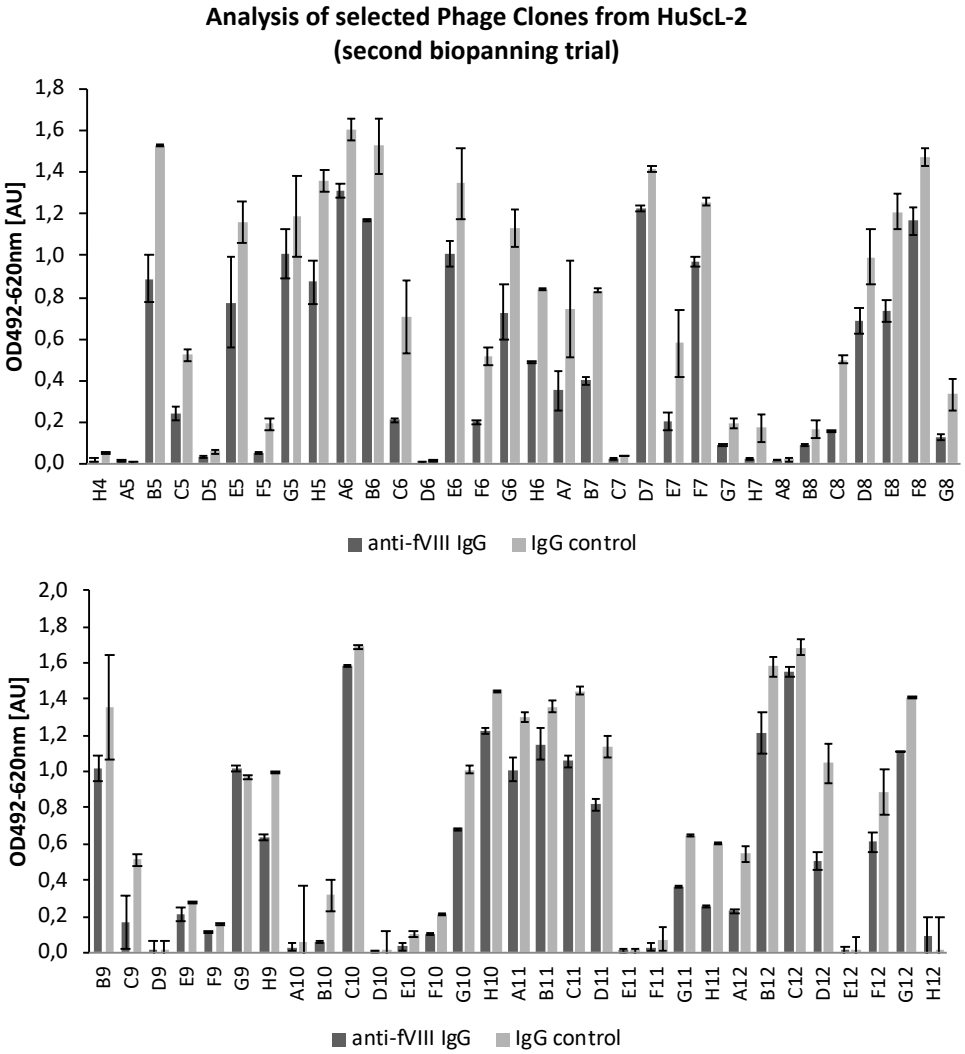

Figure 10 Analysis of selected scFv-carrying phage clones after the fourth selection round against purified anti-fVIII antibodies from human plasma. 0.1 µg purified anti-fVIII antibodies and control IgG were immobilized on microtiter plates. Supernatants of phage-infected bacterial clones were added to wells. Bound phages were detected with anti-M13 HRP-conjugate. Error bars represent the SD of the mean of duplicates.

Figure 11 compares the binding signals of the first and second biopanning with HuScL-2. In comparison, signals of round three and round four from the second biopanning were significantly reduced. This might be attributed to the use of two negative selections.

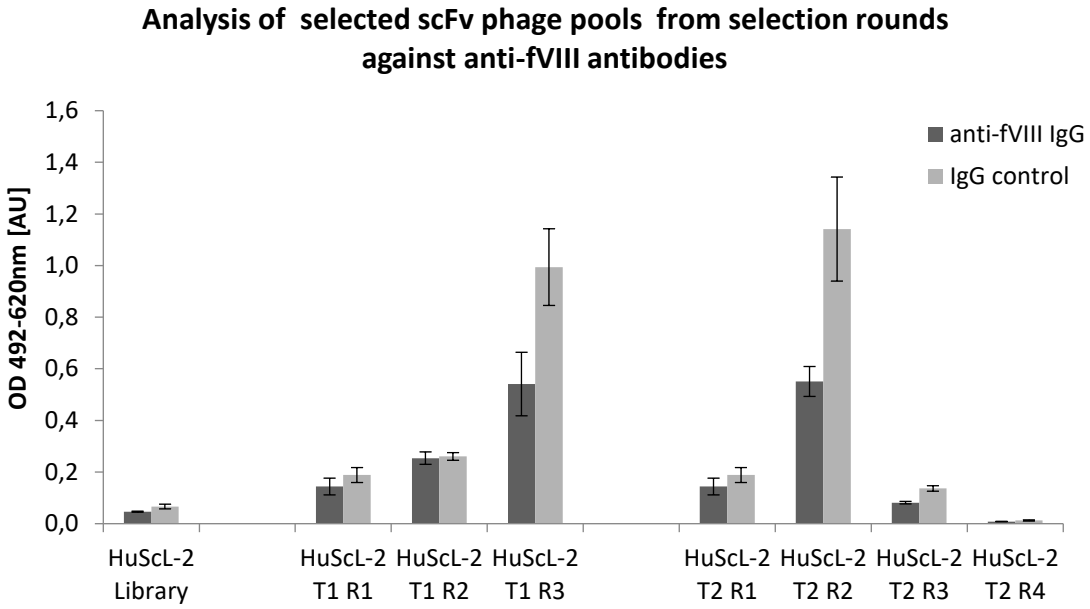

Figure 11 Analysis of pooled selected scFv-carrying phage clones against purified anti-fVIII antibodies from human plasma. 0.1 µg polyclonal anti-fVIII antibodies and control IgG were immobilized on microtiter plates. $1x10^9$ PFU from phage pools of individual selection rounds were added to wells and detected with HRP-conjugated anti-M13 antibody. HuScL-2 Library represents the binding signal of the native phage libraries. Error bars indicate SD of mean of duplicates. T1 and T2 abbreviates for the respective biopanning trial. R1-R4 describes the selection rounds.

In conclusion, biopanning against polyclonal anti-fVIII antibodies did not yield specific scFv-carrying phages from either HuScL-2 nor HuScL-3. Despite the addition of a double negative selection to eliminate isotype-binding phages, the selection of specific phages remained unfruitful.

3.2 Selection and Characterization of scFvs Specific for Anti-C1 mAbs

The second project focused on the selection of ant-idiotypic scFvs against anti-C1 domain mAbs, as this had not been done before and current findings emphasize the high importance of this domain within the immune response of HA patients to fVIII. The idea is to create an anti-idiotypic pool of synthetic mAbs that can target a number of anti-fVIII antibodies. The human mAb LE2E9 and murine mAb GMA8011 were placed as targets in phage display selections. Previous analytical findings suggest molecular and functional overlaps between the two[44]. It is assumed that LE2E9 and GMA8011 effect fVIII activity by preventing fVIII binding to vWF, increasing its clearance from the blood. Furthermore, epitope mapping of these antibodies suggests great similarities of their epitope on the C1 domain, differing solely in one crucial amino acid residue[44]. Taking this into

consideration, the paratope of both mAbs might also resemble one another. We performed two biopannings, using GMA8011 and LE2E9 as target molecules, to screen scFv phage display libraries HuScL-2 and HuScL-3, and address the theory that selected scFvs specific for one anti-C1 domain antibody could possibly cross-react with the other.

3.2.1 Affinity Selection of ScFvs Specific for Anti-C1 mAbs LE2E9 and GMA8011

To select scFvs from phage-displayed human scFv-phage libraries, we also used the phage display method, targeting human LE2E9 and murine GMA8011. The same biopanning protocol as was used for targeting anti-fVIII antibodies from human plasma was applied, using phage display libraries HuScL-2 and HuScL-3. Biopannings were performed in three sequential selection rounds, first exposing phage solutions to target mAb and then to unspecific isotype mAb for negative selection. For GMA8011, we used mIg2a as isotype control, and hIgG4 was used for human LE2E9. After three rounds of selection, we analyzed 95 scFv-carrying phage clones obtained from each biopanning. Phage clones binding isotype antibodies were labeled as unspecific binders. Phages with signals above the defined cut-off at 0.2 AU for the target mAb were identified as potential specific binders. In total, 14 phage clones bound mAb GMA8011, while 81 were unspecific isotype-binders (appendix 7.5.1). For LE2E9, six selected phages were target-specific, while 89 clones bound the isotype control (appendix 7.5.1). Subsequently, an ELISA was performed to verify the binding behavior of isolated specific binders (Figure 12). Here, precipitates of each promising phage clone were prepared and added to wells, coated with target and isotype antibody. Five out of six phages selected against LE2E9 were verified as specific binders. All 14 GMA8011-specific phage clones also proved to be target-specific.

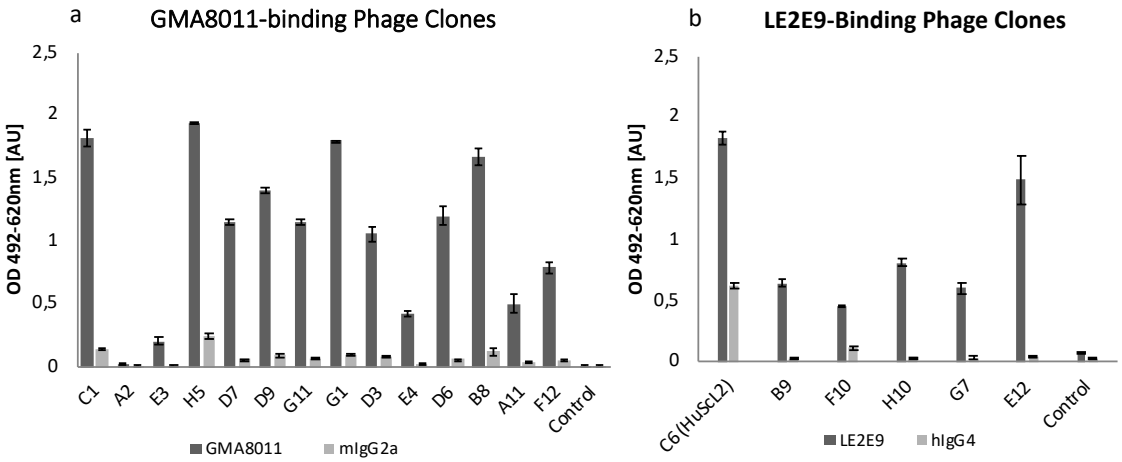

Figure 12 Binding analysis of selected phage clones against GMA8011 and LE2E9. ScFv carrying phages, identified as target-specific, were amplified and precipitated before being tested for binding to the target antibodies. 0.1 µg of (a) GMA8011 and (b) LE2E9 and isotype control mIgG2a (a) and hIgG4 (b) were immobilized on microtiter plates. 1x109 PFU of each phage clone were added to wells with respective target antibody and isotype control. Binding phages were detected using HRP-conjugated anti-M13 IgG. Green bars show binding of clones to target antibody, whereas blue bars show binding to isotype control. Error bars indicate SD from mean of triplicates.

3.2.2 Characterization of Isolated scFvs Specific for GMA8011 and LE2E9

Next, scFv-carrying phages were tested for their ability to compete with fVIII for binding to their target mAb. If a scFv was specific for the inhibitor's paratope, scFv signals would be reduced in the presence of fVIII, as fVIII would block the binding site. For this, half of the mAb-coated wells were pre-incubated with 0.5 IU fVIII (w/ fVIII), so fVIII could bind to the inhibitor. The other half of wells was not pre-incubated with fVIII (w/o fVIII). If a scFv does not bind to the fVIII-binding site of the mAb, fVIII presence would not interfere with binding and detection signals would not be reduced.

In our case, all GMA8011-specific phages revealed reduced signals in presence of fVIII, suggesting competition for the same binding site. (Figure 13 a). LE2E9-specific phages did not show reduced signals, suggesting a remote binding site, distant from the variable region of LE2E9 (Figure 13 b). Nevertheless, for analytical purposes, we decided to include LE2E9-specific phages in further experiments.

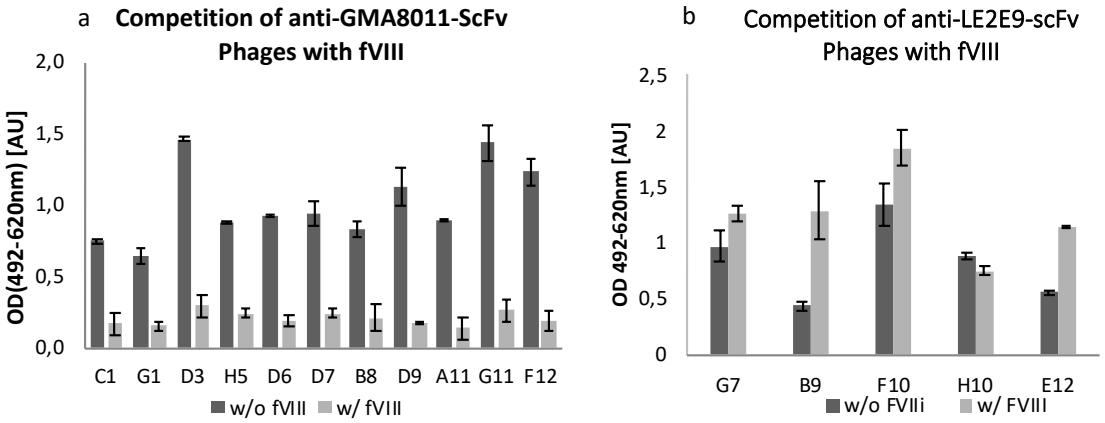

Figure 13 Binding of selected scFv-carrying phage clones to mAbs GMA8011 and LE2E9 in the presence of fVIII. 0.1 µg of GMA8011 (a) or LE2E9 (b) were immobilized on microtiter plates. A specific number of phages was added to coated wells, half of which were pre-incubated with 0.5 IU rfVIII. Errors bars indicate SD from mean of duplicates.

Vectors, carrying mAb-specific scFvs, were prepared from bacterial single clones for sequencing (see appendix for DNA sequence). Sequence analysis for GMA8011-binding clones revealed that all vectors carried identical scFv sequences. For LE2E9-binding clones, H3F10 was identical to H3E12 and H3G7 was identical to H3H10. H3B9 presented overlapping sequences, indicating that different phages were in the mix. However, separating these phages by infecting *E. coli* and testing individual clones did not prove any of the clones to be specific for LE2E9 (data not shown). Overall, through biopanning, we managed to select one GMA8011-specific clone (H2C1) and two LE2E9 -specific clones (H3G7, H3F10). In following experiments, these mAb-specific scFv-carrying phage clones were fused with Fc chain fragments and functionally analyzed.

3.3 Production of ScFv-Fc Fusion Proteins and Functional Analysis

Up to this point, scFvs had been tested still being attached to phages. The M13 phage presents the structural link between scFv and its DNA, making selection and production more efficient. This is problematic as the phage is large and carries several proteins on its surface, that could distort scFv characterization. Moreover, scFvs can only be used therapeutically in an antibody format. Therefore, we genetically fused the selected scFvs to Fc chains of human and murine origin to create different recombinant immunoglobulins. Their individual properties could then be examined.

3.3.1 Production and Purification of scFv-Fc Fusion Proteins

ScFv sequences of H2C1, H3G7 and H3F10 were genetically fused to both human IgG1- and murine IgG2c-Fc-chains, using a pCMV-mG2c-Fc and pCMV-hG1-Fc vector. In the following sections, scFvs fused to human IgG1-Fc are referred to as scFv-hFc, and scFvs fused to murine IgG2c-Fc are referred to as scFv-mFc.

ScFv-Fc proteins were expressed in transiently transfected HEK293T cells and purified from cell culture supernatants, using Protein A chromatography. Elution of each scFv-Fc from the Protein A column was achieved by pH shift. Purification was performed using an Äkta prime. An exemplary UV detection curve of the system, showing the purification of H2C1-hFc, is seen in Figure 14.

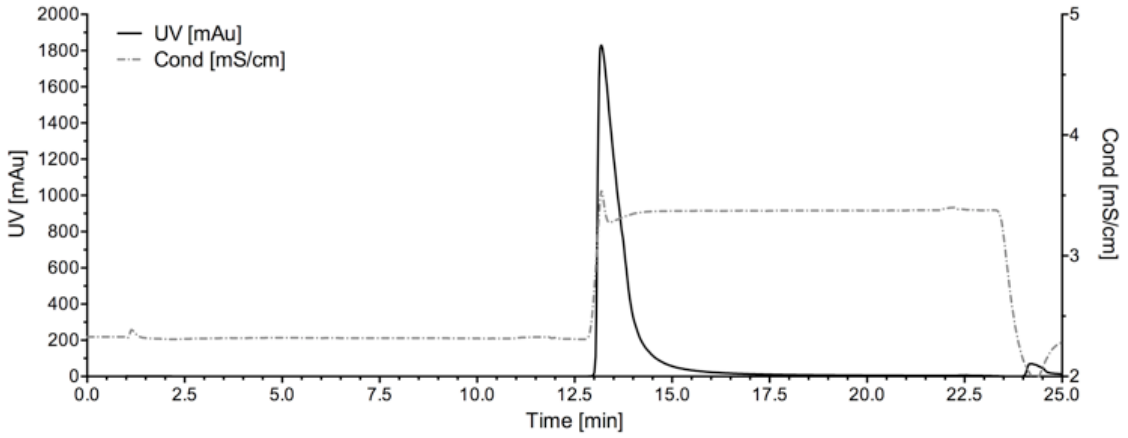

Figure 14 Analysis of H2C1-hFc purification using Äkta prime. H2C1-hFc was eluted via protein A chromatography by pH shift, implied by the change in conductance (Cond.). Protein elution was observed by measuring UV absorption. Fractions containing eluted protein were pooled and concentrations of eluted proteins were measured via spectrophotometry.

3.3.2 Functional Analysis of scFv-Fc Fusion Proteins

We analyzed the binding of H2C1-hFc to GMA8011 and H3G7-mFc and H3F10-mFc to LE2E9. For this, H2C1-hFc was used for testing with murine GMA8011 to avoid measuring errors by the detecting antibody. Likewise, H3G7-mFc and H3F10-mFc were used for testing with human LE2E9. ScFv-Fcs were prepared in a series of dilutions and incubated on ELISA plates coated with target or isotype mAb.

Results of binding analysis of H2C1-hFc are demonstrated in Figure 15, where a concentration dependent binding to both GMA8011 and mIgG2a isotype control can be observed. The binding curve of H2C1-hFc to the isotype control presents overall lower

absorption signals and a shift to the right, pointing towards a weaker binding affinity. This concludes, that H2C1-hFc does not uniquely bind its target GMA8011. These results contradict those of binding analysis of H2C1-carrying phages, which exclusively bound GMA8011 (Figure 12 a).

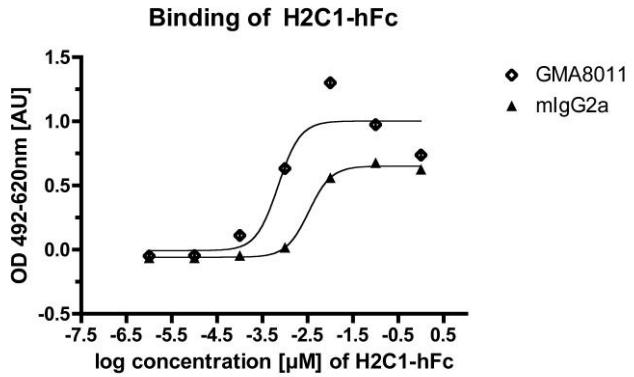

Figure 15 Binding of H2C1-hIgG1 to GMA8011. 0.1 µg of GMA8011 or mIgG2a isotype were immobilized on microtiter plates. A series of 10-fold dilutions of H2C1-hFc, starting at 1 µM, was incubated and detected using HRP-conjugated goat anti-human IgG.

H3G7-mFc bound LE2E9 in a concentration dependent manner (Figure 16 a). The binding curve for isotype control hIgG4 presented a slight rising slope with weak absorption signals. At antibody concentrations tested, we did not observe a concentration dependent binding curve here. H3F10-mFc also bound LE2E9 in a concentration dependent manner. Signals for isotype control presented a slight rising slope at growing concentrations of H3F10-mFc (Figure 16 b). In both cases, it can be argued that the weak binding to isotype control could be due to background signals from high antibody concentrations in the well. Thus, binding analysis of scFv-Fc originally selected for LE2E9 mirrored those results obtained from testing scFv-carrying phages (Figure 12b).

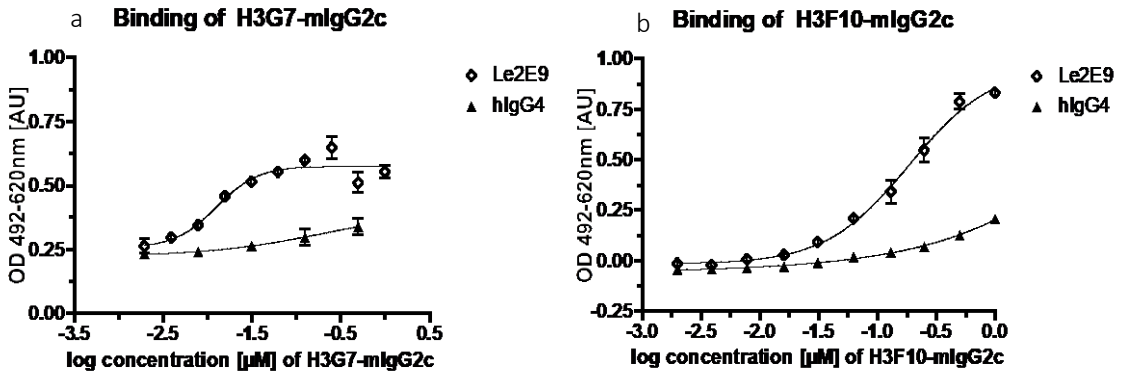

Figure 16 Binding of scFv-Fc fusion proteins to LE2E9. 0.1 µg of LE2E9 or hIgG4 isotype were immobilized on microtiter plates. A series of 2-fold dilutions, starting at 1µM, of H3G7-mFc (a) and H3F10-mFc (b) was incubated and detected using HRP-conjugated rabbit anti-mouse IgG. Error bars indicate SD from mean of duplicates.

As shown in section 3.2.2, H2C1-carrying phages competed with fVIII for binding to GMA8011. To verify these results in the context of scFv-Fc proteins, we tested whether H2C1-hFc would also compete with fVIII for binding to GMA8011. For this, fVIII was immobilized in wells of microtiter plates and a series of dilutions of H2C1-hFc was pre-incubated with constant concentrations of GMA8011 to allow complex formation. If a scFv-Fc binds the complimentary paratope of the mAb, at rising scFv-Fc concentrations, the binding of mAb to fVIII would diminish. Figure 17 displays the calculated percentage of inhibition of GMA8011 binding to fVIII at rising concentrations of H2C1-hFc. It can be observed that at 400-fold molar excess of H2C1-hFc compared to GMA8011, the scFv-Fc affects inhibitor binding to fVIII.

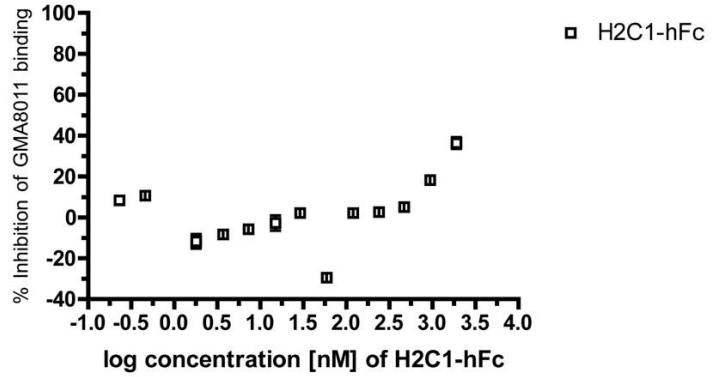

Figure 17 Impact of H2C1-hIgG1 on binding of GMA8011 to fVIII. 0.5 IU fVIII were immobilized on microtiter plates. GMA8011 was mixed with increasing amounts of H2C1-hFc and pre-incubated before being added to fVIII. GMA8011 was detected using HRP-conjugated rat anti-mouse IgG. The y-axis displays the calculated percentile inhibition of GMA8011 binding to fVIII. 0% inhibition was set for signal intensity of inhibitor binding to fVIII in the absence of H2C1-hFc. Error bars indicate SD from mean of duplicates

ScFv-Fc proteins selected for LE2E9 were also tested for their ability to impact mAb binding to fVIII, even though experiments with scFv-carrying phages did not predict scFvs to bind to LE2E9's paratope. JKH5-mFc, a scFv-Fc protein not binding to LE2E9, was used as negative control. Neither H3F10-mFc nor H3G7-mFc reduced binding of LE2E9 to fVIII. The binding curve ran in accordance to the control JKH5-mFc (Figure 18).

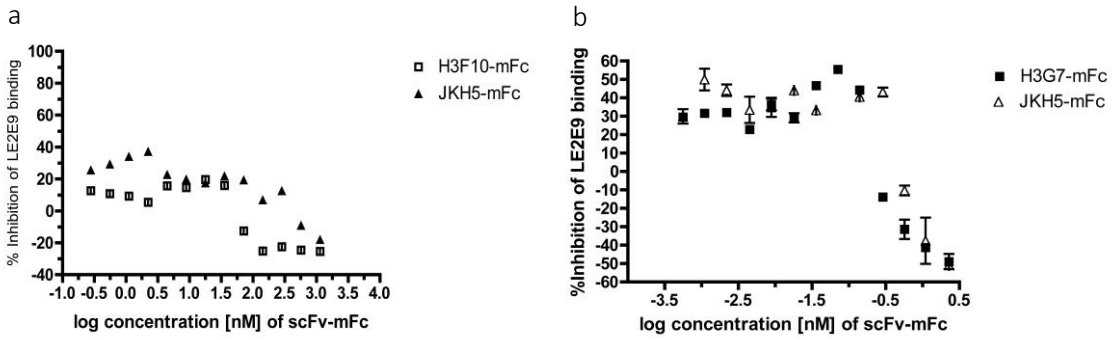

Figure 18 Impact of scFv-mFc fusion proteins on binding of LE2E9 to fVIII. 0.5 IU fVIII were immobilized on microtiter plates. LE2E9 was mixed with increasing amounts of **a) H3F10-mFc** or **b) H3G7-mFc** and pre-incubated before being added to fVIII-coated wells. LE2E9 was detected using HRP-conjugated goat anti-human IgG. The y-axis displays the calculated percentile inhibition of LE2E9 binding to fVIII. 0% inhibition was set for signal intensity of inhibitor binding to fVIII in the absence of H2C1-hFc. Error bars indicate SD from mean of duplicates.

3.4 Testing for Cross-Reactivity of scFv-Fc Fusion Proteins

Experiments with LE2E9 and GMA8011 and fVIII showed that both mAbs compete with one another in binding to fVIII. Epitope mapping revealed that they share closely overlapping epitopes on the C1 domain, only differing in one crucial amino acid residue[44]. We established the theory that the paratopes of GMA8011 and LE2E9 also share close similarities, as they recognize almost identical epitopes. Therefore, it would be conceivable that selected scFvs, specific for one of the anti-C1 domain mAbs, will also recognize the other mAb's paratope, meaning H3F10 and H3G7 will bind to GMA8011, H2C1 will bind to LE2E9.

Cross-reactivity was examined for H2C1, H3F10 and H3G7 in scFv-Fc format. On microtiter plates, we immobilized anti-C1 mAbs GMA8011 or LE2E9 and proceeded the same way as before, when testing for specific binding of scFv-Fc protein to their target antibody. To ensure the specific detection of the analyte, we used H2C1 fused to a murine IgG2c-Fc-chain for testing with LE2E9 and H3G7 and H3F10 fused to human IgG1-Fc-chains for testing with GMA8011. H2C1-mFc both bound to LE2E9 and the isotype control

hIgG4. Both bound equally in a concentration dependent manner, exemplifying no specific binding to the variable region of the target antibody (Figure 19 a). H3F10-hFc bound both GMA8011 and isotype mIgG2a and signals overall were weak, implying a weak binding affinity (Figure 19 c). A sigmoidal binding curve could not be seen at these dilutions but may be disclosed using higher concentrations of H3F10-hFc. Nonetheless, H3F10-hFc disclosed to be unspecific for GMA8011. H3G7-hFc bound GMA8011 in a concentration dependent manner. Overall signal intensity of H3G7-hFc was weak (Figure 19 b). At rising concentrations of H3G7-hFc, we detected a weak binding signal for the isotype control antibody. From this we can presume, that H3G7-hFc does not bind the paratope of GMA8011.

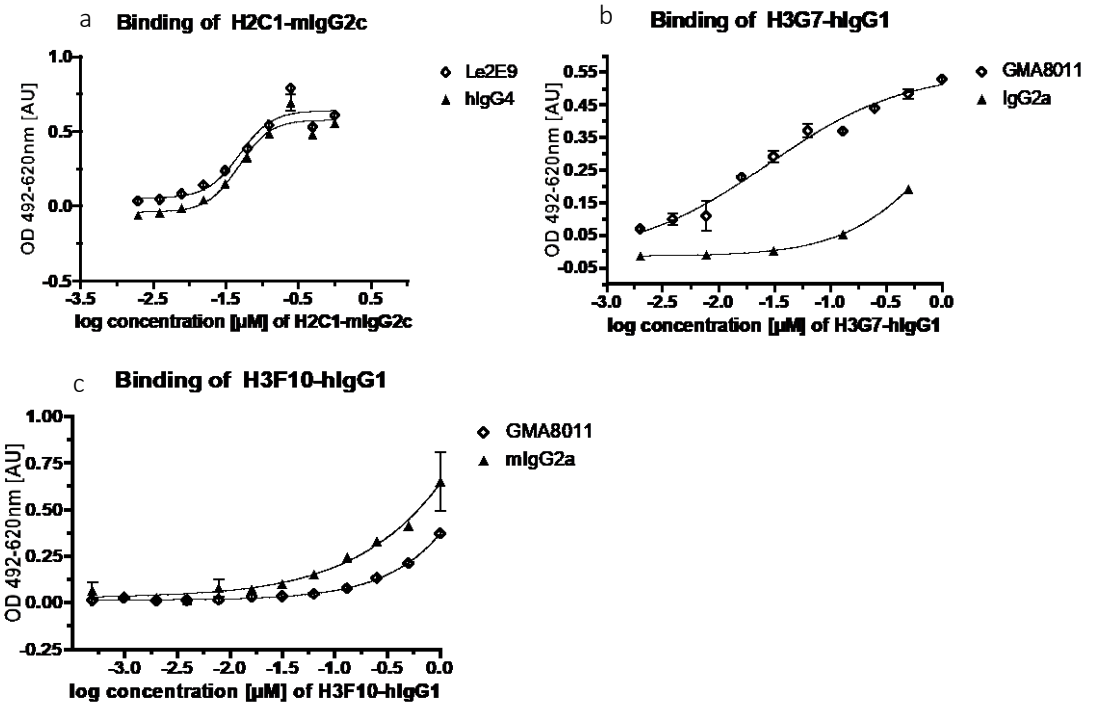

Figure 19 Cross-reactivity of selected scFv-Fc fusion proteins with anti-C1 mAbs. 0.1 µg LE2E9 or GMA8011 were immobilized on microtiter plates. Each scFv-Fc was added in increasing concentrations. a) Bound H2C1-mFc was detected using HRP-conjugated rat anti-mouse IgG. b) H3G7-hFc and c) H3F10-hFc were detected using HRP-conjugated goat anti-human IgG. Error bars indicate SD from mean of duplicates.

3.4.1 Possible Contamination of Protein A Column: Analysis of Cell Culture Supernatants

For the purification of scFv-Fc proteins we decided to recycle the Protein A column. After having purified one scFv-Fc, the column was cleansed and prepared for re-utilization, following protocol (for detail see section 2.3.1). Analysis of binding characteristics of purified scFv-Fc were contradictory to the results of scFv-carrying phage clones. During analysis, none of the purified scFv-Fc presented specific binding, but in fact all demonstrated unspecific binding towards the isotype control (Figure 15, Figure 16). For example, H2C1-mFc was purified second in row after H2C1-hFc and binding analysis of H2C1-mFc presented an identically looking binding curve to both LE2E9 and the isotype control. Here, contamination with human Fc chain from H2C1-hFc could have caused incorrect detection by the anti-mouse detection antibody and led to distorted signals. Furthermore, H3G7-mFc and H3F10-mFc did not mirror LE2E9-specific binding of respective scFv-carrying phages. If for example, H2C1-mFc molecules were not properly cleansed from the column, they could have been purified alongside H3G7-mFc or H3F10-mFc and caused the binding signal for hIgG4. Thus, H3G7-mFc and H3F10-mFc may in fact not bind the isotype control. Only the results of H2C1-hFc could not have been altered, as this was the first scFv-Fc that was purified through the Protein A column.

We took into consideration, that the purified samples may have been contaminated with previously purified scFv-Fc. To exclude that the described results from binding analysis of purified scFv-Fc (section 3.3) were distorted, due to possible contamination, we conducted an additional series of experiments. Here, we compared cell culture supernatants of transfected HEK293T cells, containing each scFv-Fc prior to purification, to compare it to the corresponding purified scFv-Fc samples (Figure 20).

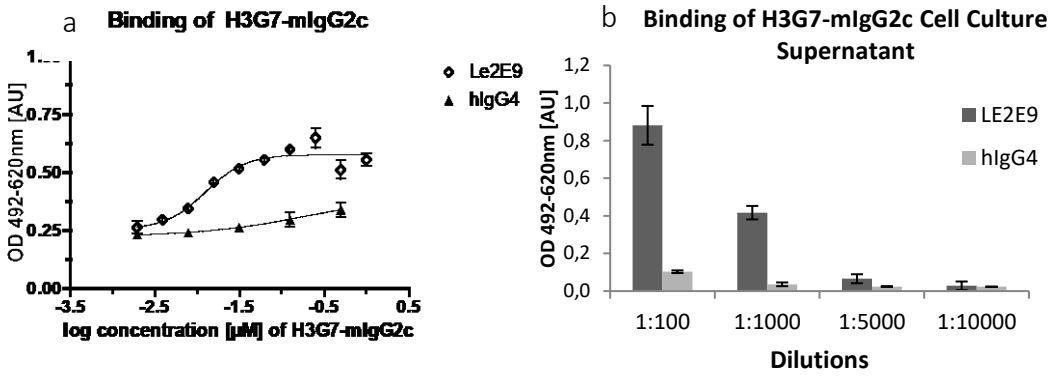

Figure 20 Comparison of binding of H3G7-mIgG2c-containing cell culture supernatant and purified H3G7-mIgG2c. 0.1 µg target antibody LE2E9 and isotype control hIgG4 were immobilized on microtiter plates. a) Binding of H3G7-mFc to LE2E9 and hIgG4. b) Binding of H3G7-mFc containing cell culture supernatant from HEK293T cells to LE2E9 and hIgG4 isotype control. Different dilutions of cell culture supernatants were added to wells with immobilized LE2E9 or hIgG4. Binding was visualized using HRP-conjugated rat anti-mouse IgG. Error bars indicate SD from mean of duplicates.

Binding analysis of cell culture supernatants of H3G7-hFc, H3F10-mFc, H3F10-hFc and H2C1-mFc were comparable to those of the corresponding purified scFv-Fc (appendix 7.5.2). As the individual binding behaviors of each were similar before and after purification, we could conclude that the binding behavior of each purified scFv-Fc was not falsified due to contamination.

4 Discussion

The development of fVIII inhibitory antibodies poses a feared complication in the treatment of hemophilia A. While mild cases of the disease only require occasional fVIII substitutions when facing surgery or injury, patients with less than 1% fVIII activity require daily infusions. This exposure to fVIII creates a 30% risk of anti-fVIII inhibitor development.

The gold standard for inhibitor treatment consists of immune tolerance induction by administering high doses of fVIII over months. Despite the observed efficacy of ITI treatment, 30% treatment failure leaves patients at high risk for bleeding and few alternatives for treatment. Therefore, it is of great interest to develop treatments against fVIII inhibitors.

4.1 Selection and Analysis of Anti-idiotypic ScFvs Specific for Purified Polyclonal Anti-fVIII Antibodies from Human Plasma

One aim in research is to find possibilities to neutralize inhibitory antibodies. In the past, our research group has invested in the selection of anti-idiotypic peptides and scFvs from synthetic phage libraries using phage display. Anti-idiotypic scFvs were successfully selected against monoclonal C2 and A2 domain-specific inhibitors[68,74]. Their neutralizing power was shown *in vitro* and *in vivo.* However, the selection of scFvs using monoclonal inhibitors is time consuming and selected scFvs were not able to neutralize inhibitor-positive patient plasma yet. Therefore, in this thesis we pursued the idea to generate a pool of scFvs directed against polyclonal anti-fVIII antibodies, purified from plasma of a human HA patient, to accelerate the selection process. A successful development of customized anti-idiotypic antibodies would open the possibility to induce immune tolerance towards fVIII in inhibitor positive HA patients individually. Such antibodies could ultimately be added as a supportive or prophylactic therapy.

In an earlier attempt, our lab used human and murine inhibitor-positive full plasma and screened it against phage-displayed libraries Tomlinson I and J. In short, magnetic beads were coupled with anti-human IgG or anti-murine IgG and hemophilic human or murine plasma were added as the target. The prepared beads were then used in a biopanning to screen libraries Tomlinson I and J. Normal human or murine plasma was used as the

negative control. The selections were unsuccessful, likely because even in inhibitor-positive patients the portion of anti-fVIII antibodies is still relatively small. Other labs have previously purified anti-fVIII antibodies using Protein G chromatography and fVIII-specific matrices[54]. Therefore, our lab developed a method to purify anti-fVIII antibodies from patient plasma using fVIII-coupled Affi-Gel® matrix, to obtain highly purified antibody concentrates. The rationale for the use of purified anti-fVIII antibodies was to decrease the diversity of the target and only present anti-fVIII antibodies during biopanning. The plasma samples chosen for this project were obtained from an anonymized patient with high Bethesda units ranging from 5000-20000 BU/ml. The reason we chose this patient, was to obtain a large quantity of inhibitors for biopanning and analysis. Thereby, we also hoped to include a representative pool of anti-fVIII antibodies, that may also occur in patients with inhibitor amounts, too low to be purified.

By explicitly isolating anti-fVIII antibodies, using a fVIII-coupled Affi-Gel® matrix column, we obtained an output of 450 µg purified anti-fVIII IgG solution with Bethesda units of 16000 BU/ml. The purification process was successful and supplied us with plenty target antibodies for phage display. Nevertheless, we cannot be certain, that we did not lose some antibody clones during purification, as analysis of purification showed that anti-fVIII antibodies were present in wash and flow through. On that note, it has to be mentioned, that through analysis of the purified anti-fVIII antibodies, we cannot know how many non-inhibitory anti-fVIII antibodies were included in the polyclonal target. Non-inhibitory anti-fVIII antibodies, binding nonfunctional epitopes, have been found in healthy humans and HA patients[65]. While their purpose is still part of research, findings suggests a link between the presence of non-inhibitory antibodies and the development of inhibitors[77]. Anti-idiotypes against non-inhibitory antibodies could therefore possibly have an immunomodulating effect, however their neutralization would not result in restoration of hemostasis.

Despite the abundance of anti-fVIII antibodies as target, screening phage libraries HuScL-2 and HuScL-3 did not single out specific binders. One explanation for this could be, that the complexity of a polyclonal antibody pool is too abundant to obtain specific binders in a scFv screening. The human immune system produces an antibody diversity of over 10^{12}, with a matching antibody for every possible antigen[11,78]. Even though, we

reduced the variability of the target down to only anti-fVIII antibodies, compared to previous experiments using full plasma, still a high number of antibody paratopes was targeted during biopanning. Simultaneously, individual antibody clones may not have been presented consistently during every selection round. This may have let to binding phages being lost during selection rounds. In addition, a weak binding affinity of matching phages could have resulted in its loss during selection. Also, the diversities of the phage libraries HuScL-2 and HuScL-3 are limited to $1.6x10^9$ and $1.42x10^9$. Possibly, the phage libraries did not hold matching phages. Under these circumstances, we have to reflect the possibility, that the amplification of binders for rare target antibodies is not possible in this setting.

Inhibitor analysis of the studied HA patient plasma revealed that anti-fVIII antibodies belonged in about 75% to subclass IgG4 and were dominantly directed against HC and C2 of fVIII molecules. This suggests that several domains are immunogenic in this patient. Also, recent publications reveal that fVIII-specific IgG4 has a higher affinity than IgG1, which may falsify the analysis of subclass distribution of the tested antibody probes[79]. If so, the IgG subclass complexity of our sample would be larger than estimated. With a smaller subset of anti-fVIII antibodies, directed predominantly against one domain, it could be anticipated that antibody variety would decrease. For example, patients with acquired HA are more likely to present anti-fVIII antibodies, directed against single domains[80]. From a clinical view point, the approach of selecting individual neutralizing scFvs would, if any, only be applicable to a small cohort of patients with a very restricted number of inhibitors.

For this project, biopanning was performed twice. The first setting consisted of three rounds of selection, applying one positive and on negative selection using the isotype control, producing no target-specific phages, but several clones binding both antibodies or simply the isotype control. The second time, settings were altered by performing four rounds with doubled negative selection in the third and fourth round, in hope that more non-specific scFvs would be eliminated. Data revealed that in comparison to the first run-through, there were 43% less scFv-carrying phages, that solely bound the isotype control. Additionally, excess phage precipitates of all rounds were individually tested for binding. It showed that phage signals for binding the isotype control in rounds, where a double negative selection took place, were decreased significantly, compared to rounds with

only one negative selection (Figure 11). This demonstrates that negative selections cleared the solution from unspecific binders and disclosed that no specific binders were included.

In both settings, examination of selected phage clones after biopanning showed that none of the selected phages bound target anti-fVIII antibodies exclusively. Instead, phage clones bound positive target and isotype control simultaneously or solely the isotype control. Kahle *et al.* argued in their paper about epitope mapping, using peptide phage display, that due to a possible growth advantage of unspecific phage clones, a selection of phages, that are not specific to dominant antibody populations, could occur[53]. This theory may also be transferred to our situation.

In contrast, the selection of peptide sequences from random peptide libraries, using the phage display method, was successful[47]. Biopannings were performed, using purified anti-fVIII antibodies[54] and full human plasma[53], to select peptide sequences that mimic fVIII epitopes of polyclonal fVIII-specific antibodies. Mühle *et al.* isolated several specific peptides, with consensus primary sequences of putative fVIII domains for two out of three tested patient plasmas. For one plasma sample, the peptides mapped partially overlapping epitopes of the C2 domain. Projected onto a three-dimensional fVIII model, the peptides resembled epitopes presented on the surface of the molecule[54]. Despite the successful selection of some specific peptides, it is noteworthy that Mühle *et al.* were unable to select peptide sequences for one of the three tested plasma samples. They suspected that the more diverse set up of anti-fVIII antibodies in this patient, that were directed against all tested domains, may have been the reason[54]. Furthermore, Kahle *et al.* also noted that the selection of peptides against plasma is indeed successful, but simultaneously challenging, as only few peptides were selected despite the presence of a polyclonal inhibitor pool[53]. They pointed out that due to a possible growth advantage of certain phage clones, a selection of phages, that are not specific to dominant antibody populations, could occur[53]. This aspect could also have impacted our selection process and possibly resulted in losing specific binders over non-specific ones.

Ultimately, the diversity of the polyclonal target and the limitations of phage-display did not lead to the selection of anti-inhibitory scFvs.

4.2 Selection and Analysis of scFv specific against anti-C1 mAbs GMA8011 and LE2E9

Despite trying several approaches of selecting neutralizing scFvs against purified anti-fVIII antibodies from human plasma, we remained unsuccessful. Therefore, we decided to take a step back and invest in the selection of scFv proteins, specific against monoclonal anti-fVIII mAbs, with the final aim to create individual combinations of therapeutic synthetic anti-idiotypes. Prior to this, anti-idiotypic scFvs were successfully selected against monoclonal antibodies, specific for fVIII domains C2 and A2[68,74]. As it has been shown that anti-C1 domain antibodies contribute to the immune response in AHA and HA patients[44,45], the neutralization of anti-C1 antibodies is important to restore fVIII activity in affected patients. Hence, the addition of anti-idiotypes, directed at anti-C1 inhibitors, to a pool of synthetically selected anti-idiotypes would be beneficial. We therefore picked mAbs LE2E9 and GMA8011, which bind the C1 domain of fVIII and mainly inhibit fVIII binding to vWF and phospholipids, and screened them against human naïve phage displayed libraries HuScl-2 and HuScL-3.

MAbs LE2E9 and GMA8011 are thought to share closely overlapping epitopes on fVIII, as they compete with one another in binding fVIII. Epitope mapping showed that binding residues of both mAbs only differ in one crucial residue[44]. In the past, synthetic scFvs selected from phage-displayed libraries were only specific to their respective mAb and did not display cross-reactivity with other inhibitory mAbs or inhibitor-positive plasma[68,74]. Hope was, that selecting against two mAbs with closely identical epitopes, namely LE2E9 and GMA8011, the chance of obtaining cross-reactive scFvs, that neutralize both mAbs, would be more likely. Besides, Kahle *et al.* observed that binding of anti-C1 antibodies from tested AHA plasmas to the C1 domain was strongly blocked in the presence of GMA8011. This suggests that the crucial residues in the epitope of GMA8011 are also recognized by anti-C1 antibodies in existing patients[44]. We therefore theorized, that anti-idiotypic scFvs for GMA8011 and LE2E9 could potentially neutralize such antibody populations in patients, if in fact they recognize the same epitope.

Screenings of phage displayed libraries were performed in three rounds against each anti-C1 domain mAb and a respective isotype antibody. Via phage display, one GMA8011-specific scFv-carrying phage clone (H2C1) was isolated. Analysis showed that

this clone bound its target mAb, but not the isotype control. H2C1 was tested for its anti-idiotypic potential to compete with fVIII for binding GMA8011 in ELISA setting. It showed reduced signals of binding GMA8011 in the presence of fVIII, which implied a binding specificity to the inhibitor's paratope (Figure 13).

H2C1 was then transformed into a scFv-Fc fusion protein and binding characteristics were reexamined. H2C1-hFc produced a concentration dependent binding curve for both GMA8011 and the isotype control mIgG2a. However, the binding curve for the isotype control presented a rightward shift with overall lower signals, indicating that H2C1-hFc binds the isotype antibody with weaker affinity. These results imply that H2C1-hFc does not exclusively bind the paratope of GMA8011. We can speculate that the binding of H2C1-hFc to both target and isotype antibody is a result of it binding to a transitory region between the framework and the complimentary determining regions (CDR) of GMA8011. Three CDRs of the heavy chain and of the light chain, which are located in the variable region of an antibody, form the specific binding regions for antibody-antigen interaction. This is also known as the paratope. The framework regions provide the structural basis of the variable domain of the antibody and are less unique than the CDRs[81]. Binding to residues in transitory regions of CDRs and framework may provide two-sided results in binding assays, on one side implying specific binding to the CDRs and on the other side binding to unspecific areas of the framework, resulting in binding the isotype antibody. This could also explain the different binding affinity to GMA8011 and isotype control mIgG2a.

Competition assays with H2C1-hFc and fVIII revealed, that at 400-fold molar excess of H2C1-hFc to the target antibody, binding of GMA8011 to fVIII was reduced by up to 40% (Figure 17). Thus, despite binding both target mAb and isotype control, H2C1-hFc is capable of preventing GMA8011 from binding to fVIII under certain conditions. However, this is not necessarily attributable to a specific competition. If H2C1-hFc, in fact, only binds in close proximity to the CDRs but actually in a transitory region, at high antibody concentrations, molecular size and steric interferences may result in a non-specific blocking of GMA8011 binding fVIII.

Several explanations for the inconsistent binding results of tested phage clones and scFv-Fc fusion proteins need to be considered. For one, steric forces, that build up

between large molecules can be the reason. Hydrophobic bonding or van der Waals forces between non-polar atoms in amino acid residues enable unspecific interaction between two molecules, while not forming a specific linkage between paratope and epitope. Van der Waals forces increase in multitude - the greater the contacting surface, the stronger the forces get. Padlan *et al*. described this reaction as hydrophobic stacking and attributed it to the amino acids tryptophan and tyrosine, which have aromatic rings, and are capable of forming hydrogen bonds, hydrophobic attraction and electrostatic interactions [reviewed in 82]. Also, arginine may exhibit a pseudo-aromatic behavior[83]. Such mechanisms have been described to be responsible for the promiscuity of antibodies, meaning their ability to bind different antigens. Examining the amino acid sequence of H2C1, only 11 tyrosine and 18 tryptophan residues in a total of 245 amino acids could be identified. When looking at the CDR regions of the DNA sequence of H2C1, we did not observe a predominance of aromatic amino acids (appendix 7.9). Thus, it cannot with certainty be claimed that hydrophobic stacking occurred.

Another explanation for the apparent GMA8001-specificity, when testing H2C1-carrying phages, could be that surface proteins of phages manipulated ELISA results. The M13 phage outer structure is formed by about 2700 copies of the major coat protein pVIII, proteins pVI, pVII, pIX and five copies of pIII, responsible for expressing scFvs and the interaction with the f-pilus of the bacterium[71,73]. The scFv is expressed in 0-5 copies attached to the pIII proteins on the phage's surface. This may have caused misleading results in testing, as while the interaction between a scFv molecule and GMA8011 may have been unspecific, several scFv copies on the phage could have interfered with binding of GMA8011 to immobilized fVIII, due to steric hindrance, and simulated competition results. The actual binding site of H2C1 may in reality be only in close proximity to the paratope of GMA8011 or include overlapping residues, as discussed above[85]. Moreover, detection of coat protein pVIII by HRP-conjugated anti-M13 IgG in ELISA may have led to exaggerated signals, as pVIII is expressed in up to 2700 copies on the phage.

Of note, it is not uncommon to encounter differences in binding specificities after cloning. In our laboratory, a scFv, specifically binding murine fVIII inhibitor ESH8 and competing with fVIII for binding ESH8, did not bind to ESH8 when fused with the human IgG1-Fc chain[74].

Biopanning against LE2E9 isolated two scFv-carrying phages (H3G7 and H3F10), which presented exclusive binding to the target mAb and not the isotype control. However, binding analysis in the presence of fVIII showed no reduction of signals, when detecting bound phage clones. This suggests that H3G7 and H3F10 do not specifically bind LE2E9's paratope. Transformed into scFv-Fc antibody format, both scFv produced high binding signals at rising concentrations for LE2E9 and weak signals for binding the isotype control. Similar as argued for H2C1-hFc, we can propose that H3F10-mFc and H3G7-mFc may bind in close proximity to the epitope of LE2E9. As mentioned, the antibody-antigen interaction is mediated by the antibodies CDRs, which are composed of 50-70 amino acid residues. However, only one-third of these residues are actually involved in the paratope and an antibody's binding pocket may contain more than one independent paratope[85]. This creates the problem, that several other residues are possible binding partners of selected scFvs, which can explain why they did not bind the isotype control, but also did not compete with fVIII for binding the paratope.

FVIII competition experiments with H3F10-mFc and H3G7-mFc were performed. Results of H3F10-mFc presented a maximum of 18 % inhibition of fVIII binding LE2E9. However, the detection curve ran in accordance to that of control antibody JKH5-mFc. Therefore, we can conclude H3F10-mFc does not specifically bind the paratope of LE2E9. Similar results were obtained for H3G7-mFc. Inhibition of up to 50% was measured at rising concentrations. However, the JKH5-mFc produced identical results, leading to the assumption that the inhibition is a result of unspecific molecular interferences.

In addition, we explored the theory that selected scFv-Fcs would show cross-reactivity for GMA8011 and LE2E9, because both mAbs recognize almost identical epitopes and thus share matching paratopes. Analysis of murine H2C1-mFc revealed binding to both LE2E9 and isotype control hIgG4. Similar results were observed for H3G7-hFc and H3F10-hFc, when tested with GMA8011. Despite both mAbs recognizing closely identical epitopes on fVIII, none of the scFv-Fc fusion protein presented cross-reactivity. However, as we already observed unspecific binding to the original targets and the isotype controls after cloning of all three fusion proteins, it was unlikely to detect cross-reactivity. Fleury *et al.* reported on two antibody Fab fragments HC45 and BH151, which recognize the same residues on X31 HA, the hemagglutinin of influenza virus X31, even though two

thirds of the interactions between paratope and epitope are different in the two complexes[84]. They showed that different atomic bonding between the residues in the two complexes resulted in the same complex formation. This indicates that a lack of compatibility does not hinder epitope recognition and provides flexibility in antibody-antigen recognition. It contradicts our theory that, due to recognizing almost identical epitopes, the paratopes of LE2E9 and GMA8011 would also match, respectively be recognized by a cross-reactive anti-idiotypic antibody. After all, we cannot conclusively say, whether a specific scFv would exhibit cross-reactivity with both GMA8011 and LE2E9.

Overall, our research did not produce specific neutralizing scFvs for LE2E9 or GMA8011. The theory that LE2E9 and GMA8011, who share almost identical epitopes, may also be detected by the same scFvs, could not be answered. A successful selection of a scFv-Fcs, cross-reactive for inhibiting both mAbs, could have implied that their paratopes also resemble one another. However, it needs to be acknowledged that despite recognizing closely similar epitopes on the C1 domain, it is not reason enough for them to show similarities in their individual paratopes, as several factors influence the antibody-antigen interaction[82]. The bonding of two proteins is more complex than the interaction of a few amino acid residues. Complex chemical reactions on the atomic level are the key operators of intermolecular interactions. Van Regenmortel *et al.* reviewed that, if an antibody is recognizing two slightly different epitopes in the same antigen, two different paratopes within the same antibody must be involved, because different atomic reactions are involved[85]. Lescar *et al.* demonstrated that two antibodies, totally different in sequence alignment, recognize the same antigenic site, partly due to different orientation of their CDR loops [reviewed in 85]. This further supports the argument that paratopes must not mirror one another despite spotting the same epitope.

5 Conclusion

This research work dealt with two projects, that focused on the selection of anti-idiotypic scFvs from human naïve phage display libraries, targeting different anti-fVIII antibodies. In a first setup, we targeted human anti-fVIII antibodies purified from human plasma. These antibodies were successfully purified from human plasma using Protein A and subsequent fVIII-coupled Affi-Gel® affinity chromatography, a method established by our laboratory. The leading idea behind this, was to simplify the target, as the use of full inhibitor-positive plasma in previous experiments, did not result in the isolation of specific scFvs. However, we were unable to isolate any anti-fVIII-antibody specific scFv. Reasons for this may have been the highly variable composition of a polyclonal antibody pool. Also, the diversity of the phage-displayed libraries is limited and probably did not include matching antibodies.

In the second project, two monoclonal inhibitory antibodies, murine GMA8011 and human LE2E9, both directed against the C1 domain of fVIII, were targeted during biopanning. In the past, C1 domain-specific antibodies were not thought to play an important immunogenic part, which was recently refuted by new findings. Therefore, this was a first attempt to select synthetic anti-idiotypes from scFv libraries against C1 domain-specific antibodies. Furthermore, these antibodies demonstrated competing behavior among each other, raising the theory of them sharing the same epitope on fVIII and therefore having a very similar paratope. If so, we were interested whether selected scFvs would be capable of neutralizing the other mAb as well. Such cross reactivity was not yet achieved by synthetic anti-idiotypic scFvs before. Biopanning, however, did not produce scFvs, that pertained their neutralizing ability as scFv-Fcs. Only H2C1-hFc, specific for GMA8011, was capable of restoring fVIII binding in the presence of the inhibitor in ELISA experiments. However, this occurred at a 400-fold excess of H2C1-hFc towards GMA8011, indicating that steric hindrance induced by high concentrations and not specific competition was responsible for the results. Most likely the scFvs bound in close proximity to the variable region of the inhibitor.

Conclusively, we have approached the goal of selecting synthetic anti-idiotypic scFvs from different angles, in polyclonal and monoclonal settings, however remained unsuccessful. In the future, using phage libraries of greater diversity or reducing the target to, for example, only the Fab-fragment of the inhibitor may improve our protocol.

6 References

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7 Appendix

7.1 List of Figures

7.2 List of Tables

7.3 Materials

The following tables list all materials and equipment used for the projects.

7.3.1 Media, Buffer, Compounds, Kits and Laboratory Equipment

Table 2: Laboratory equipment and plastic ware.

Instrument, Material	Supplier
96 well culture plates, flat bottom	Sarstedt
Cell culture/Agar dishes (10cm, 25cm)	Sarstedt
ÄKTAprime plus	GE Healthcare
Sterilizer VX-55	Systec GmbH
Biofuge fresco	Heraeus Instruments GmbH, Hanau, Germany
Biofuge primo R	Heraeus Instruments GmbH
Biosphere pipette tips, filtered, 0,5-20µl, 200µl, 1000µl	Sarstedt
CO8000 cell density meter	Biochrom
Tecan Sunrise^{TM} Microplate Reader	TECAN Group Ltd.
96 well microtiter plates Microlon 600	Greiner
ELISA-Plate-Washer Elx405 Select	BioTek Instruments Inc.
Galaxy Mini table centrifuge	VWR
Incubator Function Line	Heraeus Instruments GmbH
Lab-860 pH meter	SI Analytics
MACSmix Rotator	Miltenyi Biotec
Magnetic Particle Concentrator MPC	Invitrogen
Magnetic stirrer w/ heating plate RCT	IKA Works
Microwave	Siemens

Millex-GS syringe filter unit 0,22µm	Millipore
Minishaker MSI	IKA Works
Multichannel pipette Research® 30-300µl	Eppendorf
Multifuge 1 S-R	Heraeus Instruments GmbH
Multipipette stream	Eppendorf
Multiply 0.2ml reaction tubes	Sarstedt
NanoDrop® 1000 spectrophotometer	Thermo Fisher Scientific
Nunc Cryotube vials 1.8ml	Nalge Company
DNA engine Dyad® peltier thermal cycler	BioRad
Pipetboy acu	Integra Biosciences
Pipette Research® 0,2-10 µl, 2-20 µl, 10-100 µl, 20-200 µl, 100-1000 µl	Eppendorf
Pipette tips, 200, 1000µl	Greiner
Pipette tips, filtered, 20 µl, 200 µl, 1000 µl	Starlab
REGLO digital peristaltic pump	ISMATEC
Rotilabo 5ml reaction tubes	Carl Roth
SafeSeal® reaction tubes 1,5ml, 2ml	Sarstedt
Sartorious Universal U4800P balance	Sartorius
SealPlate® Sealing Films for ELISA	Excel Scientific
Serological pipettes Corning Stripettes 5, 10, 25, 50 ml	Corning
Shaker SM-30 with Incubation Hood TH 30	Edmund Bühler
SnakeSkin™ Dialysis Tubing, 22mm, 10000MWCo	Thermo Fisher Scientific
UV-Cuvettes semi-micro- single use	BRAND
Vortex Genius 2	VWR, USA

Water bath WB-10	PD Industriegesellschaft
Erlenmeyer flasks 50ml, 400ml	Schott Instruments
NALGENE™ Reusable Bottle Top Filter Unit	Nalge Company
CO_2 Incubator Typ BB 16 CU	Heraeus Instruments GmbH
Cell Culture Hood, HeraSafe™	Heraeus Instruments GmbH
cellulose acetate filters (0.2µm)	Sartorius
ChemiDoc XRS+ Imaging System	BioRad
Mini Trans-Blot® Cell	BioRad
Mini-PROTEAN® Tetra cell electrophoresis system	BioRad

Table 3: Buffers and compounds for IgG purification

Buffer/Compound	Composition, etc.
HiTrap Protein A HP column, 1ml	GE Healthcare #71-7002-00
NaH_2PO_4 solution, 200 mM	15.6 g $NaH_2PO_4x2H_2O$ (Merck) ad 500 ml H_2O
Protein A binding buffer (pH 7.0), 200 mM sodiumphosphate buffer	305 ml Na_2HPO_4-Solution (200 mM), 195 ml NaH_2PO_4-Solution (200 mM)
Protein A binding buffer (pH 7.0), 20 mM sodiumphosphate buffer	30.5 ml Na_2HPO_4-Solution (200 mM), 19.5 ml NaH_2PO_4-Solution (200 mM), H_2O ad 500 ml
Protein A elution buffer (pH 3.0) 0.1 M citric acid	6.23 ml citric acid (8.03 mol/l), H_2O ad 500 ml
Protein A neutralizing buffer (pH 9.0) 1 M TRIS	12.11 g TRIS (121.14 g/mol), H_2O ad 100 ml
Protein G elution buffer (pH 2.7), 0.1 M glycine	1.88 g glycine (75.07 g/mol), H_2O ad 250 ml
Vivaspin 20 concentrator, 30 kDa	Sartorius

Table 4: Buffers and compounds for Affi-Gel® coupling.

Buffer/Compound	Composition, etc.
1x Coupling buffer (Affi-Gel® kit content) + 10 mM $CaCl_2$	100 ml 10x coupling buffer (Affi-Gel® Kit, 1.1 g $CaCl_2$ (110.99 g/mol), ad 1 l H_2O; pH 5.5
Affi-Gel® Hz Immunoaffinity Kit	BioRad #153-6060
BSA-Stock, 10 %, in coupling buffer (Affi-Gel® kit content)	500 mg BSA, Coupling buffer ad 5 ml store filtrated aliquots at -20°C
Buffer A (equilibration buffer)	10 mM MES, 5 mM $CaCl_2$, 2 mM NaCl; pH 6
Buffer B (Washing buffer) for Affi-Gel® HZ	10 mM MES, 5 mM $CaCl_2$, 100 mM NaCl; pH6
Buffer C (elution buffer) for Affi-Gel® HZ	10 mM MES, 5 mM $CaCl_2$, 1 M NaCl, 50% ethyleneglycol; pH 6
Buffer D (regeneration buffer) for Affi-Gel® HZ	10 mM MES, 5 mM $CaCl_2$, 1 M NaCl, 75% ethyleneglycol; pH 6
Glycerol-Stock, 50%, in coupling buffer (Affi-Gel® kit content)	2 ml glycerine (92.09 g/mol; 1.26 g/cm^3), ad 4 ml coupling buffer; filtrated aliquots stored at 4°C
NaCl solution, 5 M	292,2 g NaCl (58,44 g/mol), ad 1 l H_2O
$NaIO_4$ solution (Affi-Gel® kit content)	25 mg $NaIO_4$, ad 1.2 ml H_2O
Poly-Prep® Chromatography Column	BioRad
Tween20 10 %	5ml Tween20, ad 50 ml H_2O
Washing Buffer for Affi-Gel®	220 mM Tris, 0.01 % Tween, 10 mM $CaCl_2$, 150 mM NaCl, 0.02 % NaN_3; pH 7.4

Table 5: Buffers and compounds for scFv-biopanning

Buffer/Compound/Media	Composition, etc.
1xPBS	1:10 10xPBS (phosphate buffered saline) in H_2O
2xYT agar	16 g trypton, 10 g yeast extract, 5 g NaCl, 15 g agar ad 1 l dH_2O, pH 7.0

2xYT agar$_{Amp/Glu}$	16g trypton, 10 g yeast extract, 5 g NaCl, 15 g agar, 5% glucose 2M, ampicillin solution (100mg/ml), ad 1 l dH_2O, pH 7.0,
2xYT medium	16 g trypton, 10 g yeast extract, 5 g NaCl, ad 1 l dH_2O, pH 7.0
2xTY$_{amp/Glu}$ medium	2xYT-Medium, 5 % glucose solution (2 M), ampicillin solution (100 mg/ml)
2xYT$_{amp/Kan}$ medium	2xYT medium, ampicillin solution (100 mg/ml), kanamycin solution (50 mg/ml)
Ampicillin solution (1000x)	100 mg/ml ampicillin sodium salt (CR) in H_2O
Blocking Buffer for Streptavidin Dynabeads® PBS with 1 % BSA, 0.05 % Tween	10% 10xPBS, 1 % BSA, 0.05 % Tween in dH_2O
Dynabeads® MyOne™ Streptavidin T1	Invitrogen, #650.02
Glucose solution, 2 M	396 g glucose, ad 1 l dH_2O
Glycerol/PBS	50% Glycerol (CR), 50% 1xPBS
rfVIII	human, recombinant fVIII; Kogenate®(Bayer)
HuScL-2 library (diversity: 1,42x10^9)	1x10^{13} PFU /ml, storage at -80°C
HuScL-3 library (diversity: 1,6x10^9)	1x10^{13} PFU /ml, storage at -80°C
kanamycin solution	50 mg/ml kanamycin sulfate in H_2O
NaCl solution, 5 M	73.05 g NaCl, ad 250 ml H_2O
NaN_3-Stock, 2 %	1 g sodium azide, ad 50 ml PBS
PBS/NaN_3, 0.02 %	5 ml 10x PBS, 0.5 ml NaN_3-Stock, ad 50 ml H_2O
PBS with $MgCl_2$ and	Gibco® by life technologies
PEG-stock, 50 %	125 g polyethylene glycol 6000, ad 250 ml H_2O
PEG/NaCl	1:1 mixture of NaCl- and PEG-stock solution
Stock buffer, 10x	0.5 g BSA, ad 50 ml 10x PBS
Top-Agar	250mg $MgCl_2x6H_2O$, 1.75 g agar, ad 250 ml YT-medium

Trypsin solution, 10 mg/ml	50 mg trypsin, ad 5 ml Tris-HCl buffer, 50 mM
Wash Buffer for Streptavidin Dynabeads®	5 ml buffer-stock solution 10x, ad 50 ml H_2O
LB medium	20 g LB medium, ad 1 L H_2O, Carl Roth
LB_{amp} agar	1 L LB Medium + 15 g Agar, ampicillin (100 mg/ml)
LB_{amp} medium	20 g LB medium, ad 1 L H_2O (ampicillin 100 mg/ml)

* All media and buffers were sterilized using a NALGENE™ Reusable Bottle Top Filter Unit with cellulose acetate filters (0.2µm). Also, bacterial media and agar were autoclaved.

Table 6: Other buffers, compounds and equipment.

Buffer/Compound	Composition, etc.
1 kb DNA ladder	GeneRuler™, Fermentas
6x DNA loading Dye (purple)	NEB
Agarose	AppliChem
$CaCl_2$ solution	100 mM $CaCl_2x2H_2O$ (AppliChem) in H_2O
$CaCl_2$/glycerol	15% glycerol (v/v) (CR) in $CaCl_2$ solution
CutSmart® Buffer	NEB
Dade® Actin® FSL	Siemens
dH_2O	Distilled Water
Ethanol (70%)	70 ml ethanol (SA), H_2O ad 100 ml
H_2SO_4	TitriPur® Merck
Isopropanol	SA
MPBST 5%	5% milk powder (w/v) (SA) in PBST
Nucleobond Kit PC500 Plasmid DNA Preparation Kit	Macherey-Nagel
OPD solution	40 mg OPD (SA) dissolved in 100 ml 0.05 M Phosphate-Citrate Buffer (SA), activated with 40µl H_2O_2

PBST	0.05% TWEEN in 1xPBS
peqGOLD Gel Extraction Kit	PEQLAB
peqGOLD Plasmid Miniprep Kit I	PEQLAB
Phusion® High-Fidelity DNA Polymerase	NEB
Quick Ligation™ Kit	NEB
Restriction Enzyme *Nco*I	NEB
Restriction Enzyme *Not*I	NEB
Roti® Gel Stain	Carl Roth GmbH
TAE-Buffer	40 mM TRIS (SA), 20 mM acetic acid (SA), 1 mM EDTA, adjust pH to 8.0

Table 7: Buffers, compounds and media for tissue culture.

Buffer/Compound/Media	Composition, etc.
DMEM - FBS	Dulbecco's Modified Eagle's Medium (Gibco) - high glucose (SA), 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin (10.000 units penicillin, 10mg streptomycin per ml (SA)), 2% L-glutamine, 200mM (SA)
DMEM - ITS	DMEM (Gibco) - high glucose (SA), 1% 10 x Insulin-Transferrin-Selenium (IITS) (Gibco), 1% Penicillin-Streptomycin (10.000 units penicillin; 10mg streptomycin per ml (SA)), 2% L-glutamine, 200mM (SA)
DPBS	Dulbecco's phosphate-buffered saline, no calcium, no magnesium (Gibco)
Polyethylenimine (PEI)	MW 40.000 linear (Polysciences Inc.)
Trypsin-EDTA solution	SA

7.3.2 Bacteria, Phages, and Cell Lines

Table 8: Bacteria, phages and cell lines

Name	Description
E. coli TG1	Suppressor strain for biopannings, component of the HuScL library kit (K12 *(lac-proAB) supE thi hsdD5/F' traD36 proA+B lacIq lacZ*M15)
E. coli Top10	Purchased at Invitrogen (F- mcrA *(mrr-hsdRMS-mcrBC) +80lacZ*M15 *lacX74 recA1 araD139 *(araleu) 7697 galU galK rpsL (StrR) endA1 nupG)
HEK 293T	Human Embryonic kidney epithelial cells tansduced with SV40 large T antigen; Reference # ATCC CRL-11268
HuScL-2 Phage Library	NEB
HuScL-3 Phage Library	NEB
KM13 helperphage	Component of Tomlinson Library Kit

7.3.3 Antibodies

Table 9: Antibodies.

Name	Description
anti-fVIII-positive patient plasma	inhibitor positive plasma from anonymized patient
Biotinylated goat anti-human IgG (H+L)	# 109-065-088, Dianova
Biotinylated rabbit anti-mouse IgG (H+L)	# 6170-08, Southern Biotech
GMA8011 (murine anti-fVIII anti-C1 domain IgG)	Green Mountain Antibodies
Human IgG isotype control	IgG-Pool from 50 healthy patients
Human IgG4 isotype control	# I 4639, Sigma Aldrich
LE2E9 (human anti-fVIII anti-C1-domain IgG)	[46]

Mouse HRP-conjugated anti-M13 antibody	# 27-9421-01, GE Healthcare
Mouse IgG2a Isotype Control	# G155-178, BD Pharmingen ™

7.3.4 Oligonucleotides

All oligonucleotides were ordered at Sigma Aldrich

Table 10: Oligonucleotides for sequencing.

Name	D	Length (bases)	T_m (°C)	GC (%)	Sequencing of	Sequence
OMHI-065	rev	20	58,9	50	scFvs in pIT2 vector	CCCTCATAGTTAGCGTAACG
OMHI-067	fow	21	76.9	66.6	scFvs in pCMV vectors	CGCAAATGGGCGGTAGGCGTG
OMHI-068	rev	18	62.2	61.1	scFvs in pCMV vectors	CCAGGAGTTCAGGTGCTG

Table 11: Oligonucleotides for Overlap Extension PCR.

scFv	Primer	Name	D	Length (bp)	T_m (°C)	GC (%)	Sequence
H2C1	a	332	out	25	77.7	60	ACTCCATGGCCGAGGTGCAGCTGTT
	b	333	inn	29	77.9	55.1	GAACCCATACCCTGAATCGCTGAGACCCA
	c	334	inn	29	77.9	55.1	TGGGTCTCAGCGATTCAGGGTATGGGTTC
	d	335	out	25	79.9	64	GCTAGCGGCCGCCCGTTTGATTTCC
H3G7	a	336	out	25	76.3	60	CACTCCATGGCCTCTGTGTTGACGC
	d	337	out	27	78.2	66.6	GCTAGCGGCCGCAGTACTATCCAGGCC
H3F10	a	338	out	26	74.4	50	CACTCCATGGCCGAAATTGTGATGAC
	b	339	inn	28	73.0	57.1	GGTACCAGGCTAACTGGCTGCTGCTAAC

	c	340	inn	28	73.0	57.1	GTTAGCAGCAGCCAGTTAGCCT GGTACC
	d	341	out	27	78.1	66.6	CTAGCGGCCGCAGTACTATCCA GGCCC

7.3.5 Vector Plasmids

Table 12: Vector Plasmids.

Name	Description
pCMV2.5-hIgG1-Fc-XP (pCMV-hG1-Fc)	Eukaryotic expression vector to express fusions of scFv with the human IgG1-Fc region
pCMX2.5-mIgG2c-Fc-XP (pCMX-mG2c-Fc)	Eukaryotic expression vector to express fusions of scFv with the murine IgG2c-Fc region

Table 13: Ligated scFv Vector Plasmids

Name	Description
H2C1-pCMV-hG1-Fc	GMA8011-specific H2C1 sequence inserted into the pCMV-hIgG1-Fc vector
H2C1-pCMX-mG2c-Fc	GMA8011-specific H2C1 sequence inserted into the pCMV-mIgG2c-Fc vector
H3G7-pCMV-hG1-Fc	LE2E9-specific H3G7 sequence inserted into the pCMV-hIgG1-Fc vector
H3G7-pCMX-mG2c-Fc -mIgG2c	LE2E9-specific H3G7 sequence inserted into the pCMV-mIgG2c-Fc vector
H3F10-pCMV-hG1-Fc	LE2E9-specific H3F10 sequence inserted into the pCMV-hIgG1-Fc vector
H3F10-pCMX-mG2c-Fc	LE2E9-specific H3F10 sequence inserted into pCMV-mIgG2c-Fc vector

7.4 Methods Supplement

Table 14: Äkta setting for IgG purification using protein A. Äkta setting for IgG purification using protein A. Conc., concentration [%] of buffer B; Flow, flow speed [ml/min]; Fractions, volume [ml] of collected fractions; Inj., valve position; Standard buffer, protein A wash buffer (2x); buffer B, protein A elution buffer.

Step	Volume	Conc.	Flow	Fractions	Buffer	Inj.	Autozero
1	0.0	0	40.0	0.0	B	Waste	N
2	35.0	0	1.0	0.0	B	Load	Y
3	45.0	0	1.0	0.0	B	Load	N
4	45.1	100	40.0	0.0	B	Waste	N
5	80.0	100	1.0	1.0	B	Load	N
6	90.0	100	1.0	0.0	B	Load	N
7	90.1	0	40.0	0.0	B	Waste	N
8	105.0	0	1.0	0.0	B	Load	N
9	110.0	0	1.0	0.0	B	Load	N

7.5 Supplemental Results

7.5.1 Analysis of Selected Phage Clones against Anti-C1 Monoclonal Antibodies

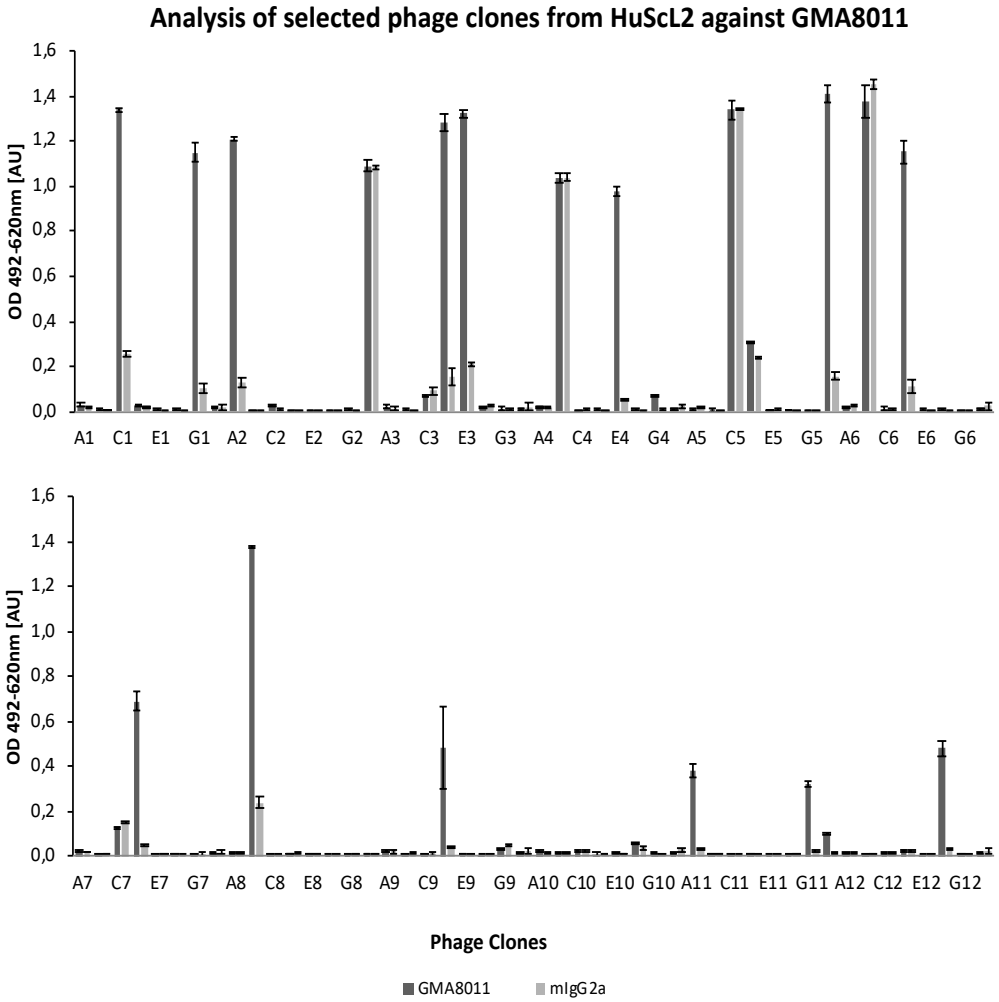

Figure 21 Analysis of selected scFv-carrying phage clones against GMA8011. 0.1 µg GMA8011 and isotype mIgG2a were immobilized on microtiter plates. Supernatants of phage-infected bacterial clones were added to wells. Bound phages were detected with anti-M13 HRP-conjugate. Error bars represent the SD of the mean of duplicates.

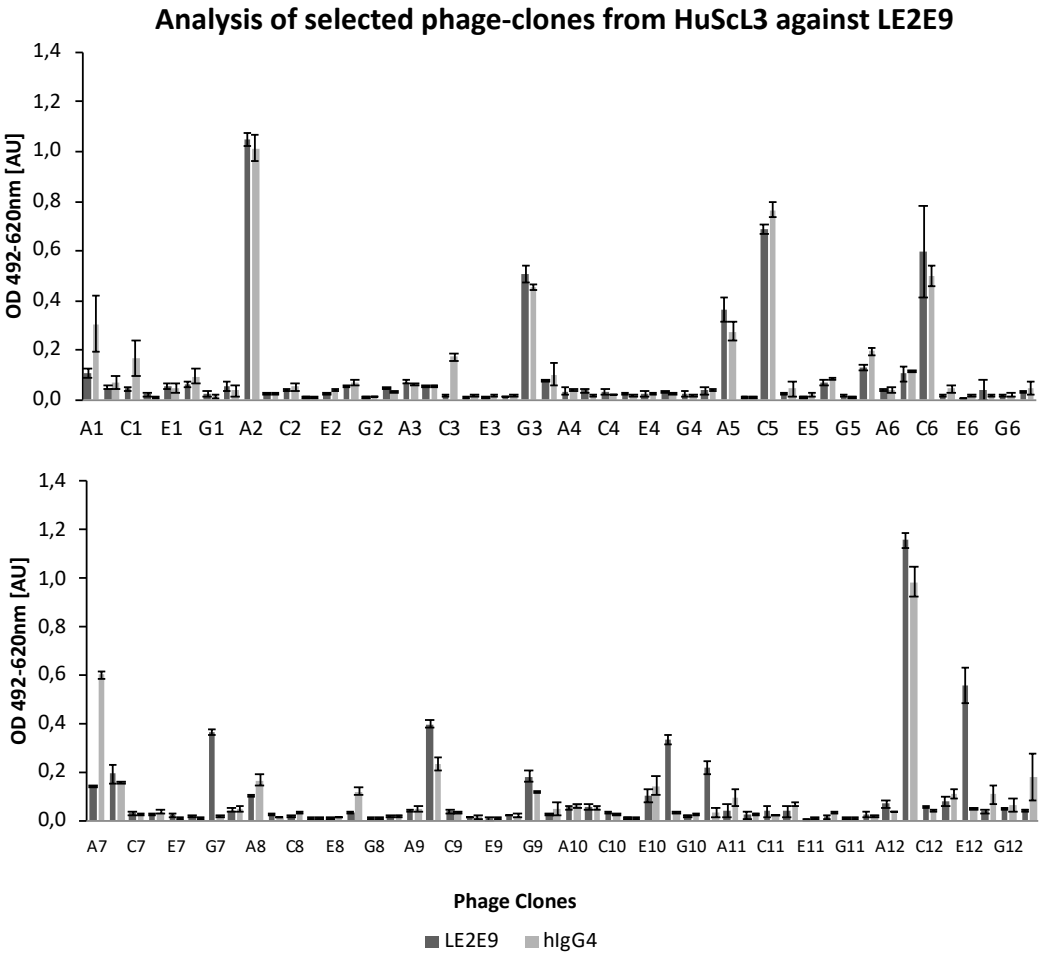

Figure 22 Analysis of selected scFv-carrying phage clones against LE2E9. 0.1 µg of LE2E9 and isotype hIgG4 were immobilized on microtiter plates. Supernatants of phage-infected bacterial clones were added to wells. Bound phages were detected with anti-M13 HRP-conjugate. Error bars represent the SD of the mean of duplicates.

7.5.2 Comparison of scFv-Fc-containing Cell Culture Supernatant and Purified scFv-Fc

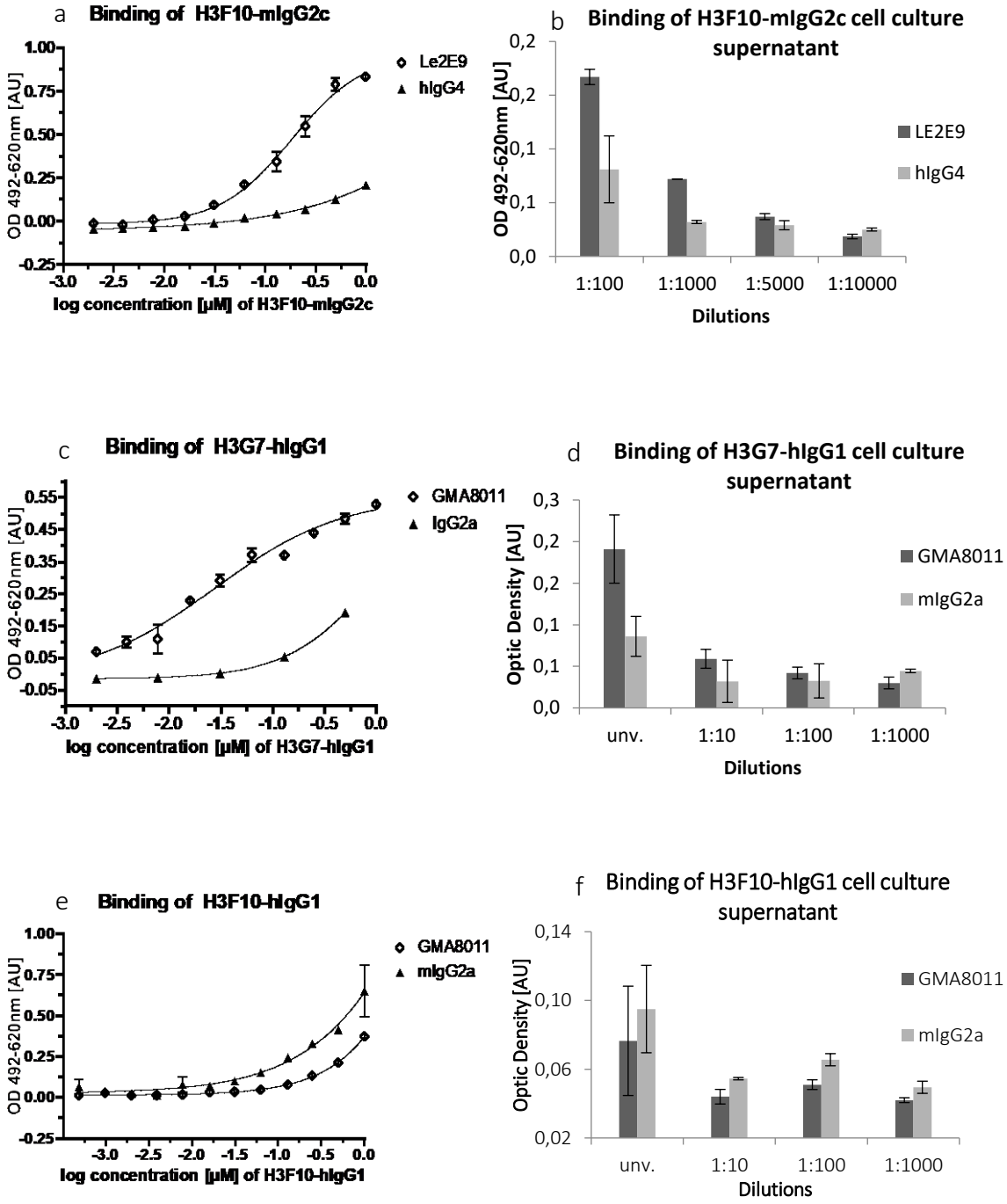

Figure 23 Comparison of binding of scFv-Fc-containing cell culture supernatant and purified scFv-Fc. 0.1 µg target antibody GM8011 or LE2E9 and respective isotype control were immobilized on microtiter plates. Different dilutions of scFv-Fc-containing cell culture supernatants were added to wells. Binding was visualized using HRP-conjugated rat anti-mouse IgG or goat anti-human IgG (right column). Error bars indicate SD from mean of duplicates. Results of cell-culture supernatants were compared to results from analysis of purified scFc-Fc (left column).

7.6 Abbreviations

Table 15: Abbreviations

Abbreviation	Long Version
Ab	Antibody (antibodies)
APC	Antigen presenting cell
aPCC	Activated prothrombin complex
Asn	asparagine
AU	Absorption units
CDR	Complementary determining region
CR	Carl Roth
DMEM	Dulbecco's modified Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
ELISA	Enzyme-linked Immunosorbent Assay
fIX	Factor IX
For	Forward
fow	forward
fV	Factor V
fVII	Factor VII
fVIII	Factor VIII
$fVIII^{-/-}$	fVIII knockout
fX	Factor X
g	Gravitational constant ($9.81 m/s^2$)
HC	Heavy Chain of fVIII
hG1-Fc or hFc	Fc part of human IgG1
HRP	Horse raddish peroxidase
Hu2	Naive phage display library HuScL-2
IgG	Immunoglobuline G
IgM	Immunoglobulin M
ITI	Immune tolerance induction
IU	International units
IVIg	Intravenous immunoglobuline

LC	Light Chain of fVIII
mAb (mAbs)	Monoclonal Antibody (antibodies)
mG2c-Fc or mFc	Fc part of murine IgG2c
MPBST	PBST with 5% milk powder
NaCl	Sodium chloride
OD_{600}	Optical density (492-620 nm)
OPD	O-phenylenediamine
PBS	Phosphate buffered saline
PBST	PBS with 0.05% Tween20
pCMV-hG1-Fc	pCMV2.5-hIgG1-Fc-XP
pCMX-mG2c-Fc	pCMX2.5-mIgG2c-Fc-XP
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PFU	Plaque forming units
R1	Biopanning Selection Round 1
R2	Biopanning Selection Round 2
R3	Biopanning Selection Round 3
R4	Biopanning Seleciton Round 4
rev	Reverse
rfVIIa	Recombinant activated fVII
rfVIII	Recombinant fVIII
RT	Room temperature
scFv	Single chain variable fragment
scFv-Fc	ScFv-Fc fusion protein
scFv-hFc	*Single chain fragment variable* human Fc chain fusion protein
scFv-mFc	*Single chain fragment variable* murine Fc chain fusion protein
SD	Standard deviation
T_{regs}	Regulatory T cells
T1	Biopanning - first trial
T2	Biopanning - second trial
TCR	T cell receptor
TF	Tissue Factor
vWf	Von Willebrandt Factor
w/ fVIII	Pre-incubated with fVIII

w/o fVIII	Wells without fVIII

7.7 Supplier

Table 16: Information on Suppliers and Service Providers

Supplier	Headquarters
AppliChem	Darmstadt, Germany
Biochrom	Cambridge, UK
BioRad	München, Germany
BioTek Instruments Inc.	Vermont, ME, USA
BRAND	Wertheim, Germany
Carl Roth (CR)	Karlsruhe, Germany
Corning	NY, USA
Edmund Bühler	Hechingen, Germany
Eppendorf	Hamburg, Germany
Excel Scientific	Victorville, CA, USA
Fermentas	Waltham, MA, USA
GE Healthcare	Freiburg, Germany
Greiner	Kremsmünster, Switzerland
Heraeus Instruments GmbH	Hanau, Germany
IKA Works	Staufen, Germany
Integra Biosciences	Zizers, Switzerland
Invitrogen	Darmstad, Germany
ISMATEC	Grevenbroich, Germany
Merck	Darmstadt, Germany
Millipore	Billerica, MA, USA
Miltenyi Biotec	Bergisch Gladbach, Germany
Macherey-Nagel	Düren, Germany
Nalge Company	Penfield, NY, USA
New England Biolabs (NEB)	Schwalbach, Germany
PD Industriegesellschaft,	Puschwitz, Germany
Polysciences Inc.	Warrington, PA, USA

PEQLAB	Erlangen, Germany
Sarstedt	Nümbrecht, Germany
Sartorius	Göttingen, Germany
SCHOTT Instruments	Mainz, Germany
SI Analytics	Mainz, Germany
Siemens	Germany
Sigma Aldrich (SA)	St. Louis, MO, USA
Starlab,	Milton Keynes, UK
Systec GmbH	Linden, Germany
TECAN Group Ltd.	Switzerland
Thermo Fisher Scientific	Waltham, MA, USA
VWR	Radnor, PA, USA
BD Pharmingen ™	San Diego, CA, USA
Dianova	Hamburg, Germany
Southern Biotech	Birmingham, Great Britain

7.8 Plasmid Maps

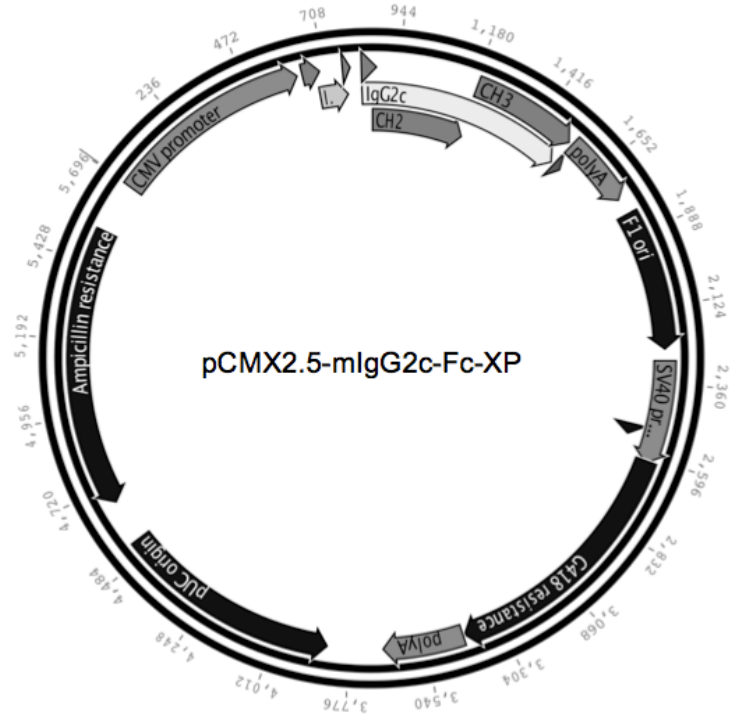

Figure 24: Plasmid map of pCMX2.5-mIgG2c-Fc-XP coding for Fc-region of murine IgG2c. ScFv sequences were ligated into the vector using restriction enzymes *Nco1* and *Not1.*

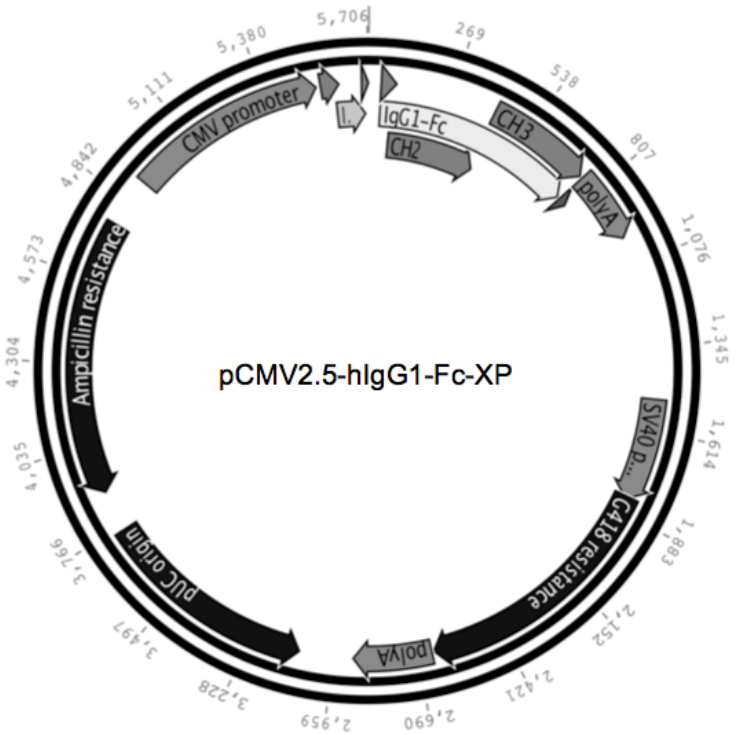

Figure 25 Plasmid map of pCMV2.5-hIgG1-Fc-XP coding for Fc-region of human IgG1. ScFv sequences were ligated into the vector using restriction enzymes *Nco1* and *Not1.*

7.9 DNA Sequence of ScFv H2C1

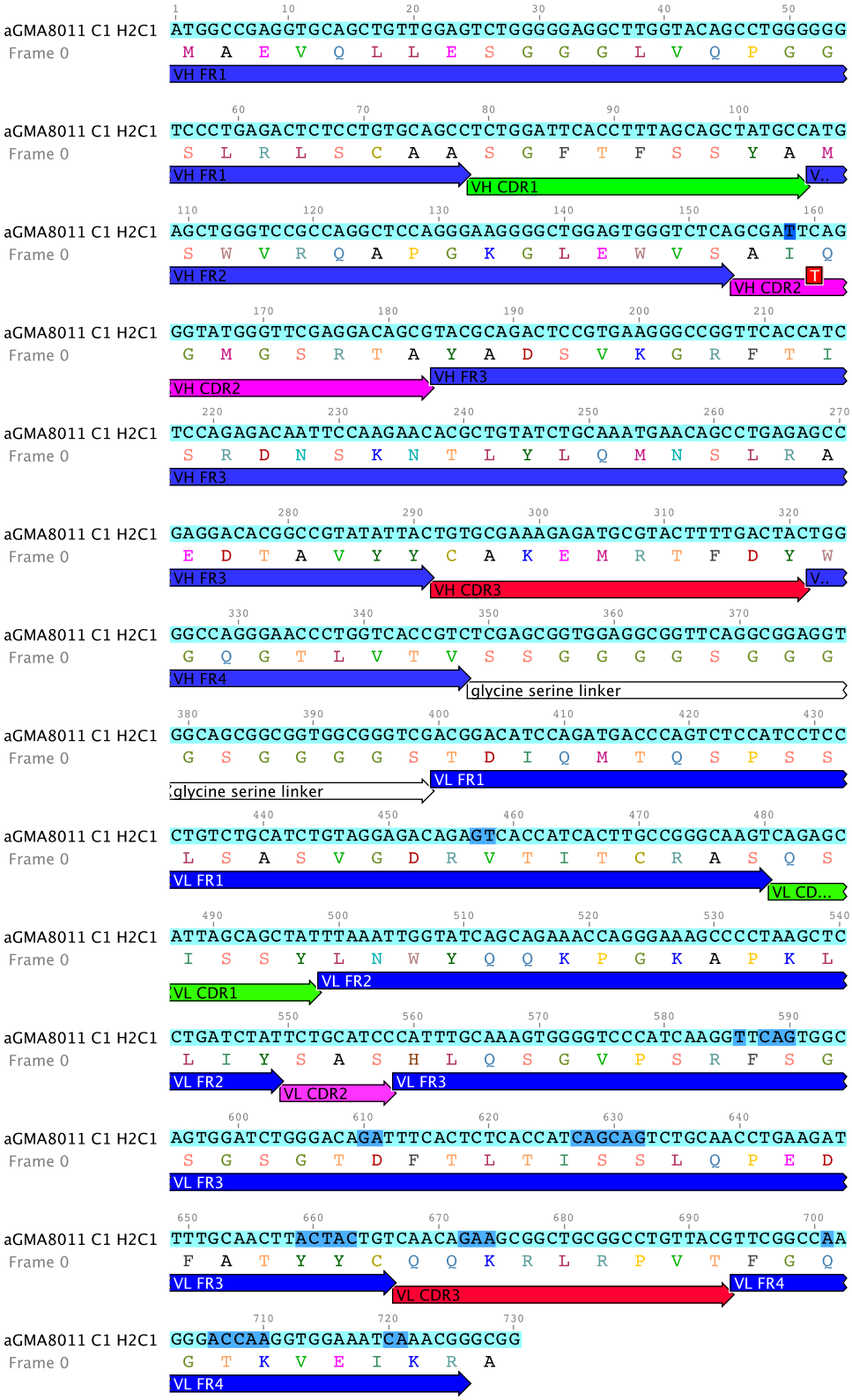

Figure 26 DNA Sequence of H2C1-hFc. DNA was extracted from *E. coli* using the GeneJET Plasmid Miniprep Kit. DNA was sent in for sequencing.

Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

Exploration of anti-idiotypic antibodies for elimination of fVIII inhibitors in hemophilia A patients.

am Zentrum der Kinder- und Jugendmedizin des Universitätsklinikum Frankfurt unter Betreuung und Anleitung von Dr. Dr. Christoph Königs mit Unterstützung durch Dr. Kerstin Brettschneider ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

_____ _____ (Ort, Datum) (Unterschrift)