Pathway to tolerance in hemophilia

Michela Leoni

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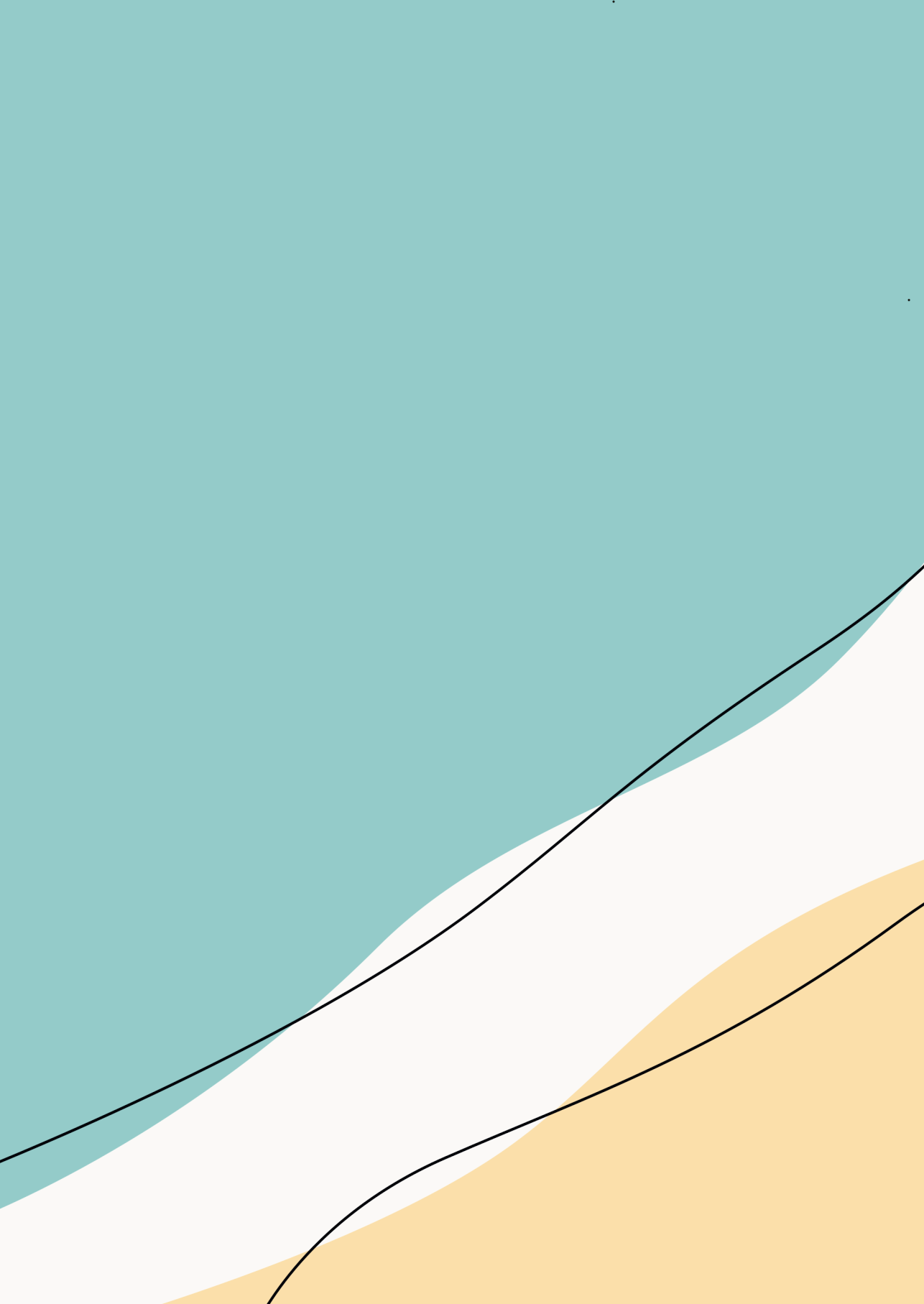
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Chapter 1
General Introduction



Hemophilia

Hemophilia is a hereditary X-linked recessive bleeding disorder and it is caused by the lack or deficiency of factor VIII (FVIII) in hemophilia A or factor IX (IX) in hemophilia B[1]. Due to the X-linked nature, hemophilia A and B mostly affect males; women are heterozygous and, in most cases, they experience milder symptoms as they have one unaffected gene on the other X chromosome[2]. In women, one of the X chromosomes is randomly inactivated during embryonic development[3]. This may result in lower levels of circulating FVIII or FIX, dependent on the distribution of inactivated X chromosomes[4]. When the X chromosome with the affected gene is inactivated less than the chromosome with the unaffected gene, women have reduced FVIII and FIX levels, below 50 IU/Dl, and are classified as mild hemophilia patients. When levels are normal they are classified as carriers[2]. Clinical symptoms of hemophilia include large joint bleeds (ankles, elbows and knees) that can develop to arthropathy, but also life-threatening bleeds, both as spontaneous bleeds and in connection to surgery or injuries[5,6].

Functional role of factor VIII and factor IX in blood coagulation

Hemostasis is the process that controls bleeding in the event of vascular injury[7]. The coagulation system assures timely arrest of bleeding which is mediated and is highly dependent on the coordinated activation of blood coagulant factors that include FVIII and FIX. Anti-coagulant pathways in conjunction with fibrinolysis are in place to prevent excessive thrombus formation and to dissolve clots in an ordered manner.

Blood coagulation has been historically divided into an intrinsic and extrinsic pathway but nowadays, due to a better understanding of the properties of the proteins involved, the model has changed and it has been divided in three phases: initiation, amplification and propagation[8,9].

Initiation – The main actor of the first phase of coagulation is tissue factor (TF). TF is expressed by perivascular cells but it is exposed to blood flow only upon vascular injury. TF acts like a trigger for blood coagulation, due to its ability to bind to FVII[10]. Binding of FVII to TF promotes autocatalytic conversion of FVII to FVIIa which together can activate FIX to FIXa and FX to FXa. Newly generated FXa can bind to FVa which allows for conversion of prothrombin into thrombin. The amount of thrombin formed in the initiation phase is minimal due to the low levels of FVa in circulation[11].

Propagation – Newly generated thrombin can activate FV to FVa and FVIII to FVIIIa. In addition, thrombin has been shown to activate FXI into FXIa which allows for generation of FIXa[12,13]. Furthermore, through its binding to the PAR-1 receptor thrombin promotes platelet activation and aggregation which coincides with exposure of phosphatidylserine on the platelet surface[14]. FIXa together with FVIIIa forms the tenase complex that can activate FX to FXa at a rate that is 50 fold higher compared to the extrinsic TF-FVIIa complex. The newly generated FXa and FVa assemble into the so-called prothrombinase complex which rapidly converts prothrombin into thrombin. The resulting thrombin burst allows for the massive conversion of soluble fibrinogen into the insoluble fibrin, which promotes stabilization of the platelet plug generated during primary hemostasis. The clot is further stabilized by cross-linking of fibrin by the transglutaminase FXIIIa which is also generated from its precursor FXIII during the thrombin burst[9].The contact activation pathway, previously defined as intrinsic pathway, instead has been considered as not being essential for manteining hemostasis, it can however contribute to thrombosis[15]. Binding of FXII to negatively charged surfaces such as polyphosphate and collagen promotes its autoactivation[15]. FXIIa than activates FXI to FXIa, which in turn activates FIX to FIXa that can be incorporated in the tenase complex with FVIII, ultimately resulting in the formation of fibrin clots[16]. A recent study indicated that FXII dependent fibrin formation contributes to innate immunity by enhancing trapping of pathogenic bacteria[17].

Tight regulation of pro-coagulant pathways is important to avoid excessive coagulation which results in thrombosis. Anti-coagulant pathways composed of serine protease inhibitors like antithrombin and tissue factor pathway inhibitor (TFPI) directly target the active site of serine proteases involved in blood coagulation. Additional control is provided by the protein C pathway that serves to inactivate the cofactor FVIII and FV[18]. Antithrombin, a member of the serpin super family, principally acts by inhibiting FXa, FIXa and thrombin[19]. Heparin promotes the activity of antithrombin by exposing its reactive center loop, crucial for rapid inactivation of serine proteases like FXa and thrombin[19]. TFPI acts on the complex TF-FVIIa in a FXa dependent manner and protein S has been shown to act as co-factor for TFPI[20,21]. The protein C pathway regulates procoagulant pathways in a different manner. Protein C is first converted by thrombin into activated protein C (APC) which subsequently can proteolytically inactivate FVa and FVIIIa[22,23]. Interestingly, activating of protein C is also linked to cytoprotective signaling through PAR-1 expressed on endothelial cells[24].

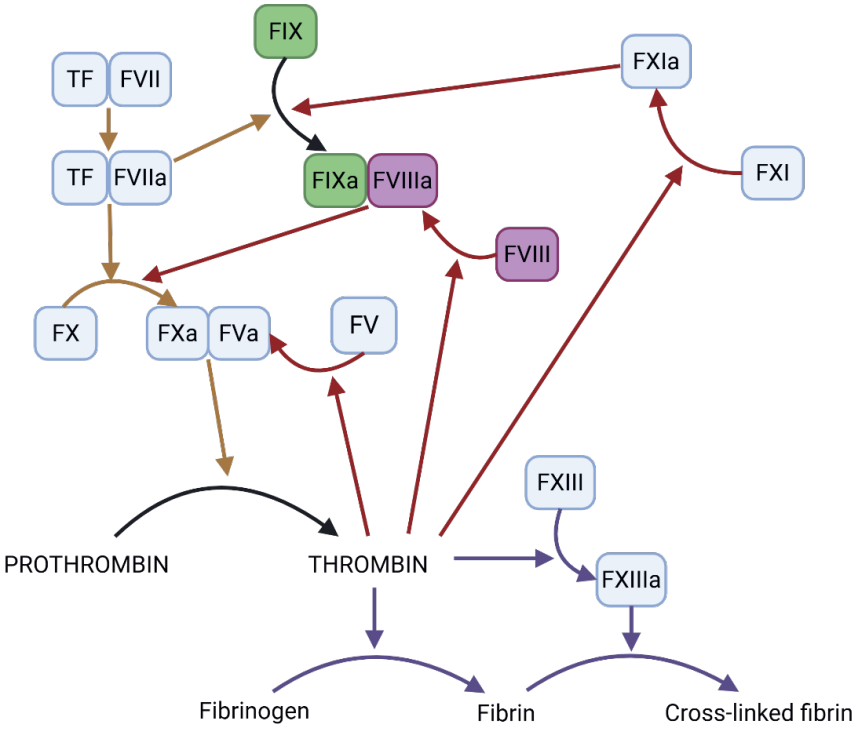


Figure 1. Coagulation cascade. Schematic representation of the coagulation system with steps happening during each phase with a different colored arrows, respectively, initiation phase in yellow, propagation phase in red, after thrombin burst in purple and in multiple phases in black.

Hemophilia A

The average incidence of hemophilia A is 1 in 5000 male births, however it has been documented that the incidence various considerably over different countries [25]. This number is difficult to estimate correctly due to the difficulty in access to adequate diagnosis and registration, the incidence of hemophilia A is probably underestimated[25,26]. The classification of hemophilia severity is based on the percentage of FVIII activity in plasma relative to that measured in plasma of non-affected individuals. FVIII is a trace protein, the normal concentration of FVIII in plasma being 0.1 µg/mL[1]. The concentration of FVIII is expressed in international units (IU) of activity per mL of plasma or in percentage of normal activity. Based on FVIII activity in plasma, patients are classified as mild (FVIII activity between 5 and 40%), moderate (FVIII activity between 1 and 5%,) and severe (FVIII activity less than 1%)[27]. Usually, FVIII circulates in complex with von Willebrand factor

(VWF) and only 3-4% of FVIII has been proposed to circulate as "free" non-VWF bound FVIII[28]. The association of FVIII to VWF increases the half-life of the protein, that is only 2 hours when in circulation in the absence of VWF[28].

Molecular genetics of hemophilia A

The F8 gene is located at position Xq28, on the distal end of the long arm of the X chromosome and is composed of 26 exons[29]. Mutations in the F8 gene that cause severe hemophilia are mostly comprised of large deletions, gene arrangements and nonsense mutations; however, also missense mutations have been linked to severe hemophilia A[30]. An inversion in intron 22 is the principal mutation found in severe hemophilia A patients (40% of cases)[31]. Intron 1 inversion accounts for almost 5% of mutations in severe hemophilia A patients[32,33]. An elegant study has shown that intron 22 inversions originate from recombination events in male sperm cells[34]. In non severe hemophilia A patients usually point mutations and missense mutations are present.

Biosynthesis and structure function of FVIII

F8 gene encodes for a 2332 amino acid protein; it is mainly produced in the liver by a specific cell type, liver sinusoidal endothelial cells (LSEC). Analysis of isolated LSEC, mouse models and single cell transcriptomics have convincingly demonstrated that FVIII is not expressed in hepatocytes or Kupffer cells but selectively in LSEC[35–38].

FVIII contains a signal peptide of 19 amino acids that is removed upon its translocation to the endoplasmic reticulum. FVIII in its unprocessed form is composed of A1, *a1*, A2, *a2*, B, *a3*, A3, C1 and C2 domains. The B-domain is heavily glycosylated and has been shown to be dispensable for FVIII activity[39]. The A domains have been implicated in binding to FIXa and FX whereas the C1 and C2 domain are primarily involved in binding of FVIII to phospholipid membranes[40]. The *a1*, *a2* and *a3* regions have been implicated in thrombin activation of FVIII; these regions are rich in negatively charged amino acids and also contain sulfated tyrosines[41]. Sulfation of Tyr1680 in the *a3* region is crucial for binding of FVIII to VWF[42].

Prior to secretion, FVIII is cleaved at Arg1648 resulting in the formation of a heterodimer composed of an A1,*a1*,A2, *a2*, B domain heavy chain and an *a3*, A3, C1 and C2 domain light chain. FVIII circulates in complex with VWF in an inactive form, upon thrombin activation the protein is cleaved at Arg372, Arg740 and Arg1689. The cleavage at Arg1689 is necessary for the release of VWF, while the cleavage on Arg372 result in the formation of hetero-trimeric FVIII which can bind

to activated FIX via its A2 and A3 domains[40]. The C1 and C2 domains interact with negatively charged phospholipids on the membrane of activated platelets allowing for the assembly of the tenase complex[40].

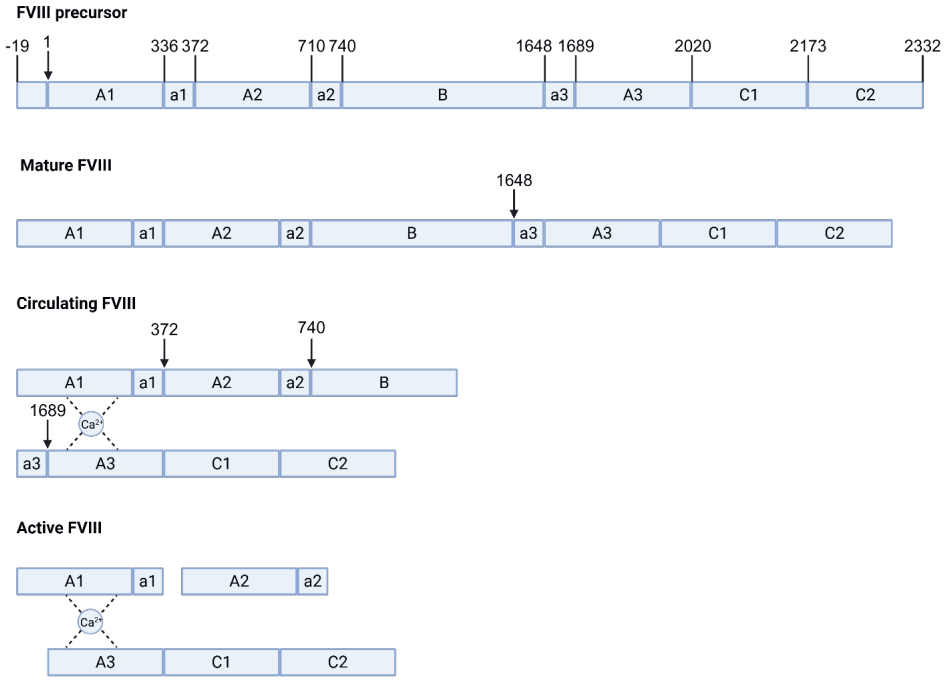


Figure 2 Factor VIII domain structure and intracellular and extracellular processing. Upper panel: domain structure of FVIII including the amino-terminal 19 amino acid signal peptide which is removed during entry in the endoplasmic reticulum. Cleavage at Arg1649 in the Golgi apparatus generates a heterodimer composed of a heavy (A1-a1-A2-a2-B) and light (a3-A3-C1-C2 light chain that circulates in blood tightly bound to VWF. Thrombin activation coincides with proteolytic processing at Arg1689 which allows for dissociation of FVIII from VWF and binding of FVIII to phospholipids. Cleavage at Arg372 and Arg740 generates catalytically active FVIIIa that converts FX into FXa.

Hemophilia B

The incidence of Hemophilia B is 1 in 30000 males. The normal concentration of FIX in circulation is 50 times higher than that of FVIII, around 5 µg/mL[1]. Patients are classified as severe, moderate and mild depending on the remaining FIX

activity, respectively <1%, 1%-5% and 5%-40%. The half life of FIX is between 16 and 18 hours[43,44].

F9 gene and mutations

The F9 gene is located in the Xq27 of the X chromosome, contains 8 exons and is around 34 kb[45,46]. The type of mutations present in the F9 gene strongly correlate with the severity of symptoms in the patients. Currently, over 1500 mutations in the F9 gene have been identified, missense mutations have been observed in the majority of patients[47,48]. In a subset of patients, deletions of the F9 gene have been observed[48]. Mutations are distributed all over the coding sequence of FIX[47,49]. Hemophilia B Leyden is a subtype of hemophilia B, the patients suffering from hemophilia B Leyden have a mild to severe bleeding phenotype at birth but the FIX levels increase after puberty, with some of the patients even reaching normal levels. Hemophilia B Leyden is caused by mutations in the promoter region from c.-50 to c.-18 of the F9 gene[49]. It was shown in a mouse model for hemophilia B Leyden that growth hormone levels were inducing a rise of FIX during puberty but it is not yet clear how the growth hormone regulates the transcription factors that binds to that promoter region[50].

Interestingly, a gain of function mutation, R338L, has been identified in a patient with venous thrombosis which gave rise to a hyper functional FIX with activity *in vitro* that is 5 to 10 times higher than wild-type FIX. This mutant FIX has been classified as FIX Padua. Patients with FIX Padua have normal levels of FIX antigen; the R338L mutation has been shown to enhance the binding of FIXa to FVIIIa resulting in enhanced formation of FXa[51]. Patients with FIX Padua do not have a bleeding tendency but are at risk for developing venous thrombosis[51,52].

Biosynthesis, structure and function of FIX

FIX is produced by hepatocytes in the liver and its precursor contains 461 amino acids. FIX contains a signal peptide of 28 amino acid and a propeptide of 18 amino acid residues. The mature protein is composed by 415 residues and contains in a Gla domain (47-92), two epidermal growth factor (EGF) like domains, EGF1 (93-129) and EGF2 (130-171), a linker sequence (171-191), an activation peptide (192-226) and a serine protease domain (227-461)[53–55]. The Gla domain contains 12 glutamic acid residues that are converted by γ-glutamyl carboxylase (GGCX) to γ-carboxyglutamates (Gla) using vitamin K hydroquinone as cofactor [56]. The propeptide is necessary for γ-carboxylation of glutamic acids because it acts as the primary binding site of GGCX[57]. The Gla domain mediates the binding of FIX to phospholipid membranes in a calcium dependent manner[55]. In addition it has been proposed that the Gla domain interacts with the phospholipid binding C1 and/or

C2 domains of FVIII[53,55]. The EGF1 domain of FIX interacts with either FIXa or the TF/FVIIa complex, specifically TF, during FIX activation[58]. The EGF2 domain is responsible for the correct assembly of the Xase (FIXa/FVIIIa) complex on activated platelets [59,60]. In order to be converted from its zymogen to the active form, the activation peptide of FIX needs to be cleaved[55]. The first cleavage occurs between Arg191–Ala192 and the second between Arg226–Val227 (UniProt numbering). Following activation (activation peptide cleavage) the light chain (N-terminal) and heavy chain (C-terminal) are kept together by a disulfide bond between the linker sequence and the serine protease domain (Cys178-Cys335)[55].The serine protease domain contains the catalytic triad His267, Asp315, and Ser411. The serine protease domain mediated the binding to FVIII, in particular the A2 domain of FVIIIa interact with an exposed alpha helix in this domain. Additionally, the serine protease domain also contains a FX recognition site[54,61].

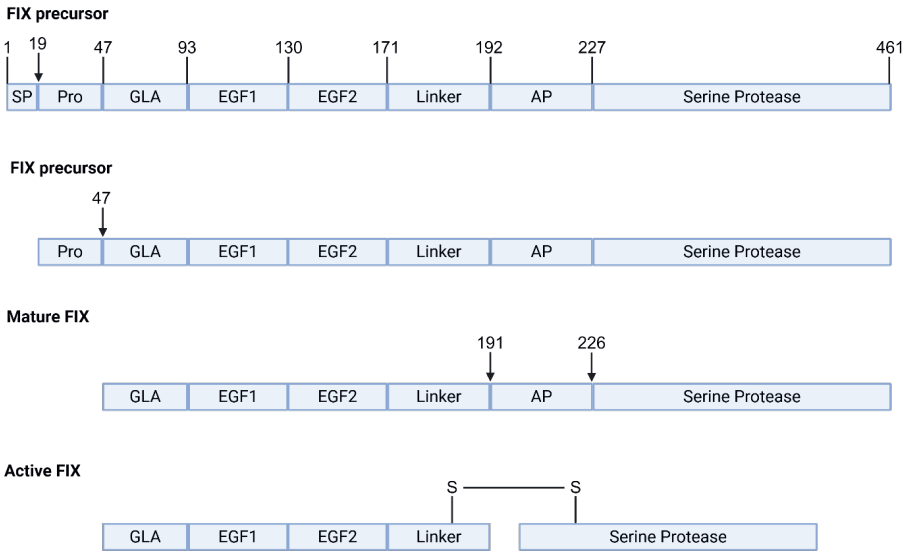


Figure 3. Factor IX domain structure and processing steps. Upper panel: domain structure of FVIII including the amino-terminal 19 amino acid signal peptide which is removed during entry in the endoplasmic reticulum. In the endoplasmic reticulum glutamic acid residues are γ-carboxylated. Cleavage at Arg46 by furin removes the propeptide and generates mature FIX which is released in the circulation. Activation of FIX by FXIa or the TF-FVIIa complex removes the activation peptide and results in a disulfide bond stabilized FIXa which can assemble with FVIIIa in the tenase complex.

Management of bleeding in hemophilia

Treatment of patients with hemophilia A and B is based on supplementation of coagulation FVIII or FIX to re-establish hemostasis[1]. In the last 40 years of the previous century patients were infused with plasma derived products, at first with either plasma or cryoprecipitate and later with plasma derived coagulation factors concentrates[62]. In the '80s, 60% of hemophilia A patients became infected with HIV following treatment with contaminated factor concentrates prepared from large numbers of donors. An even higher percentage contracted hepatitis C. Since then, virus inactivation processes have been applied to plasma derived product but apprehension for new unknown pathogens has remained, especially in families impacted by contaminated-plasma products in the past. This apprehension coincided with the molecular cloning of FIX and FVIII and provided a strong impetus for the medical community to push for a switch from plasma derived factor concentrates to recombinant products[43,63]. These efforts quickly resulted in the availability of recombinant concentrates that were based on production of FVIII and FIX in mammalian cell lines. The half life of intravenously infused FVIII ranges from 8 to 12 hours so at least 3 intravenous injections per week are necessary for prophylactic treatment of patients, while for FIX the half life is 18-24 hours[1,43]. To limit the number of injections required to maintain therapeutic levels of FVIII in circulation, extended half life (EHL) products have been developed. One strategy was to increase the half life by PEGylation of FVIII and FIX, this increases the hydrodynamic volume of the molecule reducing renal filtration and clearance[1]. Another strategy is the addition of an Fc portion of IgG to FVIII and FIX, this enables FVIII-Fc and FIX-Fc to be recycled via the neonatal Fc receptor thereby prolonging there circulatory half-life. Also, albumin fusion has been used for half-life extension of FIX[64]. The above modifications have resulted in a half-life extension of 1.5-2.0 for FVIII whereas a 4 fold increase was observed for FIX-Fc, FIX-albumin and PEGylated FIX[64.]

The limited gain observed for FVIII is due to its binding partner VWF which regulates clearance of FVIII[28]. To tackle this limitation a VWF independent FVIII variant was designed in which FVIII-Fc was fused to the D'D3 domains of VWF[65]. The D'D3 domains contains the binding site for FVIII and therefore the resulting FVIII-Fc-D'D3 fusion (designated BIVV001 or efanesoctocog alfa) cannot interact with circulating endogenous VWF. In addition to the D'D3 domains of VWF, XTEN polypeptides were added to further enhance its pharmacokinetics proprieties. All together, these modification results in a half-life of efanesoctocog alfa that is 3 to 4 times higher when compared to rFVIII[66]. The extended half-life of efanesoctocog alfa reduces the number of infusions to 1-2 per week[43,44,67].

Non replacement therapies are defined as therapies that do not involve FVIII or FIX infusions[1]. Non-replacement therapies can be classified as either FVIII mimetics, which are mainly composed of bispecific monoclonal antibodies, or rebalancing agents that (partially) restore thrombin formation in patients with hemophilia. Emicizumab is a bispecific monoclonal antibody that targets FIXa and FX, thus bridging the two factors and mimicking the function of FVIII, thereby restoring hemostatic function in patients with hemophilia A[68] . Emicizumab is injected subcutaneously providing ease of administration, especially in children. The efficacy of emicizumab has been documented in numerous studies and has greatly improved the quality of life of hemophilia A patients. Importantly, *in vitro* and *in vivo* findings show that the hemostatic efficacy of emicizumab compares unfavorably with that of FVIII. Therefore, breakthrough which may require treatment with FVIII or activated FVII are still observed [68].

A second-generation bispecific antibody designated Mim8 builds on the same principle as emicizumab. Interestingly, Mim8 targets the same helix in the protease domain of FIXa that also interacts with FVIII[55,69]. The in vitro efficacy of Mim8 compares favorably with respect to emicizumab; lower levels of Mim8 are needed to restore thrombin generation in *in vitro* studies[68]. Clinical studies show that Mim8 can be administered either weekly or once every month providing adequate protection from bleeding[68,70]. An improved, re-engineered version of emicizumab designated NXT007 was recently reported[69]. Based on in vitro and in vivo findings it was reported that NXT007 was more than 20-fold more potent when compared to emicizumab. A recently published clinical study showed the clinical efficacy of NXT007[71]. Remarkably, anti-drug antibodies were observed in 73% of patients following treatment; anti-drug antibodies did not cross-react with emicizumab and were reported not to effect pharmacokinetics of NXT007in the majority of patients [71].

Rebalancing agents inhibit anti-coagulant pathways thereby restoring the normal hemostatic balance in patients with hemophilia. Two monoclonal antibodies inhibiting TFPI (concizumab and marstacimab) provide hemostatic efficacy through sustained production of thrombin[72,73]. Fitusiran is a siRNA that interferes with the production of antithrombin[74]. Thrombotic complications have been reported and therefore further dose optimization is needed for this siRNA based therapy[1,75]. Finally, serpinPC is a bio-engineered antitrypsin like protease that inhibits activated protein C[66].

Gene therapy for hemophilia

AAV based gene therapy for both hemophilia A and B have been successfully developed[1,76]. Roctavian (valoctogene roxaparvovec) is based on an AAV5 virus which expresses a B domain deleted FVIII variant under the control of a liver specific promoter that drives expression in hepatocytes[77,78]. High supraphysiological levels of FVIII, that declined over time, were observed in patients with hemophilia A[79]. The main side effect of this innovative strategy is the increase of liver transaminase, this is, most likely, due to liver toxicity. Reports on vector integration have raised concern with respect to potential genotoxicity of AVV5 based gene therapy for hemophilia A[80]. Therefore, further clinical development of Roctavian has currently been halted[81]. Another approach uses a lentiviral vector encoding the F8 transgene ET3 (a bioengineered hyperactive FVIII variant) transduced in hemopoietic stem cells (HSCs)[82]. In a similar approach, lentiviral vectors expressing FVIII under the control of a platelet-specific promoter have been used for ex-vivo gene therapy employing mobilized hematopoietic stem cells in hemophilia A patients with inhibitors[83]. A reduction in annual bleeding rate was observed in this clinical study. The burden of treatment of these *ex-vivo* approaches is quite high when compared to the currently available factor and non-factor replacement therapies.

AAV based gene therapy approaches have been successfully developed for hemophilia B patients and are approved by both EMA and FDA[84]. Instead of wild type FIX, the hyperactive FIX Padua has been used in these vectors to further increase FIX activity levels[85]. Both AVV5 and AAV8 have been successfully used and for both delivery systems the transgene is expressed under control of a strong liver specific promoter[86,87]. So far, AAV based gene therapy for hemophilia B patients has been demonstrated to be more successful than for hemophilia A; stable levels of FIX have been achieved in most gene therapy trials[88,89]. This is probably due to the intrinsic ability of AAV5 and AAV8 and other AAV variants to efficiently target liver hepatocytes which provide the natural site of synthesis for FIX. In contrast, FVIII is produced in LSEC which are not targeted by AAV5. It also needs to be emphasized that pre-existence of antibodies directed to specific AAV serotypes used in gene therapy trials have been used as an exclusion criteria with the exception of the AAV5 based product Etranacogene Dezaparvovec (which is currently been marketed as Hemgenix)[90]. Despite its success in restoring FIX expression levels in patients with hemophilia B, uptake of gene therapy has been slow[91]. This has been attributed to pricing issues as well as concerns with respect to long-term side effects of AAV-based gene therapy such as potential genotoxicity. Based on these considerations Pfizer as decided to discontinue their FIX gene

therapy program[92] despite the fact that stable therapeutic FIX levels were achieved with a relatively low vector dosage [86,87].

Inhibitor development in hemophilia

Incidence and reaction

A major complication of current FVIII and FIX based therapies for hemophilia is the development of inhibitory antibodies in a subset of patients[1]. Replacement factor therapy is rendered ineffective when an immune response develops to FVIII or FIX. Approximately 20 to 40% of patients with hemophilia A develop inhibitors following treatment with FVIII[93,94]. Inhibitor development in hemophilia A has been linked with genetic risk factors as well as treatment-related factors[95]. Importantly, the majority of inhibitors in patients with hemophilia A develop within first 30 or 50 exposure days (ED) whereas inhibitors usually do not develop after 75 ED[96].

The incidence of inhibitors in hemophilia B patients is in the range of 1%-4%[97]. Other than developing antibodies that interfere with the treatment, hemophilia B patients can also develop allergic or anaphylactic responses following FIX infusion, the reason for this is not completely understood. In hemophilia B patients the main subclass of antibodies identified are IgG1 and IgG4. In addition, IgE have also been identified and linked to allergic reactions. The allergic reaction is probably mediated by IgE directed against FIX. One of the reason proposed for the anaphylactic is the diffusion of FIX in the extracellular space[98,99].

Risk factors and properties of inhibitors in hemophilia

F8 genotype is closely linked to the risk of inhibitor development[100,101]. Large deletions, inversion as well as nonsense and frameshift mutations are linked to increased risk for inhibitor development[100]. The increased risk of inhibitors in this group of patients is due to the absence of endogenous FVIII. Autoreactive B cells are removed from the total B cells repertoire during their maturation in the bone marrow[102]. Similarly, autoreactive T cells binding strongly to peptides derived from self-antigens are removed by negative selection in the thymus[103]. In many patients with hemophilia A endogenous FVIII is not present and therefore FVIII reactive B and T cells are not removed. Upon exposure to therapeutic FVIII, FVIII-derived peptides are presented on T helper cells and antibody-producing B cells are expanded[104,105]. The development of antibodies against therapeutic FVIII is quite complex and entails shuttling of exogenous FVIII through different cellular compartments in the spleen. In this secondary lymphoid organ, FVIII can be

captured by marginal zone macrophages (MZM), marginal metallophilic macrophages (MMM) and marginal zone B cells. These cells shuttle FVIII without processing it to dendritic cells that can present peptides derived from FVIII on majority histocompatibility complex II (MHC II) which allow for their presentation to $CD4^+$ T cells. This results in the priming of the T cells and their subsequent differentiation in T follicular helper cells. Activated $CD4^+$ T cells can stimulate FVIII specific B cells to differentiate into both memory cells and antibody secreting cells [106]. Anti-FVIII antibodies produced by antibody secreting cells recognize infused FVIII and can inhibit its function and accelerate its clearance from the circulation. The final result is that FVIII replacement therapy is not effective anymore. These antibodies are defined as inhibitors[107,108] . Particularly interesting is that FVIII seems to have intrinsic immunomodulatory properties, indeed, when other exogenous proteins are infused at the same dosage they do not follow the same trafficking pathway observed for FVIII but seem to be directed to the red pulp instead of the white pulp of the spleen and fail to result in the production of antibodies[106].

Together with F8 mutation other risk factors have been identified in hemophilia A patients and specifically are polymorphisms in MHC class II proteins but also in genes encoding proteins involved in immunomodulatory pathways such as in TNFα, CTLA4 and IL10[109–111].

Similar to hemophilia A, the majority of hemophilia B inhibitor positive patients do not express endogenous FIX due to the presence of large deletions or nonsense mutations in the *F9* gene[112]. For missense, splice site or promoter mutations the risk of inhibitor development is 0.4%; 5% of patients with nonsense and frameshift mutations develop in inhibitors. The missense variant Glu66Asp in the GLA domain of FIX has also been associated with inhibitor development[47]. Anti-FIX antibodies that develop in patients with hemophilia B are primarily directed towards the Gla domain and the serine protease domain but not the two EGF like domains[113].

Treatment of inhibitors in hemophilia

In patients, inhibitors are measured with the Nijmegen-Bethesda assay and the titer is measured in Bethesda Units (BU); patients are divided in low (<5.0 BU) or high titer (≥5.0 BU) inhibitors. Bleedings in hemophilia patients with inhibitors are treated with bypassing agents that are not as effective as FVIII or FIX concentrates. For prophylaxis, non replacement theraoy can be used in the form of bispecific antibodies in hemophilia A (Emicizumab, Denecimig) and rebalancing agents in hemophilia B (Marstacimab, Concizumab)[68,69,72]. During bleeds, patients with low titer inhibitors can be treated with high doses of FVIII or FIX[114,115].

The only method until now to eradicate inhibitors is immune tolerance induction (ITI). This method consists of administration of high doses of either FVIII or FIX at relatively short intervals. Patients with high inhibitors titer have been subjected to this therapy since the ’70, and, overall, the treatment has been successful for two third of the hemophilia A patients while this number is lower for hemophilia B patients[116,117]. Currently, the precise mechanism by which ITI results in eradication of immune responses remain elusive mostly due to the lack of adequate model systems and the limited availability of patient samples[106]. Overall, ITI still leaves a lot of unknows that, combined with the high burden on patients and the high costs, makes prophylaxis employing non replacement therapies such as emicizumab and concizumab the preferred treatment for patients with inhibitors[116–118].

Thesis aim and outline

One of the main unmet challenges for hemophilia care is the development of antibodies against either FVIII or FIX. Our current knowledge as to why anti-drug antibodies develop in a subset of patients is incomplete. More knowledge on this topic is needed since many patients that are currently treated with non-factor replacement therapy receive FVIII for treatment of breakthrough bleeds as well as during surgery[119]. This thesis explores the uptake, processing and presentation of coagulation factor FVIII and FIX and provides examples of innovative delivery systems that may be utilized for inducing and/or restoring tolerance. The current chapter **(Chapter 1)** offers an overview on hemophilia A and B in conjunction with the development of anti-drug antibodies.

Chapter 2 provides a review on the properties of liver sinusoidal endothelial cells (LSEC) that are primarily responsible for the production of FVIII. LSEC express numerous scavenger receptors on their surface that interacts with plasma proteins as well as viruses. Additionally, the cells can also act as antigen presenting cells due to their ability to present antigen derived peptides on MHC class I and class II. Importantly, LSEC have been suggested to promote tolerance by a variety of mechanisms[120].

In **Chapter 3** the focus is on a stabilin-2, a scavenger receptor that is highly expressed on LSEC. In this Chapter we show that stabilin-2 can bind to FVIII. Moreover, we characterize the kinetics of binding between these two proteins and we show that the Link domain of stabilin-2 contributes to the binding of FVIII. These findings are in accordance with previous literature that revealed that LSEC are involved in the clearance of FVIII. In view of the synthesis of FVIII by LSEC our findings may suggest a feedback loop for the production and clearance of FVIII.

In **Chapter 4** we explore a novel way to induce tolerance to FVIII, delivering it to antigen presenting cells in a tolerogenic manner. We developed novel recombinant proteins that contain both the A2 domain as well as the light chain and the C2 domain of FVIII as well as the phosphatidylserine binding protein Annexin A5. We used red blood cells as a model to illustrate how FVIII-AnnexinA5 fusion proteins can bind to phosphatidylserine exposing cells and can subsequentially be endocytosed and processed by macrophages. Using mass-spectrometry we demonstrated that macrophages can present FVIII peptides derived from FVIII-AnnexinA5 fusions on HLA-DR. Phosphatidylserine targeting FVIII-AnnexinA5 fusion proteins provide an interesting new potential strategy for the induction of tolerance to FVIII in hemophilia A patients.

Chapter 5 focuses on the identification of FIX derived peptides presented by antigen presenting cells, in order to better understand the mechanisms that contribute to inhibitor development in hemophilia B. FIX was incubated with monocyte derived dendritic cells and peptide presented on HLA-DR were identified by mass-spectrometry. Interestingly, we found that one peptide derived from the protease domain of FIX was promiscuously presented on MHC class II. We speculate that this particular FIX derived peptide contributes to the onset of inhibitor development in patients with hemophilia B.

FVIII inhibitors form immunocomplexes with FVIII, in **Chapter 6** we explore the binding of a patient derived monoclonal antibody to Fc receptors. We show that following modification of six amino acids enhance its binding to FcγRIIb. The potential outlook of this research is a FcγRIIb-engagement induced switch in the response of dendritic cells from an inflammatory to a more tolerogenic phenotype. We also document the binding of FVIII-Fc to the different classes of human Fc receptors.

In the final part of the thesis, **Chapter 7**, we discuss the results obtained in the context of current challenges for treatment of patients with hemophilia. In the last twenty years many advances have been made with respect to treatment of hemophilia but not all questions have yet been fully addressed. In this part of the PhD thesis we highlight remaining questions on the role of LSEC and stabilin-2 in the clearance as well as inhibitor development in hemophilia A. Furthermore, we discuss current knowledge gaps with respect to inhibitor development in hemophilia B. Furthermore, we provide an overview of innovative strategies that could potentially be used to further improve treatment of hemophilia patients with inhibitors.

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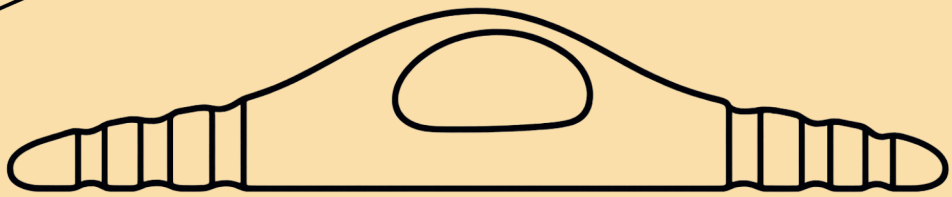
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Chapter 2

Liver sinusoidal endothelial cells provide a highly developed exchange platform in the liver promoting clearance and tolerance



Michela Leoni and Jan Voorberg

Manuscript in preparation

Abstract

Liver sinusoidal endothelial cells (LSEC) provide a highly efficient exchange platform that, due to their characteristic fenestration, allows for transport of metabolites and proteins from blood to the Space of Disse, providing access to hepatocytes and hepatic stellate cells. The primary role of LSEC is to capture ligands from circulating blood through endocytosis mediated by a large and diverse repertoire of scavenging receptors. Their unique endocytotic properties are emphasized by their ability to process internalized antigens and present antigen derived peptides on major histocompatibility class I and II. The specific microenvironment, the expression of immune regulatory receptors such as PD-L1 and low levels of co-stimulatory molecules like CD80 and CD86, position LSEC as an unique antigen presenting cell which promotes the formation of regulatory CD4+ T cells. A unique hallmark of LSEC is the expression of the inhibitory FcγRIIb receptor, which scavenges circulating antibody-antigen complexes, thereby preventing immune activation by reducing the binding to high affinity activating FcγR's on macrophages and other immune cells. The synthesis of blood coagulation factor VIII (FVIII) provides a unique specialized feature of LSEC which is not shared with other populations of endothelial cells. The highly specialized role of LSEC in liver regeneration, immune regulation and clearance of blood derived proteins positions them as an unique population of organ specific endothelial cells. Their unique functional properties and receptor signature allows for the development of targeted therapies that are not only limited to immune modulation but also involve gene-editing as well as supplementation approaches to restore synthesis of FVIII.

Introduction

Oxygen rich blood enters the liver via the hepatic artery whereas the portal vein transport nutrients retrieved from the digestive tracts[1]. The liver is organized in hepatic hexagonal lobules which are highly vascularized, thereby allowing for efficient provision of nutrients and oxygen to the hepatic tissue. These so-called hepatic sinusoids provide an intricate network of small vessels within the hepatic lobules and transport blood to the central hepatic vein (Figure 1). Exchange of nutrients and clearance of potentially harmful compounds from blood are mediated by liver sinusoidal endothelial cells (LSEC) which provide a highly specialized liver-specific population of endothelial cells that are equipped with unique functional properties. LSECs lack a basal lamina, this is essential for their filtering capability, permitting direct access to the Space of Disse, allowing for direct exposure of blood components to microvilli of hepatocytes, hepatic stellate cells (HSC), which control liver homeostasis and store vitamin A in lipid droplets[2] , and Kupfer cells (KC), a subpopulation of macrophages that resides in the liver. LSECs line the hepatic sinusoids and, together with KCs, phagocytose portal blood residing pathogens and toxins, providing a first layer of protection for the liver. Even though the volume of LSECs is only 3% of the total volume of the liver, their specific morphology makes them cover a surface area that has been calculated to be of around 210 m^2 in an average human liver[3–5]. Single cell transcriptomics has provided evidence for a zonal distribution of different LSEC populations along with the graded microenvironment created by the liver microvasculature linking the portal vein and hepatic artery towards the central vein (Figure 1C)[6–8]. In addition, an unique microRNA signature that distinguish them both by other liver cells and also other endothelial cells, has been identified for LSEC[9]. One of the fundamental characteristics of LSECs morphology is their fenestration, this cells type has fenestrations of 100-150 nm in diameter. The fenestrae allow for the passage of small and medium size components from the capillary to the Space of Disse, this is further facilitated by the absence of basal lamina[10]. The fundamental cytokine controlling fenestration is bone morphogenic protein 9 (BMP9), in KO mice the absence of BMP9 leads to a loss of fenestration already in 2-week-old pups and later in life leads to LSECs capillarization, fibrosis and inflammation[11]. Possibly BMP9 acts by regulating the expression of plasmalemmal vesicle associated protein (PLVAP) which controls fenestration[12]. GATA4 is also a target of BMP9, this is particularly important since GATA4 has been previously identified as master transcription factor for LSECs differentiation[13,14]. During liver development GATA4 has been shown as responsible of the discontinuous nature of LSECs, this is necessary for hepatic stellate cells transmigration during fetal development[15]. Lack

of fenestration in LSECs leads to impaired passage of macromolecules, accumulation of chylomicron remnants but also an increase in plasma levels of LDL and eventually results in liver damage[14]. The morphology of LSECs is fundamental for their function but it is quickly lost *in vitro*, cells in fact switch to a cobblestone morphology and towards a confluent endothelial cell phenotype[16]. This mirrors what happens *in vivo* during liver cirrhosis and hepatocellular carcinoma[16]. One of the genes identified as important in this process is Ehd3, usually overexpressed in LSEC, and responsible for trafficking of Stabilin-1. Wtn-2 is another gene that is down-regulated during culture *in vitro* and that has been shown to be a positive regulator of VEGF receptors[17]. VEGF-A and its stimulation of nitric oxide-dependent signaling has been shown to be necessary for maintenance of LSEC differentiation. LSECs loss of differentiation is followed by activation of HSCs and onset of fibrosis[18]. Expression of CD31 (PECAM-1) in cell to cell junctions is also a symptom of de-differentiation, indeed when cells still show fenestrations CD31 is expressed in the cytosol of the cells, while it migrates towards the cells-cells junctions once cells are cultured *in vitro* in a similar manner to other types of endothelial cells. De-differentiation signal are provided in a paracrine manner by both hepatocytes and HPCs, one of the signals is VEGF[19]. TSP-1 levels have also been shown to have a role in LSECs differentiation and defenestration, this works both by paracrine and autocrine pathways since TSP-1 can be released both by HSCs and immature LSECs[20]. Loss of fenestration in LSECs could also interfere with AAV gene therapy, due to the decrease in permeability of LSECs and impaired transfer of AAV to hepatocytes[21].

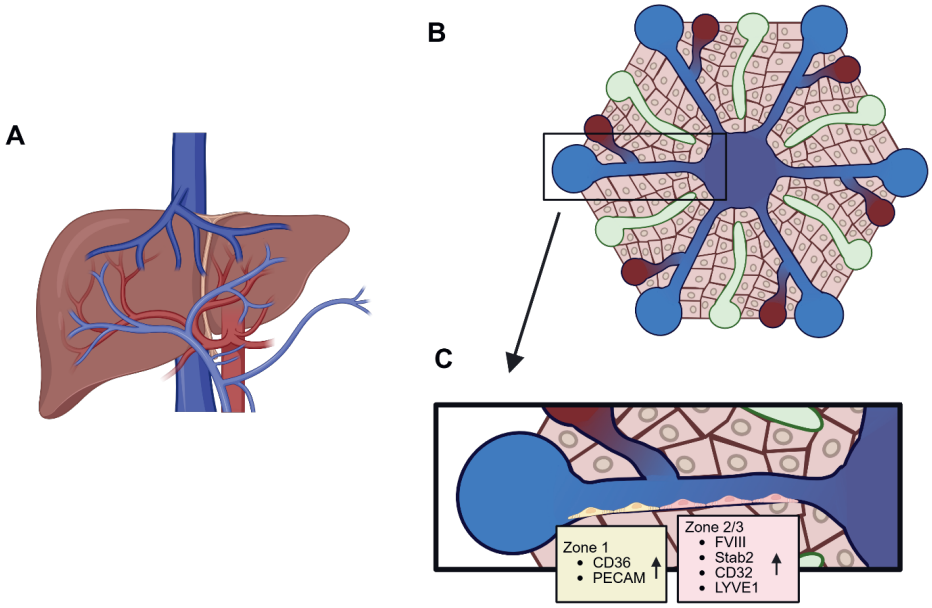


Figure 1. Zonal distribution of LSEC in the liver. (A) Schematic overview of different blood vessels in the liver. The portal vein (shown in dark blue) and hepatic artery (shown in red) provide oxygen rich blood to the liver. The portal vein also transports nutrients from the gastro-intestinal tract to the liver. Blood from the portal vein and the hepatic artery enters the liver sinusoids and is collected in the central vein (shown in dark blue). (B) Schematic overview of a lobe of the liver showing hepatocytes surrounding the bile duct. Liver sinusoids are lined by sinusoidal liver endothelial cells that due to their fenestrae and lack of basement membrane allow for access of oxygen and nutrients to the Space of Disse. (C) Zonal distribution of LSEC in the liver. Zone 1 LSEC express scavenger receptors like CD36 whereas zone 2/3 LSEC express FVIII, CD32, LYVE1 and stabilin-2.

Scavenging roles of LSEC

The endocytic capacity of LSECs is well recognized and characterized, LSECs are more efficient in antigen uptake compared to hepatic dendritic cells, spleen macrophages or Kupffer cells[22–24]. The high endocytic activity of LSECs is due to the presence of multiple scavenger receptors on their surface[25]. Scavenger receptors are cells surface membrane proteins involved in the promiscuous binding of a wide range of ligands that include blood derived proteins, but also microbial pathogens, leading to their uptake and elimination. Currently, scavenger receptors are divided into 8 main classes (from SR-A to SR-H) plus 4 additional ones

that contains proteins with scavenger receptor like activity (SR-I to SR-L)[26]. Of these, LSECs express under physiological conditions SR-A (SCARA1), SR-B1, SR-B2 (CD36), SR-E3 (mannose receptor/CD206), SR-H1 (stabilin-1/CLEVER/FEEL-1), SR-H2 (stabilin-2/HARE/FEEL-2) and FcγRIIb. Endocytic processes involving the scavenger receptors, with some exceptions, utilize clathrin-dependent pathways as opposed to the caveolae-mediated endocytosis[27,28].

SR-A is expressed in macrophages, Kupffer cells, brain microglia, astrocytes, vascular smooth muscle and LSECs. In LSECs, SR-A is mainly responsible for the uptake of acetylated LDL (acLDL) but also oxidated LDL (oxLDL)[29]. Apart from ac/oxLDL, SR-A is also able to bind to other ligands such as advanced glycation end (AGE) products; studies in SR-A deficient mice show that the removal of these compounds is not exclusively dependent on SR-A[30].

Both SR-B1 and SR-B2 (also called CD36) are expressed at high levels in LSECs, where they co-localize with caveolin on the membrane, this suggests that endocytosis of SR-B1 and SR-B2 is mediated via a caveolin-dependent pathway in LSECs[31,32]. SR-B1 is known to be an HDL receptor whereas SR-B2 contributes to lipid metabolism through the uptake of long-chain fatty acid from the circulation[33,34]. Class B type I scavenger receptor, SR-B1 is responsible for the binding to HDL, this receptor has a large extracellular domain and is anchored on the cell membrane by two transmembrane domains that are flanked by small N- and C-terminal cytoplasmic domains[34,35]. SR-B1 was believed to be mostly expressed in hepatocytes but more recent studies established that SR-B1 is primarily expressed on the LSECs surface[31]. SR-B1 is involved in HDL clearance but can also bind to LDL, VLDL oxLDL and apoptotic cells, in addition in LSECs it is believed to mediated LPS clearance[36,37]. CD36 is a class B scavenger receptor and in LSECs is involved in the uptake of HDL but also binds to LDL, VLDL and apoptotic cells; it does not have a role in the metabolism of AGE modified proteins in LSEC[38,39].

SR-E1 (also known as LOX-1) is the main responsible for the uptake of oxLDL but also has a variety of other ligands such as apoptotic cells, C-reactive protein, phosphatidylserine, platelets, bacteria[40]. SR-E3, known as mannose receptor, belongs to the C-type lectin family. Mannose receptor has an important role in the uptake and degradation of denatured collagen[41]. In general, SR-E3, has numerous other ligands such as tissue plasminogen activator, lysosomal hydrolases but also pathogens[42,43].

Two prominent receptors present on the surface of LSEC are stabilin-1 and stabilin-2, also called FEEL1, CLEVER-1 (stabilin-1) and FEEL-2, HARE (stabilin-2)[22].

Both stabilin-1 and stabilin-2 are responsible for the uptake of oxLDL but also of several other macromolecules. Stabilin-2, specifically, can bind hyaluronan and AGE containing proteins[28]. Both stabilin-1 and stabilin-2 are involved in the uptake of oxLDL, while highly oxidized LDL aggregates are taken up by both KCs and LSECs, mildly oxidized LDL, the more physiological low molecular weight forms of mildly oxLDL are selectively endocytosed by LSECs most likely through preferential binding to stabilin-1[44]. Long term treatment with LDL can induce senescence in endothelial cells in vitro, this is at least partly due to ROS production after LDL endocytosis via LDLR and signal transduction via both the p53 and p16-pRb pathways[45]. Both stabilin-1 and stabilin-2 can also bind phosphatidylserine (PS) and have, therefore, been implicated in phosphatidylserine mediated clearance of red blood cells[46]. Stabilin-2 is expressed in the spleen, liver, lymph node and at lower levels in placenta, skeletal muscle, pancreas, thymus, heart and bone marrow[47]. One of the main ligands of stabilin-2 is hyaluronic acid; stabilin-2 was originally identified as hyaluronic acid receptor for endocytosis (HARE). The specific domain that can bind hyaluronan is the Link domain; homologous Link domains are also present in CD44 and TSG-6[48]. Stabilin-2 is present in LSECs in two isoforms, a 190kDa and 315 kDa isoform, it has been shown that proteolytic processing of full-length stabilin-2 generates lower molecular weight form of 190 kDa[48–51]. Stabilin-2 is also able to bind FVIII and VWF, offering a possible interestingly mechanism for the regulation of both FVIII production and clearance by LSEC[52]. Apart from stabilin-2, LSEC express a wide variety of clearance receptors which includes CLEC4M[22]. Interestingly, CLEC4M has also been shown to bind to von Willebrand factor (VWF), both in its free form but also in complex with FVIII; this interaction seems to be dependent on the presence of N-glycans on the FVIII-VWF complex[53,54].

Role of LSEC in immunity and tolerance

The high endocytic capacity ideally position LSEC as professional antigen presenting cells[10]. Similar to other populations of endothelial cells LSEC express MHC class I which allows for presentation of peptides derived from processing of viral and also soluble antigens through cross-presentation to CD8+ T cells[10]. In contrast to other populations of endothelial cells, LSEC constitutively express MHC class II which allows for peptide presentation to CD4+ T cells[10]. Importantly, MHC class II presentation has been proposed to result in the conversion of naïve CD4+ T cells into regulatory CD4+ T cells[10]. Crucial for the induction of stable populations of Treg, is the exposure to TGF-beta, that LSEC expose in a latent conformation bound to Glycoprotein A repetition predominant (GARP)[55,56]. Latent TGF-beta is

generated by furin mediated cleavage of its precursor pro-TGF-beta once it is bound to GARP in the Golgi apparatus; the amino terminal latency inducing chain (LAP) remains connected to the carboxy-terminal chain thereby keeping TGF-beta in a latent conformation[57,58]. Once mature, GARP localizes latent TGF beta on the cells surface, TGF-beta is released following binding of the GARP/LAP complex to integrin αVβ8. In LSEC, GARP localizes TGF-beta, it is not clear yet if the TGF-beta is synthesized by LSEC or derived from the hepatic microenvironment. The large endocytic capacity of LSEC allows for prolonged exposure to antigen specific CD4+ T cells that interact with peptides originating from LSEC processed antigens that are presented on MHC class II (Figure 2). The combination of these two signals allows the differentiation of naïve T-cells, TGF-beta binds to its receptor on the T cell surface, the signal is transduced by the SMAD proteins that drive de-methylation of the promoter region of Foxp3 and allow for the transcription of this master regulator of Treg induction[56–60]. The expression of the programmed death ligand 1 (PD-L1) on the surface of LSEC provides an inhibitory signal for conversion of naive (antigen specific) CD4+ T cells to an effector CD4+ T helper phenotype, thereby promoting formation of antigen specific Tregs[61,62]. The lack of co-stimulatory receptors such as CD80/CD86 and others, which provide crucial signals for activating CD4+ as well as CD8+ T cells through cognate binding to CD28, further promote the generation of CD4+ (as well as CD8+) T cells with a regulatory phenotype[62]. The metabolic profile of LSEC allows for the generation of an immunosuppressive microenvironment, created by its ability to release IL-10, retinoic acid and downregulation of the generation of metabolites of the tryptophan pathway through expression of indolamine 2,3-dioxygenase (IDO)[63]. LSEC express the inhibitory Fc gamma receptor IIb (CD32; FcγRIIb) a low affinity receptor for immunoglobulin-containing immune complexes that, by virtue of the its immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail, allows for activation of tyrosine phosphatases, like SHIP-1, to interfere with tyrosine phosphorylation of activating receptors[27,64]. High levels of CD32 on LSEC contribute to its endocytic profile and have been proposed to result in the clearance of small size immune complexes[65].

Additionally, LSEC also influence the effect of neighbor cells to activate CD8 T cells. In the hepatic sinusoid also dendritic cells are present but upon physical contact with LSEC they seem to lose specifically the ability to prime naïve CD8 T cells. The dendritic cells are still able to process antigens and present them on MHC class I, the cells can also stimulate already activated CD8 T cells. This action was lost as soon as the dendritic cells were not in physical contact with LSECs[66]. Using the same mechanism of CD8 T cell interaction, LSEC could also present circulating

tumor antigens on MHC class I and lead to development of CD8 T cell tolerance. This represent an additional modality of tumor immune escape[67].

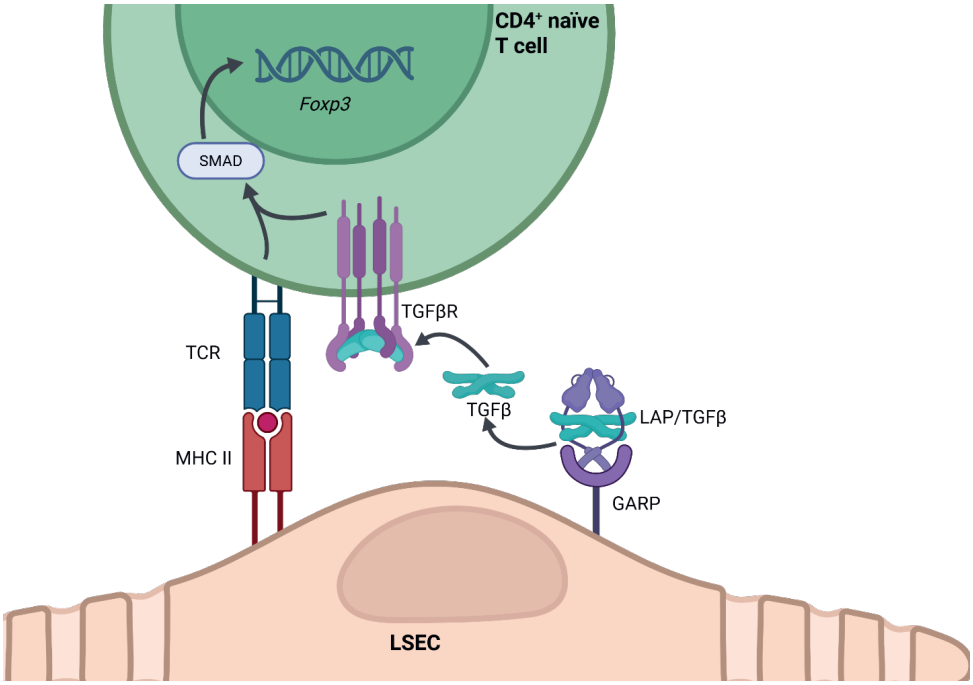


Figure 2. LSEC promote formation of regulatory T cells. GARP (short for Glycoprotein A repetition predominant) on the surface of LSEC binds active TGF-beta in complex with the latency inducing chain (LAP) of TGF-beta. Binding of GARP/TGF-beta complex to integrin αVβ8 results in release of active TGF-beta which binds to the TGF-beta receptor on CD4+ T cells where it is induces SMAD signaling finally resulting in expression of the Treg specific transcription factor Foxp3.

FVIII synthesis by LSEC

Single cell transcriptomics have shown zone-specific expression profiles of LSEC that differ based on their proximity to the portal and central vein[7,68]. Zone 1 LSEC located in proximity to the portal vein express proteins involved in angiogenesis and metabolic processes whereas LSECs in zones 2 and 3 are enriched in scavenging receptors like Lyve-1, CD32B and ficolin-3 (Figure 1C)[7,68]. Early studies by the Lollar group have shown that LSEC cells express FVIII[69]. Originally, hepatocytes were proposed to be the major site of FVIII synthesis. An elegant study however revealed that while endothelial cells transplant can correct bleeding phenotype in mice, hepatocytes transplants can not[63]. This finding was

confirmed in a follow-up study which demonstrates production of human FVIII in a immunodeficient uPA deficient mice upon transfer of human LSEC transplant in mouse followed by human FVIII detection[62]. Employing an in vivo approach Fah and co-workers and Everett and co-workers employed conditional mouse models to show that endothelial cells are the primary site of FVIII synthesis[61,70]. In an elegant approach LSECs generated from iPSCs were shown to synthesize FVIII[71]. Studies by Singer and co-workers and the Pratt-laboratory showed that LSEC derived from iPSC of patients with hemophilia A display absence of FVIII expression and/or altered biosynthesis of variant FVIII[72]. Interestingly, human pluripotent stem cell derived LSECs progenitor were shown to correct the bleeding phenotype in mice when engrafted in an immune-compromised hemophilia A mouse model[73]. Altogether these findings have provided overwhelming evidence that LSECs provide a major site of FVIII synthesis. A recent study employed single cells analysis to confirm that FVIII is synthesized in LSEC[74]. Analysis of zone-specific expression revealed that FVIII transcripts were primarily detected in zone 3 which is localized immediately adjacent to the central vein of the haptic lobules[74]. A set of microRNAs has been identified (miR-122-5p, miR-214-3p, miR-204-3p, and miR-2682-5p) as having a crucial role in the regulation of F8 transcription both acting through suppression but also on F8 transcription factors[75]. The same study also underlays how the FVIII expression was not the same in all LSECs cells but quite different, this is probably correlated to the position of the cells in the organ[9,75]. Loss of endothelial fenestration and capillarization also affect FVIII production[21].

The unambiguous localization of FVIII in LSEC has promoted efforts to explore the potential of LSEC directed gene therapy for hemophilia A. In a recent study it was shown that a lentiviral vector co-expressing the baculo-virus derived surface protein GP64 combined with CD47 (which blocks entry into Kupfer cells and other macrophage populations) efficiently transduces LSEC in vivo resulting in therapeutic levels of FVIII in mouse models for hemophilia A[76]. In addition, lipid nanoparticles specifically targeting LSEC have recently been explored for CRISPR-Cas9 mediated gene correction in hemophilia A mice[77]. In a separate approach the promoter of stabilin-2 has been used in lentiviral vector-mediated expression of FVIII to target LSECs specifically for the expression of the protein and the result was the successful and durable recovery of the hemophilia A phenotype in mouse. An additional potential benefit of specifically targeting this cell type is the tolerogenic nature of the cells, indeed mice expressing the transgene didn't develop anti-FVIII antibodies[78].

Apart from FVIII synthesis, LSEC have also been shown to contribute to the clearance of FVIII as well as the FVIII-VWF complex[79] . Single nucleotide polymorphisms in the clearance receptors CLEC4M, SCARA-5 and stabilin-2, which are expressed on LSEC, are associated with circulating levels of FVIII and VWF in the CHARGE study[80]. Based on biochemical data as well as in vivo studies, SCARA5 was suggested to have a limited role in clearance of FVIII and/or the FVIII/VWF complex[81]. Also, the glycan receptor CLEC4M was shown to bind to FVIII both a VWF dependent as well as VWF independent manner[53]. In line with the original observations reported in the CHARGE study stabilin-2 was shown to contribute to the clearance of the FVIII-VWF complex[82]. Interestingly, a reduced immune response to FVIII was observed in stabilin-2 deficient mice. These findings suggests that stabilin-2 mediated internalization modulates immunogenicity of the FVIII/VWF complex[52].

Perspective; Future outlook

LSECs are a complex cell type that thanks to their localization, the morphology and the proteins on their surface make them fundamental in the control of the immune response against antigens that circulate in the body. Additionally, thanks to their ability to produce FVIII but also to the presence of receptors that are able to interact with it on the surface, LSECs also play a relevant role in hemostasis. Mutations in any of the proteins on the surface of these cells could cause defects in the clearance of specific antigens, the redundance of some of those receptors could probably be explained as a way to ensure adequate clearance of a wide array of ligands that include oxidized lipids, pathogens and apoptotic cells. Studies on LSECs are currently hampered by their rapid de-differentiation in in vitro cultures. Single cell sequencing has allowed for recognizing different LSECs population with different functional properties in the liver. Recently, protocols for the differentiation of LSEC starting from induced pluripotent stem cells (iPSC) have been successfully developed. This opens the possibility of analyzing LSEC derived from specific patient groups. Additionally, gene editing of iPSC would allow for functional studies on this enigmatic population of fenestrated endothelial cells in the liver.

Author contributions.

ML and JV wrote the manuscript. ML prepared the figures.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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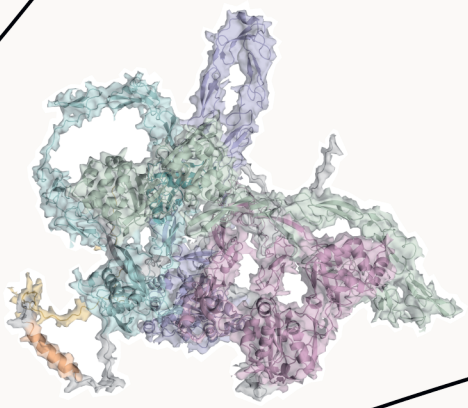
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Chapter 3

Binding of blood coagulation factor VIII to stabilin-2



Michela Leoni*, Zsolt Szeles*, Paul Kaijen, J. Arnoud Marquart, Karin Fijnvandraat and Jan Voorberg, on behalf of the EDUC8 consortium.

*These authors contributed equally to this work and share first authorship

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Abstract

Background: Promiscuous binding of blood coagulation factor VIII (FVIII) to multiple cell surface receptors promotes its clearance from the circulation as well as its ability to interact with different populations of antigen presenting cells.

Objectives: In this study we focused on binding of FVIII to stabilin-2 which is preferentially expressed on liver sinusoidal endothelial cells (LSEC). Here, we set out to determine which domains of stabilin-2 are involved in binding to FVIII.

Methods: A truncated version of stabilin-2 lacking the transmembrane and cytoplasmic domain was expressed in ExpiCHO cells. The truncated stabilin-2 protein was secreted into the medium and purified employing a carboxy-terminal Strep-Tag. We also expressed the isolated Link domain in ExpiCHO cells. Inclusion of a spacer region containing 3 intramolecular disulfide bonds was required for expression of the Link domain of stabilin-2.

Results: Using an ELISA based set-up it was shown that FVIII binds with high affinity to the extracellular and the Link domain of stabilin-2. We subsequently performed surface plasmon resonance to analyze the kinetics of binding of the extracellular domain of stabilin-2 to FVIII. A rapid association of stabilin-2 to immobilized FVIII was observed followed by a relative slow dissociation phase. Kinetic analysis suggested the presence of multiple binding sites of FVIII on stabilin-2. Surface plasmon resonance revealed the presence of a single high affinity binding site for FVIII in the Link domain of stabilin-2.

Conclusion: Our results show that FVIII binds to stabilin-2 and this binding is partly mediated via its Link domain.

Keywords: factor VIII, stabilin-2, hemophilia A, FVIII inhibitors, endocytosis, clearance

Introduction

The X-linked bleeding disorder hemophilia A is caused by mutations in the gene encoding blood coagulation factor VIII (FVIII)[1]. Multiple treatment options are currently available for prevention and treatment of bleeding symptoms in patients with hemophilia A [2,3]. Classically, replacement therapy composed of intravenous administration of FVIII concentrates is being used to correct the bleeding tendency in patients with hemophilia A. This approach is limited by the relatively short half-life of FVIII which requires frequent administration of FVIII [4] . Over the past decades, several bioengineered FVIII variants have been designed with improved circulatory half-life which include the recently developed Efanesoctog alfa or BIVV001. BIVV001 is a bioengineered FVIII variant which contains the D'D3 domains of von Willebrand factor (VWF) fused to a Fc portion that is linked to the C2 domain of FVIII [5]. Additional modifications include two XTEN-polypeptides that increase the hydrodynamic radius of BIVV001 which may further help in increasing its circulatory half-life [5]. Despite the pronounced effects on FVIII half-life, it has not yet been established whether BIVV001 has the potential of reducing the risk of inhibitor development in previously untreated persons with hemophilia [6]. FVIII independent products have further revolutionized current strategies for treatment of patients with hemophilia A [3]. In particular the bispecific antibody emicizumab is now widely used for the treatment of patients with hemophilia A [7]. Once weekly or even bi-weekly subcutaneous administration of emicizumab has significantly reduced the therapeutic burden of hemophilia care [7]. In view of the limited efficacy of emicizumab when compared to FVIII, in promoting factor Xa generation, FVIII is still required for treatment of breakthrough bleeding and in case of trauma or surgery [8]. In the context of acute bleeds which often coincide with tissue damage exposure to FVIII may provoke inhibitor development [9].

Exposure to FVIII under conditions of trauma and surgery may present with an increased risk of inhibitor development [10]. This has raised the question of whether patients receiving emicizumab would need to be tolerized by frequent administration of low dosage of FVIII [9]. Classically, patients who developed inhibitors are treated through immune tolerance induction (ITI) therapy which comprises frequent administration of high dosages of FVIII [8]. Currently, ITI is initiated once inhibitors have developed in patients with hemophilia A. The current protocol for ITI is composed of frequent administration of high dosages of FVIII that induce tolerance through mechanism that are incompletely understood [11]. Further improvement of protocols for tolerance induction requires detailed knowledge on the interaction of FVIII with antigen presenting cells as well as FVIII specific B cells and T cells [11].

The FVIII-VWF complex is cleared by macrophages in the liver and spleen [12–14] primarily through the low density lipoprotein receptor LRP-1 (low density receptor protein-1) [15,16] but there is also evidence for the involvement of macrophages receptors SR-A1 (scavenger receptor), MGL (macrophage galactose-like lectin) and Siglec-5 in this process[17–19].

Other than hepatocytes and macrophages, the VWF-FVIII complex has been reported to be cleared by liver sinusoidal endothelial cells (LSEC). Specifically, it has been associated with three surface receptors: SCARA-5 in the spleen and CLEC4M and stabilin-2 in the liver[20]. The presence of clearance receptors that can endocytose FVIII in liver sinusoidal cells is particularly interesting since these cells are also the main site of FVIII synthesis [20–23]. CLEC4M can bind VWF both when it is in complex with FVIII and when it is not but this is significantly attenuated when N-linked glycans are removed from the complex [24,25]. This interaction however is difficult to study in vivo because of the absence of an orthologous protein in mouse [26,27]. SCARA-5, expressed on splenic sinusoidal endothelial cells, can also bind to the FVIII-VWF complex in a VWF dependent manner [20,28,29].

Stabilin-2 is a transmembrane receptor that, in humans, is encoded by the gene STAB2 located in chromosome 12. According to single cell transcriptomic data in mouse, stabilin-2 is located on liver endothelial cells, spleen and partly in the testis [30]. Stabilin-2 has been confirmed to be expressed principally in spleen, liver, lymph node in humans and at lower levels in heart, placenta, skeletal muscle, pancreas, thymus, and bone marrow [31]. One of the principal ligands of stabilin-2 is hyaluronic acid, from which it also takes one of its first names, HARE, hyaluronic acid receptor for endocytosis [32]. Stabilin-2, together with stabilin-1, is also involved in the phosphatidylserine (PS) mediated clearance of red blood cells in the liver [33]. Stabilin-2 is composed of 16 epidermal growth factor (EGF) like and 7 fasciclin (FAS1) domains. In addition, stabilin-2 contains a so-called Link domain, which is composed of two α-helices and two antiparallel β-sheets that cover a hydrophobic core. This structural feature is commonly present in C-type lectins and is consistent with the known ability of the Link domain to interact with hyaluronan [34–36] In this study we explored the contribution of the different domains of stabilin-2 in binding FVIII. We also defined binding characteristics of FVIII to different stabilin-2 domains using surface plasmon resonance. The current work provides insight into the binding mechanism of FVIII to stabilin-2, thereby contributing to further elucidating the pivotal role of stabilin-2 in the clearance of FVIII. The results of the current study may also help to gain insight into the

proposed role of stabilin-2 in antigen presentation of FVIII in the context of inhibitor development in hemophilia A.

Materials and methods

Molecular cloning of stabilin-2 subunits

The plasmid containing human stabilin-2 cDNA (gene STAB2) was kindly provided by Prof. Dr. Julia Kzhyshkowska (Heidelberg University)[31]. The sequence contains the SNV rs7306642 that encodes the polymorphism P2039T (frequency in global population 7%)[37]. The extracellular domain of stabilin-2 and the Link domain were amplified using AccuPrimeTM Pfx (ThermoFisher) and primers GTTCCACCGGTGACCCGGAGCAGCTGCCCATT, GTGACCTCGAGCCCCACTGCATGAGAAGCCA, GTTCCACCGGTGACGTAGGGGTTCGAGATTGCAG and GTGACCTCGAGCGGGTGCTTTTAAAGGCCTGG. The fragment was inserted in pcDNA 3.1 vector (+) containing the murine immunoglobulin kappa light chain signal peptide (METDTLLLWVLLLWVPGSTG) and a V5 plus Twin-Strep-Tag using the restriction sites inserted in the primers, AgeI and XhoI [38]. The expression construct of extracellular domain (EC-Stab2) encodes for amino acids Val66 to Pro2449. For the Link domain two expression constructs were generated: X-Link-Stab2 and Link-Stab2. X-Link-Stab2 encodes for amino acids Gly2198 to Arg2292. A second extended Link domain construct, Link-Stab2, was designed based on the known three-dimensional structure of the Link domain of CD44 [39] and included Pro2166 to Gly2312 of stabilin-2. The expression construct of the CD44 Link domain (Link-CD44) encodes for the amino acids Ala19 to Val178.

Expression and purification of the proteins

ExpiCHO cells were cultured and transfected at 37°C, 5% CO2, 125 rpm, according to instructions of the manufacturer (ThermoFisher) using 0.8 µg of DNA per mL of culture. Twenty hours post transfection, enhancer and feed were added and a temperature shift was induced by incubating the cells at 32°C, 5% CO2, 120 rpm at this time-point. Cell density and viability was monitored throughout the experiment. Cell culture supernatant was collected by centrifugation for 30 minutes at 4000 g, 7 days after transfection. A final concentration of 10 mM benzamidine was added to the supernatant and aliquots were stored at -30°C until use. Expression was confirmed through Western blot using anti-V5-HRP (1:15000; Invitrogen, R961-25). Proteins were purified using StrepTrap XT prepacked chromatography columns (Cytiva) installed in the AKTA pure (GE Healthcare) purification system. Culture supernatant was filtered through 5.0 and 0.45 µm

filters (Durapore, Merck) and loaded on the StrepTrap XT column. Following loading, the column was washed employing a buffer composed of 100 mM Tris-HCl, 150 mM NaCl (pH 8.0). Bound protein was eluted using 100 mM Tris, 150 mM NaCl, 50 mM biotin pH 8.0); eluted fractions were dialyzed to Hepes buffer (25 mM HEPES, 150 mM NaCl, pH 7.4). The protein concentration was measured by Bradford method using the Protein Assay kit (Bio-Rad), following the manufacturer's instructions. Purified proteins were snap frozen and stored at -80°C. The purified Link domain was deglycosylated using the Protein Deglycosylation Mix II (New England Biolabs) under non-denaturing reaction conditions according to the instructions of the manufacturer. To check the efficiency of the reaction, the starting material and the product was analyzed with Western blot using anti-V5-HRP (1:15000).

Binding of FVIII to EC-stab2, Link-stab2, and Link-CD44

We used 96 wells Nunc MaxiSorp plates (ThermoFisher) that were coated with 5 µg/mL of EC-Stab2, Link-Stab2 or Link-CD44 diluted in coating buffer (0.05 M $NaHCO_3$, pH 9.8) overnight at 4°C. Plates were washed five times with PBS-0.1 % Tween-20. The plates were incubated with blocking buffer (20 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 2 % BSA, pH 7.4) for one hour at 37°C. A range of FVIII (Takeda) concentrations (0 – 15 nM) were pre-incubated together with a fixed concentration of CLB-CAg117-HRP (0.25 µg/mL) in incubation buffer (20 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 0.5 % BSA, 0.05 % Tween-20, pH 7.4) for 30 minutes at 37°C. The plates were washed, then, the FVIII-CLB-Cag117-HRP complexes were added to the plates and incubated for 1.5 hour at 37°C. The plates were washed five times; after the last washing step, bound CLB-CAg117-HRP was visualized employing TMB substrate solution (0.1 mg/mL TMB in DMSO, 0.11 M sodium acetate, pH 5.5, 0.0045% H_2O_2). The reaction was stopped after 8 minutes by the addition of 100 µL 1 M H_2SO_4.

Competition assays with dextran sulfate (Sigma-Aldrich, D8906), heparin (Sigma-Aldrich, H4784), hyaluronan (Abcam, ab143634) and monoclonal anti-FVIII antibodies. The same conditions were applied as above with the following modifications. The plates were coated with 1 µg/mL of EC-Stab2 or Link-Stab2 for these assays. Subsequently, 1 nM of FVIII and CLB-CAg117-HRP (0.25 µg/mL) were incubated together in incubation buffer for 30 minutes at 37°C. Meanwhile, dilution series were prepared for the anti FVIII antibodies (0 - 1.3 µM), hyaluronan (0 - 2.5 mg/mL), heparin (0 - 0.5 mg/ml) and dextran sulfate (0 - 4.1 mg/ml). After

preincubation, the same volume of FVIII-CLB-CAg117-HRP and diluted competitor were pipetted together into the wells. The plates were developed for 15 minutes. The antibodies used are CLB-CAgA directed towards the A3 domain of FVIII and human monoclonal antibody VK34 directed towards the A2 domain [40,41].

Binding of FVIII to deglycosylated Link-Stab2. The same conditions described for the binding assay were applied except for the concentration of the protein in the coating step (1 µg/mL). Additionally, the developing time was increased to 30 minutes.

For all binding experiments the OD was measured at 450nm with SpectraMax (Molecular Devices) and data was processed using the SoftProMax software and analyzed with GraphPad Prism 10.3.1.

Surface plasmon resonance

Two setups were used for monitoring the interaction of FVIII and stabilin-2 using surface plasmon resonance (SPR) on Biacore T200 (Cytiva). In the first setup, EC-Stab2 was diluted in SPR buffer (20 mM HEPES, 150 mM NaCl, 5 mM CaCl2, 0.05 % Tween-20, pH 7.4) then flowed over (10 µL/min) the Strep-Tactin XT-modified sensor chip (Xantec Bioanalytics) until it reached a density of 2200 RU. FVIII (0, 1.2, 3.7, 11, 33, 100 nM) was diluted in SPR buffer then flowed (30 µL/min) over the reference and the EC-Stab2 coated channels for 360 seconds followed by a dissociation step of 720 seconds. Regeneration of the channels was performed employing a 30 seconds injection of 10 mM glycine (pH 1.5). In the second setup, antibody EL14-IgG1 [42] was immobilized by amine coupling chemistry on a CM5 sensor chip that was used for single cycle kinetics experiments at a density of 2800 RU. First, FVIII was flowed over the chip at a concentration of 5 nM for 300 seconds (5 µL/min); this resulted in an increase of 1430-1440 RU. Subsequently, increasing concentrations of the analyte, EC-Stab2 or Link-Stab2, were injected. At the end of each injection series SPR buffer was flowed over the channels for 30 minutes to monitor dissociation of bound ligands. Channels were regenerated with two 20 seconds injections of 0.85% phosphoric acid. The sensorgram data were analyzed with the BIAevaluation software and GraphPad Prism 10.3.1.

Results

Construction and expression of soluble stabilin-2 derived fragments

To study the interaction of FVIII to stabilin-2, we generated fragments corresponding to the extracellular domain of stabilin-2 (EC-Stab2) as well as its Link domain (Link-Stab2). The EC-Stab2 fragment (encoding amino acid sequence Val66 to Pro2449) was expressed in Expi-CHO cells essentially as described previously[38]. Monitoring of expression of the EC-Stab2 fragment yielded a band migrating at the expected molecular weight of around 250 kDa (Figure 1D). The design of the Link domain construct is shown in Figure 1. Based on the domain structure listed in UniProt a construct (X-Link-Stab2) was designed encompassing Gly2198-Arg2291 (Figure 1A). Monitoring of expression unexpectedly revealed that the Link domain containing construct was not present in conditioned medium of transfected ExpiCHO cells (Figure 1D). These results suggest that the Link domain designed is not efficiently produced and released from transfected ExpiCHO cells. We subsequently designed a second Link domain containing construct based on the three-dimensional structure of the Link domain containing fragment of CD44 [39]. An alignment of the hyaluronic acid binding domain of CD44 and the corresponding part of stabilin-2 is shown in Figure 1B. Based on this alignment we designed an extended Link domain (Link-Stab2) ranging from Pro2166-Gly2312 (Figure 1A). The resulting Link domain includes flanking regions containing three additional cysteine residues on each side. Expression of the extended Link domain in Expi-CHO cells revealed that is was efficiently secreted by ExpiCHO cells (Figure 1D). We employed AlphaFold to design a three-dimensional model of the extended Link domain which is displayed in Figure 1C. This resulting model suggests that the amino-terminal and carboxy-terminal extensions of the Link domain of stabilin-2 form two anti-parallel β-sheets that are stabilized by two disulfide bonds.

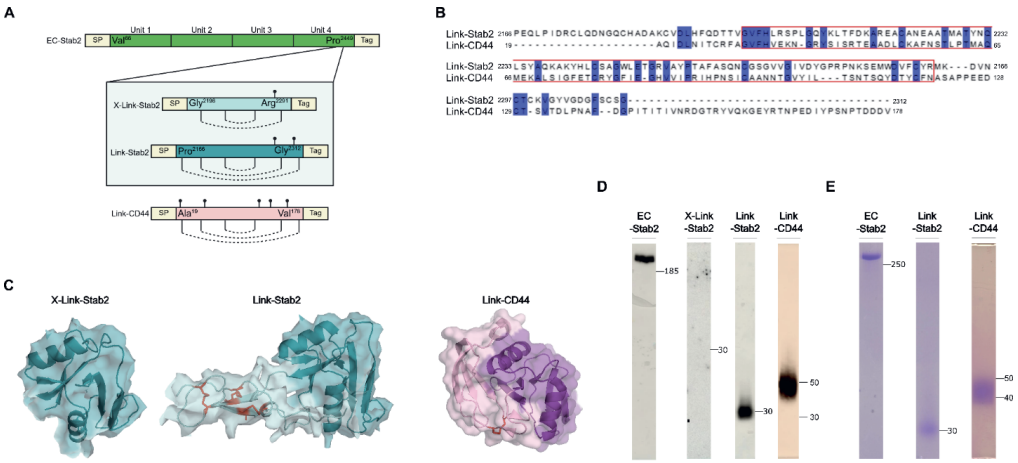


Figure 1: Expression of fragments of stabilin-2. (A) Schematic representation of construct for expression of stabilin-2 fragments. For the expression of the fragments we included a signal peptide at the N-terminal and a tag at the C terminal that contains the V5 and TwinStrep sequences. The amino acid included for the extracellular domain of EC-Stab2, X-Link-Stab2, Link-Stab2 and Link-CD44 are also shown. (B) Link domain of stabilin-2 and CD44 pair-wise alignment, the Link domain has been enclosed in the red area. (C) AlphaFold model of the extracellular domain of X-Link-Stab2, Link-Stab2 and Link-CD44. The structure of X-Link-Stab2 is represented here in teal; Link-Stab2 contains additional amino acids (light blue), the cysteine amino acid in this part are represented in red. The Link domain of CD44 is shown in purple; the additional amino acids are colored light pink, with cysteine residues in this region shown in red. (D) Stabilin-2 fragments expression in Expi-CHO cells. The expression media of cells transfected with different stabilin-2 fragments (EC-Stab2, X-Link-Stab2, Link-Stab2) and CD44 (Link-CD44) has been collected at day 7 and blotted using anti-V5 antibody. (E) Purified fragments of stabilin-2 and CD44. EC-Stab2, Link-Stab2 and Link-CD44 have been purified and run on polyacrylamide gels and blotted with imperial protein stain.

Binding of FVIII to soluble stabilin-2 fragments

To study the interaction between FVIII to the extracellular and extended Link domain of stabilin-2 we purified EC-Stab2 and Link-Stab2 from conditioned medium of transiently transfected ExpiCHO cells (Figure 1E). Purified EC-Stab2 was immobilized at a concentration of 5 µg/ml on microtiter wells and binding of increasing concentrations of FVIII was evaluated employing a peroxidase labelled CLB-CAg117 directed towards the C2 domain of FVIII. Dose-dependent binding of FVIII to immobilized EC-Stab2 was observed. Addition of 3 nM FVIII resulted in saturation of binding; half-maximal binding was observed at 0.7 nM FVIII (Figure

2A). These findings show that FVIII can bind with high affinity to stabilin-2. Next we also explored the binding of FVIII to immobilized Link domain. Dose-dependent binding was also observed for the Link domain (Figure 2A). Half-maximal binding was observed at 1.0 nM FVIII, suggesting that the Link domain of stabilin-2 can also bind FVIII with high affinity.

The Link domain contains two N-linked glycans that are attached to Asn2198 and Asn2292. Purified Link domain was incubated with a mixture of PNGase F, β1-4 Galactosidase S, O-Glycosidase, α2-3,6,8,9 Neuraminidase A, β(1-4)Galactosidase S and β-N-Acetylhexosaminidasef. Two bands were observed consistent with the presence of two N-glycan in the Link domain (Supplementary Figure 1B). We next assessed the binding of FVIII to de-glycosylated Link employing an ELISA based assay. Binding of FVIII to the de-glycosylated Link fragment was similar to that observed for the non-modified Link domain (Supplementary Figure 1C).

The Link domain of stabilin-2 is homologous to the Link domain of CD44 [39]. Therefore, we assessed whether CD44 could also bind to FVIII. In contrast to the Link domain of stabilin-2, no binding of FVIII to the Link domain of CD44 was observed (Figure 2A). We further assessed the effect of increasing concentrations of hyaluronic acid, dextran sulfate and heparin, known ligands of stabilin-2, on the binding of FVIII to the extracellular domain of stabilin-2 (Figure 2B, 2C and 2D). Increasing concentrations of hyaluronic acid and heparin partly decreased the binding of FVIII to immobilized extracellular stabilin-2 domain. Pronounced inhibition of FVIII binding to stabilin-2 was observed in the presence of high concentrations of dextran sulfate. Interestingly, at low concentrations of dextran sulfate, enhanced binding of FVIII was observed (Figure 2B). These findings suggest that the binding site for FVIII in the Link domain of stabilin-2 overlaps with that of dextran sulfate. We also explored the ability of a panel of monoclonal antibodies directed towards different domains of FVIII to interfere with binding to stabilin-2. Human monoclonal antibody VK34 directed towards residues Arg503-Ile527 in the A2 domain of FVIII partially inhibited the binding of FVIII to EC-Stab2 as well as FVIII binding to the Link-Stab2 (Supplementary Figure 1). Monoclonal antibody CLB-CAgA directed towards amino acid residues Glu1830-Lys1837 in the A3 domain of FVIII partially competes with binding of FVIII to EC-Stab2 and Link-Stab2 domain (Supplementary Figure 2). The concentrations of CLB-CAgA and VK34 antibodies needed to partly inhibit the binding to stabilin-2 were quite high. These findings indicate that binding of FVIII to stabilin-2 is mediated by multiple domains.

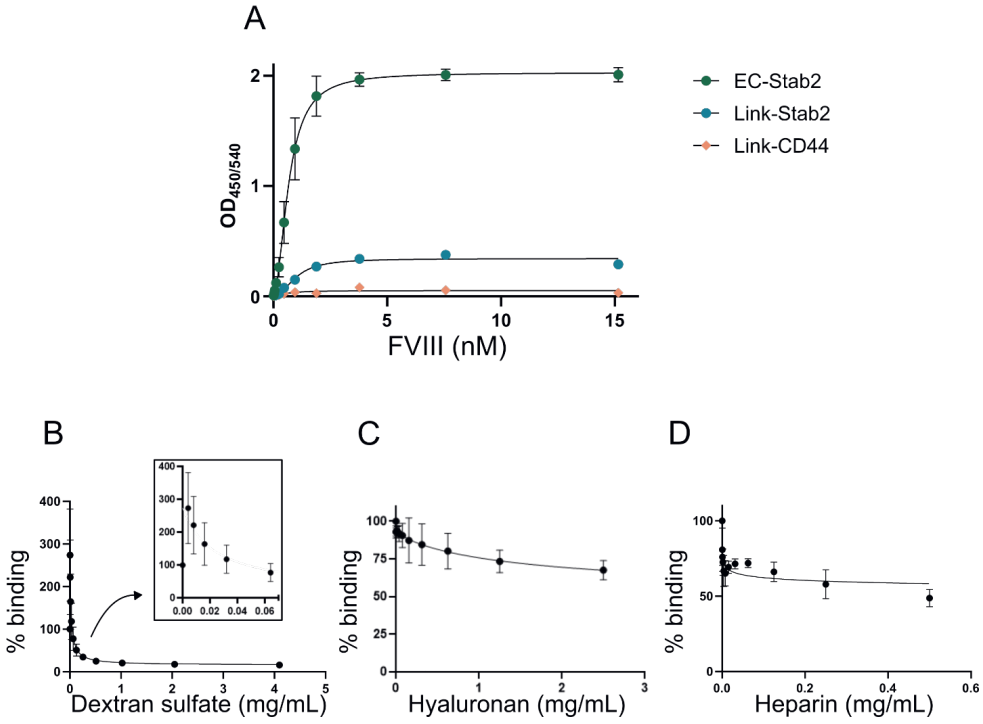


Figure 2: Binding of stabilin-2 fragments to FVIII. (A) Binding of EC-Stab2, Link-Stab2 and Link-CD44 to FVIII. EC-Stab2, Link-Stab2 and Link-CD44 have been immobilized to the ELISA plate and FVIII has been added at different concentrations; signal was detected using CLB-CAg117-HRP antibody. (B) Inhibition of the FVIII-stabilin-2 binding by dextran sulfate. After immobilization of EC-Stab2 on the ELISA plate, FVIII was added together with different concentrations of dextran sulfate. The signal was detected with CAg117-HRP antibody. (C) Inhibition of the FVIII-EC-Stab2 binding by hyaluronan. After immobilization of EC-Stab2 on the ELISA plate, FVIII was added together with different concentrations of hyaluronan. The signal was detected with CAg117-HRP antibody. (D) Inhibition of the FVIII-EC-Stab2 binding by heparin. After immobilization of EC-Stab2 on the ELISA plate, FVIII was added together with different concentrations of heparin. The signal was detected with CAg117-HRP antibody. The data were normalized and fitted using sigmoidal 4 parameters.

Surface plasmon based assessment of the interaction between FVIII and stabilin-2

We employed surface plasmon based analysis to quantify in more detail the interaction between stabilin-2 and FVIII. The following set-up was used. EC-Stab2 was immobilized on the sensor chip via its StrepTag as described in the Materials and Methods section. Different concentrations of FVIII ranging from 0.4 to 100 nM were flowed over the sensor chip. A dose-dependent binding of FVIII was observed

under these conditions (Figure 3). The signals obtained reached a maximum of 280 resonance units (RU) at the highest concentration of FVIII. These results show that FVIII binds to the extracellular domain of stabilin-2. We also studied binding of FVIII to immobilized Link-Stab2 domain using SPR. No significant binding was observed, most likely due to poor accessibility of binding sites on the StrepTag immobilized Link domain.

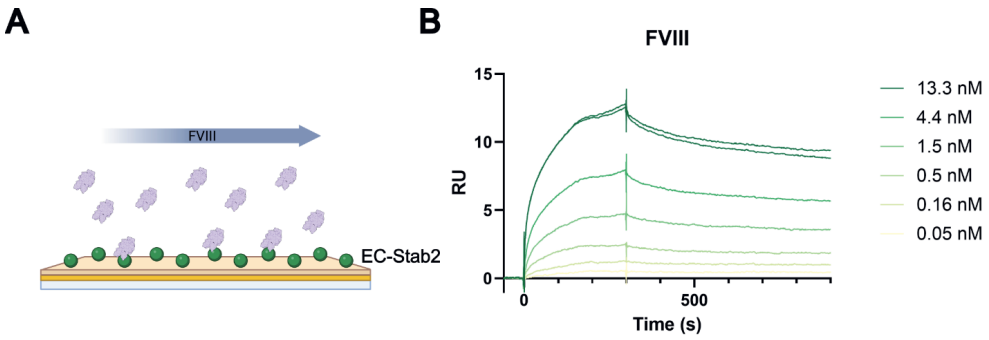


Figure 3: FVIII binds to stabilin-2 in a dose dependent manner. (A) Schematic representation of the SPR experiment. EC-Stab2 has been immobilized to the chip via the StrepTag, FVIII at different concentrations is flowed over the chip. (B) Dose dependent binding of FVIII. Six different concentrations of FVIII (0.4 to 33.3 nM) were flowed over the chip in random order; the association phase lasted 360 s and the dissociation phase (buffer flow) 720 s. After each cycle, EC-Stab2 was removed with a regeneration step.

An alternative set-up was also employed to study the binding of FVIII to EC-Stab2 and Link-Stab2. Human monoclonal antibody EL-14, directed towards the C2 domain of FVIII, was immobilized on a CM5 sensor chip. This particular antibody was selected due to its high affinity for FVIII; in particular, the k_{off} of FVIII for EL-14 has been shown to be very low [43]. FVIII was immobilized on EL-14 until a value of approximately 1000 resonance units (RU) was reached. Subsequently, increasing concentrations of EC-Stab2 and Link-Stab2 were added. A progressive increase in binding of EC-Stab2 to the immobilized FVIII was observed. The maximal signal obtained was 110 RU upon injection of 25 nM EC-Stab2 on immobilized FVIII (Figure 4B). A similar pattern was observed upon the addition of increasing concentrations of Link-Stab2. A maximal signal of 75 RU was obtained following the addition of 100 nM of Link-Stab2 (Figure 3C). These data confirm dose-dependent binding of EC-Stab2 and Link-Stab2 to FVIII.

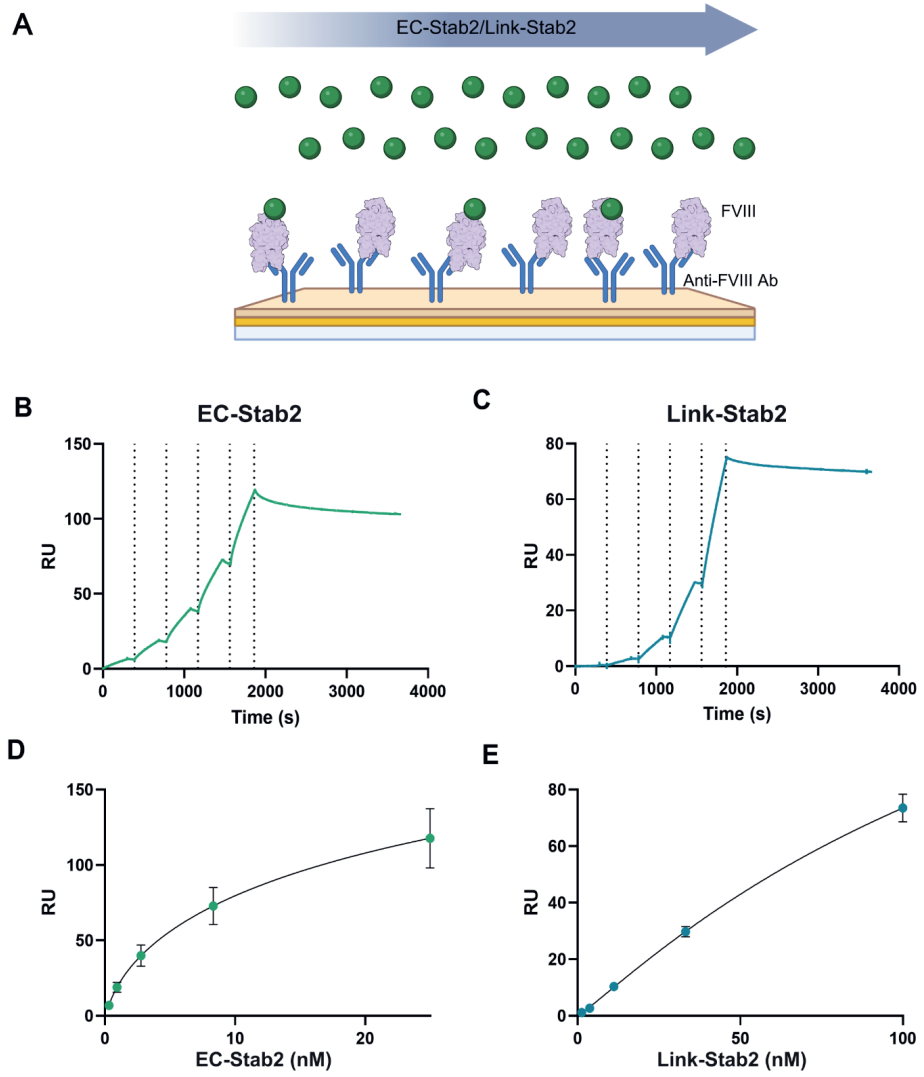


Figure 4: Single cycle kinetics of stabilin-2 fragments binding to FVIII. (A) Schematic representation of the experiment, anti-FVIII antibody (EL14) was immobilized on chip, subsequentially FVIII was captured by the antibody and finally either the extracellular domain of stabilin-2 or the Link domain were flowed over. (B and C) FVIII was captured on the chip and subsequentially EC-Stab2 (0,31nM, 0.93nM, 2.78nM, 8.33nM and 25nM) or Link-Stab2 (0.34nM, 3.70nM, 11,11nM, 33.33nM and 100nM) were added through five consecutive injections; the beginning of the injections is highlighted by the dashed lines. The sensograms shown is an average of two different cycles. (C and D) The response units reached at the end of each injection have been graphed and the values interpolated.

Discussion

Non-factor replacement therapies have revolutionized the treatment of hemophilia A over the last decade [44]. In addition, AAV mediated gene therapy approaches have been developed for hemophilia A and B [45]. Building on increased knowledge on the clearance of FVIII, bioengineered FVIII variants with an improved circulatory half-life have also been successfully developed [6]. Classically, the development of FVIII inhibitors is viewed as the major complication in hemophilia care. FVIII administration is currently still needed for treatment of breakthrough bleeds in patients receiving non-factor replacement therapy, potentially putting these patients at risk of developing inhibitors [9]. Both inhibitor development and FVIII clearance are promoted by the promiscuous binding of FVIII to multi-ligand receptors expressed on LSECs, hepatocytes, macrophages and other populations of antigen-presenting cells [46]. In this paper, we show that stabilin-2, an LSEC surface receptor, can bind to FVIII alone, without VWF. Moreover, we identify the Link domain of stabilin-2, previously shown to contain a binding site for hyaluronic acid, as a key determinant for FVIII binding. Macrophages in both the liver and spleen have been determined as responsible for the clearance of the FVIII-VWF complex through the receptors LRP1, Siglec-5, SR-A1 and MGL [47]. Multiple receptors, including LRP1 and the LDLR, are involved in the clearance of FVIII by hepatocytes [48]. Interestingly, ASPGR has been shown to interact with the heavily glycosylated B domain of FVIII [49]. The receptors identified as responsible for clearance in LSECs are CLEC4M, SCARA-5 and stabilin-2 [47].

Stabilin-2 is a complex protein of 315 kDa that has originally been identified as a receptor for hyaluronic acid [50]. More recently, stabilin-2 was also shown to bind to phosphatidylserine, thereby promoting clearance of apoptotic cells [51]. Interestingly, polymorphic sites within the gene encoding stabilin-2 were linked to circulating FVIII levels in the CHARGE study [20,52]. An elegant study by Swystun and co-workers revealed that stabilin-2 regulates in vivo half-life and immunogenicity of the FVIII-VWF complex [29]. Currently, structural determinants on stabilin-2 for binding to FVIII have not yet been established. To study the interaction of stabilin-2 with FVIII we chose to focus on the extracellular domain and the Link domain of stabilin-2. Unexpectedly, we were unable to express a Link domain containing fragment encompassing residues Gly2198-Arg2291. An extended Link domain containing fragment composed of residues Pro2166-Gly2312 was however efficiently expressed in Expi-CHO cells (Figure 1D). Comparison of the Link domain of stabilin-2 to that of CD44 revealed that in both cases there were Cys residues in the N and C terminal of the domains (Figure 1C).

We hypothesize that the formation of intramolecular disulfide bonds between the N and C terminal cysteines are necessary for the correct folding of the Link modules with N and C terminal extensions [53].

Swystun et al. previously showed with experiments in mouse the relevance of stabilin-2 in binding the FVIII-VWF complex and not FVIII alone [29]. Here, we show that the extracellular domain of stabilin-2 can bind to FVIII alone in vitro (Figure 2A and 3). In this study we show that the Link domain of stabilin-2 mediates the binding to FVIII (Figure 2A and 4C). A previous study showed a greatly reduced endocytosis of hyaluronic acid when compared to HEK293 cells expressing wild type stabilin-2 [54]. Our studies show that increasing concentrations of hyaluronic acid only partly inhibits the binding of FVIII to stabilin-2 while the addition of dextran sulfate, another known binder of stabilin-2, seems to have a more pronounced effect (Figure 2B and 2C). Heparin also has only a partial effect on the binding (Figure 2D). Interestingly, at low concentrations dextran sulfate seems to enhance the binding of FVIII to stabilin-2. We hypothesize that this could be due to a change of conformation in the extracellular domain of stabilin-2 that may render the Link domain more accessible to FVIII. In the Link domain of stabilin-2 two consensus sites for N-linked glycosylation are present at Asn2280 and Asn2296 [55]. N-linked glycans in the Link domains of CD44 have been implicated in the binding of hyaluronic acid [56]. Removal of the N-linked glycan at amino acid position Asn2280 of the Link domain of stabilin-2 has been shown to abrogate in vitro binding of hyaluronic acid [55]. In the current study we show that binding of FVIII is not affected by de-glycosylation of the Link domain. Apparently, N-linked glycans do not modulate the binding of FVIII to the Link domain of stabilin-2. Altogether, these data suggest that different amino acids contribute to the binding sites of FVIII and hyaluronan in the Link domain of stabilin-2. In vivo studies by Swystun and co-workers showed that co-administration of hyaluronic acid and FVIII prevented the development of FVIII inhibitors in a mouse model for hemophilia A. This finding suggests that binding of FVIII to stabilin-2 provides a crucial step in the development of an immune response to therapeutic FVIII. Our results show that FVIII binds to stabilin-2, and that this binding is partly mediated via its Link domain. Modification and/or shielding of binding sites of FVIII on stabilin-2 may represent a novel strategy to prevent inhibitor development in hemophilia A.

Conflict of interest:

The authors have no conflicts of interest to declare.

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Author contributions:

ML, ZS and JV designed the study. ML and ZS performed the experiments. ML and JV wrote the first draft of the manuscript. ML performed the data analysis. PK and AM assisted with the SPR experiments. KF provided clinical input for study and manuscript. All authors critically reviewed and approved the manuscript.

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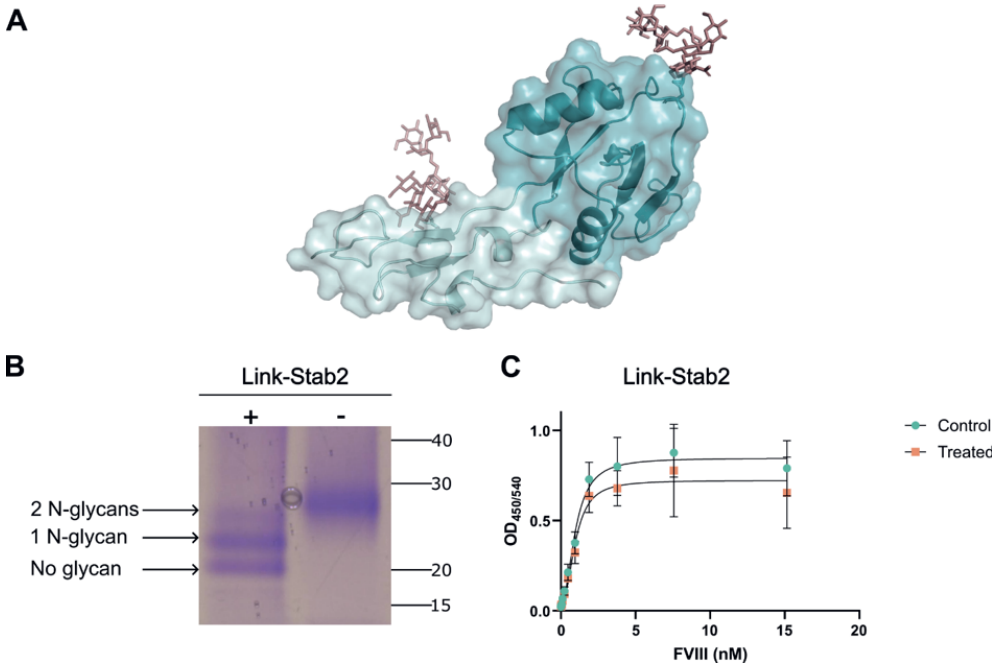
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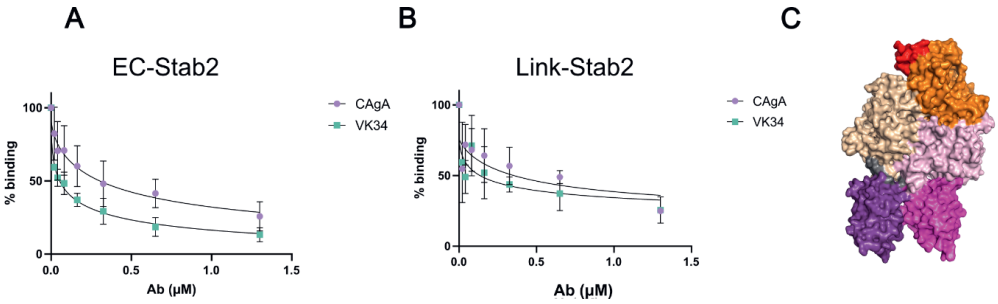
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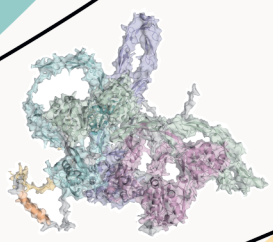
Supplementary Data



Supplementary Figure 1. The glycans on the link domain do not have a role in FVIII binding. (A) Highlight of glycosylation sites on the link domain of stabilin-1. (B) De-glycosylation of Link-Stab2. The protein underwent deglycosylation treatment. The reaction product (+) has been run on gel together with non-treated Link-Stab2 (-) as a control. (C) De-glycosylated link binding to FVIII. The de-glycosylated link domain (treated) and normal Link-Stab2 have been immobilized on an ELISA plate and different concentrations of FVIII have been added. The signal has been detected with CLB-CAg117.



Supplementary Figure 2. Specific FVIII antibodies inhibit its binding to stabilin-2 fragments. (A) Antibody specific inhibition of FVIII binding to the extracellular domain of stabilin-2. The extracellular domain of stabilin-2 has been immobilized on the ELISA plate. Subsequentially, FVIII and different concentrations of the antibodies VK34 and CAgA have been added to the plate. The signal has been detected using CAg117. (B) Antibody specific inhibition of FVIII binding to link domain. The extracellular domain of stabilin-2 has been immobilized on the ELISA plate. Subsequentially, FVIII and different concentrations of the antibodies VK34 and CAgA have been added to the plate. The signal has been detected using CAg117. (C) Epitope of VK34 and CLB-CAgA. The epitope of the two antibodies has been colored in red in the 3D model of FVIII.

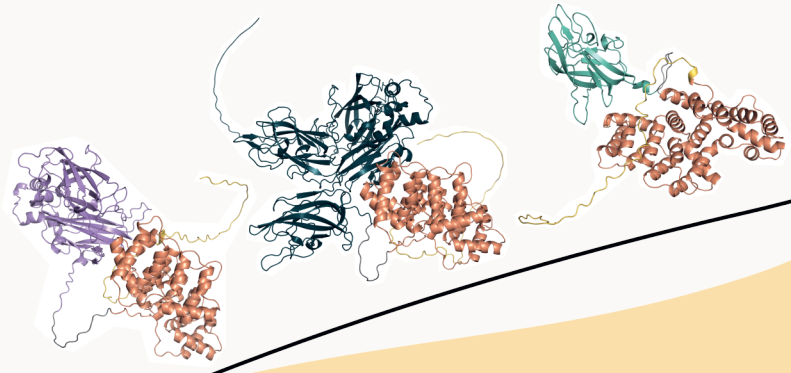






Chapter 4

MHC class II presentation of FVIII-AnnexinA5 fusion proteins internalized by antigen presenting cells



Miranda M[†], **Leoni M**[†], van der Zwaan C, van Bruggen R, Reutelingsperger CPM, Fijnvandraat K, Hoogendijk AJ, van den Biggelaar M and Voorberg J

[†]These authors contributed equally to this work and share first authorship

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Abstract

Introduction:

The development of neutralizing antibodies (inhibitors) against coagulation factor VIII (FVIII) remains the most serious complication in the treatment of hemophilia A. While immune tolerance induction (ITI) is the standard strategy to eliminate these antibodies, it fails in approximately 30% of patients with severe hemophilia A, underscoring the need for innovative approaches to promote FVIII-specific tolerance.

Methods:

To address this challenge, we generated fusion proteins composed of A2, A3-C1-C2 (light chain, LCh), and C2 domains of FVIII linked to Annexin A5 (AnxA5), a protein that binds phosphatidylserine (PS), a hallmark of apoptotic cells.

Results:

ELISA confirmed high-affinity binding of all fusion proteins to immobilized PS. To model PS exposure *in vitro*, red blood cells (RBCs) were treated with phorbol 12-myristate 13-acetate (PMA), leading to the release of PS-exposing microvesicles. Flow cytometry showed that FVIII-AnxA5 fusion proteins selectively bound to PS-exposing microvesicles but not to intact RBCs. Using mass spectrometry-based immunopeptidomics, we demonstrated that macrophages pulsed with FVIII-AnxA5 fusion proteins efficiently processed and presented FVIII-derived peptides on HLA-DR molecules.

Conclusions:

These findings suggest that FVIII-AnxA5 fusion proteins can engage apoptotic cell clearance pathways to facilitate antigen presentation in a potentially tolerogenic context. This strategy may offer a novel means of inducing immune tolerance to FVIII in hemophilia A.

Keywords

FVIII, annexin A5, antigen presentation, phosphatidylserine, red blood cells

Introduction

Hemophilia A is a bleeding disorder caused by a mutation in the *F8* gene, resulting in a deficiency of functional clotting factor VIII (FVIII) (1). The deficiency or dysfunction of FVIII impairs the formation of a stable fibrin clot, leading to prolonged bleeding episodes. The cornerstone of hemophilia A treatment is replacement therapy, which involves the administration of FVIII to restore normal hemostasis (1). Over the past decade, bioengineered FVIII molecules with extended half-life (EHL) have been developed to reduce the burden of frequent intravenous injections, improving patient adherence and quality of life (2–4). Despite these advancements, the primary complication of replacement therapy remains the development of anti-FVIII antibodies (so called FVIII inhibitors), which occur in 25-40% of patients with severe hemophilia A (5, 6). FVIII inhibitors significantly reduce the efficacy of FVIII infusions thereby severely complicating managements of bleeds (7). To overcome this challenge, bypassing agents such as activated prothrombin complex concentrate (aPCC) and recombinant activated factor VII (rFVIIa) have been employed to restore hemostasis through FVIII-independent pathways (8, 9). Non-factor replacement therapies have emerged as effective prophylactic options. Emicizumab, a bispecific monoclonal antibody that bridges activated factor IX and factor X, functionally mimics FVIII and even secure hemostasis in patients with high-titer inhibitors (10–13). Building on its excellent clinical profile in inhibitor patients as well as its convenient relative infrequent subcutaneous administration emicizumab is now widely used for prevention of bleeds in patients with hemophilia A (14, 15). While emicizumab is effective for routine prophylaxis, FVIII infusions remain necessary to treat breakthrough bleeding or to prevent bleeding during surgery (16). It is currently not clear whether infrequent exposure to FVIII puts these patients at risk of inhibitor development (10). Currently there are limited clinical protocols available for restoring tolerance in patients with hemophilia A; immune tolerance induction (ITI) remains the only established strategy to eradicate FVIII-specific antibodies. ITI involves high-dose, repeated FVIII infusions to induce a state of peripheral immune tolerance (17). Although effective in many cases, ITI is a costly and time-consuming treatment that requires frequent infusions and fails in approximately 30% of patients (18, 19). Clearly, there is a need for alternative tolerization strategies to protect patients receiving emicizumab, or other non-factor replacement therapies, from the risk of inhibitor development following breakthrough bleeds or surgical interventions (6).

FVIII inhibitory antibodies predominantly belong to the IgG subclasses, with IgG4 and IgG1 being the most abundant (20–22). This pattern reflects the role of $CD4^+$ T

cells in driving the FVIII inhibitor response. A recent study by Kaczmarek et al. revealed that FVIII is recognized by marginal zone B cells and marginal metallophilic macrophages (23). These antigen presenting cells (APCs) transport FVIII to the white pulp, where conventional dendritic cells (cDCs) prime helper T cells, which subsequently differentiate into follicular helper T (Tfh) cells. This process promotes T-cell proliferation and antibody production. In contrast, a less immunogenic protein, such as chicken ovalbumin (OVA), is taken up primarily by red pulp macrophages (RPMs) and does not trigger the development of inhibitory antibodies (23).

Under quiescent conditions, approximately one million cells per second die through apoptosis in the human body (24). The engulfing of dead cells by professional phagocytes, known as efferocytosis, is essential to clear apoptotic cells and maintain homeostasis (24–26). Efferocytosis occurs in all major tissues and organs, ensuring that processes such as removal of aged neutrophils and red blood cells (RBCs) and clearance of negatively selected thymocytes are executed rapidly (27). These clearance processes are performed by professional phagocytes, such as macrophages and dendritic cells (DCs), and non-professional phagocytes, such as fibroblast and epithelial cells (24). Apoptotic cells expose 'eat me' signals on their surface, which are sensed by phagocytes through their receptors and bridging molecules (28–30). The main cell surface receptors involved are the low-density lipoprotein receptor-related protein 1, T cell immunoglobulin mucin receptor (TIM) 1, TIM3, TIM4, adhesion G protein-coupled receptor B1, stabilin-1 and stabilin-2, while among the main bridging molecules there are protein S, milk fat globule epidermal growth factor 8 (MFG-E8) and vitamin K-dependent protein growth arrest specific 6 (Gas6) (24, 31). The most well characterized "eat-me" signal is phosphatidylserine (PS), which is found in the inner leaflet of living cells and is exposed externally via the action of caspase-regulated flippase during apoptosis (32, 33). PS is recognized directly by PS binding receptors (e.g. stabilin-1, stabilin-2, TIM4) or indirectly by bridging mediators (31).

A crucial aspect of efferocytosis is the clearance of senescent erythrocytes, a process essential for erythropoietic homeostasis and systemic iron recycling (27). Removal of senescent RBC from the circulation occurs through phagocytosis, which takes place mainly in macrophages of the spleen, but also in the liver and the bone marrow (34). Red pulp macrophages (RPMs) in the spleen and Kupffer cells (KC) in the liver are specialized in recognizing and engulfing aged RBCs (34, 35). Under physiological conditions, RBCs express CD47, which interacts with signal regulatory protein α (SIRP-α) on RPMs and KCs, delivering a 'don't eat me' signal that prevents premature phagocytosis (36, 37). As RBCs age, they

progressively accumulate removal signals including conformational changes in CD47, oxidation of proteins and lipids, loss of membrane deformability, activation of adhesion molecules, and PS exposure (38). PS is recognized by a specialized set of receptors predominantly expressed on RPMs and liver macrophages, including TIM4, CD36, and TAM receptors (39, 40). Following engulfment, the enzyme heme oxygenase-1 (HMOX-1) catalyzes the breakdown of heme thereby promoting iron recycling and protecting tissues from heme-induced toxicity (35, 41). Furthermore, RPMs are pivotal in maintaining immune tolerance, as their ability to phagocytose self-antigens leads to the deletion of antigen-specific T cells (35, 42). This function is enabled by their expression of MHC class I and II molecules, alongside the absence of costimulatory molecules like CD80 and CD86 (35). RPMs also promote the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β, and enhance Foxp3 expression in CD4+ T cells, thereby fostering the generation of regulatory T cells (35, 42). Similarly, KCs exert their tolerogenic activity, which is crucial for preventing undesired immune responses under physiological conditions, by suppressing T cell activation through the production of prostaglandin E2 (PGE2), 5-deoxy-delta12,14-PGJ2 (15d-PGJ2), IL-10, and TGF-β (43–46).

In this study, we designed fusion proteins by combining FVIII domains with Annexin A5 to specifically target PS exposing apoptotic cells. We evaluated the binding of FVIII-AnxA5 fusion proteins to RBC derived PS exposing microvesicles. In parallel we also assessed MHC class II presentation of FVIII-AnxA5 fusion proteins by macrophages. Our findings suggest that Annexin A5-fused antigens can engage apoptotic pathways to modulate immune responses, highlighting their potential for immune regulation.

Materials and methods

Subjects

Blood was drawn from healthy volunteers in accordance with Dutch regulations and following approval from Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn, EDTA anticoagulated blood by separation over a Ficoll-Paque TM PLUS gradient (GE Healthcare). Red blood cells (RBCs) were isolated from freshly drawn, citrate anticoagulated blood by centrifugation at 105 g for 5 minutes (Eppendorf 5417R Refrigerated Centrifuge). After removing the platelet-rich plasma (PRP) and PBMC, erythrocytes were washed twice with saline-adenine-glucose-mannitol (SAG-M; 150 mM NaCl, 1.25 mM adenine, 50 mM glucose, 29 mM mannitol; Compoflex®, Fresenius Kabi) and resuspended in SAG-M. Final concentration of RBCs was determined with an Advia 2120 (Siemens Medical Solutions Diagnostics).

Reagents

Annexin AnxA5, A2- AnxA5, C2- AnxA5 and A3-C1-C2 (light chain, LCh)- AnxA5 encoding cDNAs were ordered from GENEWIZ/Azenta, StrepTrap XT 1mL prepacked columns from Cytiva, Brain PS L-α-phosphatidylserine from Avanti Polar Lipids, anti-V5-HRP Antibody from Invitrogen, CD14 microbeads, and manual MACS Magnetic Separators for cell separation from Miltenyi; M-CSF from Peprotech; Human Serum Albumin (HSA) (200 g/l) from Sanquin; ExpiCho Expression System, IMDM, RPMI 1640, UltraPure™ 0.5M EDTA (pH 8.0), Alexa Fluor 555 labeling kit, Alexa Fluor 647 labeling kit, Vybrant™ DiD Cell-Labeling Solution, and Vybrant™ DiO Cell-Labeling Solution from ThermoFisher. InVivoMAb anti-human/monkey HLA-DR (L243) was obtained from BioXCell. Purified monoclonal antibody L243 was coupled to CNBr Sepharose 4B at a final concentration of 2 mg/ml. Phorbol 12-myristate 13-acetate (PMA), L-glutamine and Penicillin Streptomycin Solution, Pen-Strep (10,000 units penicillin and 10 mg streptomycin/mL) from Sigma.

FVIII-AnxA5 fusion proteins design and expression

Five constructs were designed and synthesized: three FVIII-Annexin V (AnxA5) fusion proteins (A2-AnxA5, C2-AnxA5, and LCh-AnxA5), AnxA5 alone, and the FVIII light chain (LCh) alone. All constructs were cloned into the pcDNA3.1(+) vector. Each fusion protein included an N-terminal mouse Igκ-chain signal peptide (METDTLLLWVLLLWVPGSTGD) to promote secretion (47). The synthetic FVIII-derived regions included the A2 domain (Ser392–Pro758), the C2 domain (Ser2192

–Tyr2350), and the light chain (LCh, Glu1668–Tyr2350) were ordered at Genewiz and inserted into the pcDNA3.1(+) construct using AgeI and XhoI. These domains were linked via a flexible $(GGGGS)_3$ GS linker to human AnnexinA5 (UniProt ID: P08758). A C-terminal V5 epitope tag (GKPIPNPLLGLDST) and Twin-Strep tag (WSHPQFEKGGGSGGGSGGSAWSHPQFEK) were included in the constructs to allow for protein detection and purification. Proteins were expressed in ExpiCHO using the High Titer expression protocol with 1 µg of DNA per mL of cell culture as recommended by the manufacturer. Five days after transfection cell culture supernatant was collected by centrifugation at 4000g for 30 minutes at 4°C. A final concentration of 10 mM benzamidine was added to the supernatant and aliquots were stored at −30 °C until use. Expression was confirmed by Western blot using anti-V5-horseradish peroxidase (HRP) (1:15000; Invitrogen, R961-25). The proteins were purified from the media using StrepTrap XT prepacked column in the ÄKTA pure™ chromatography system (Cytiva). The proteins were washed with 100mM Tris-HCl, 150mM NaCl, pH 8.0, and eluted in 100mM Tris-HCl, 150mM NaCl, 5mM $CaCl_2$, 100mM biotin, pH 8.0; the buffer was exchanged to 25mM Hepes, 150mM NaCl, 2mM $CaCl_2$, pH 7.4 by dialysis overnight. The success of the purification was assessed by SDS page gel and Coomassie brilliant blue (CBB) staining. The concentration of the protein was determined by Bradford assay using the Protein Assay kit (Bio-Rad), following the manufacturer's instructions. The proteins were aliquoted, snap-frozen and kept at -80°C until use.

FVIII-AnxA5 fusion proteins interaction with PS

Polysorp microtiter plates (Nunc) were coated with PS (2 µg/mL) in ethanol and incubated overnight at room temperature until complete evaporation. The plates were then blocked with blocking buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3% BSA) for 1 hour at room temperature. Following blocking, each of the four AnxA5 fusion proteins was individually diluted in sample buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$) and added to separate wells, then incubated for 1 hour at room temperature. After incubation, anti-V5-HRP detection antibody (1:2000 dilution in sample buffer) was added and incubated for 1 hour at room temperature. For detection, the plate was developed with TMB substrate solution (0.1 mg/mL TMB in DMSO, 0.11 M sodium acetate, pH 5.5, 0.0045% H_2O_2) for 10 minutes. The reaction was then stopped by adding 100 µl of 1 M H_2SO_4. Optical density (OD) was read at 450 nm with a 540 nm reference on a SpectraMax Plus plate reader. Data were analyzed with GraphPad Prism v10.3 (GraphPad Software). Data were fitted by nonlinear regression to a four-parameter logistic (4PL) sigmoidal dose–response model (variable slope) to determine EC_{50} values.

FVIII-AnxA5 fusion proteins interaction with RBC

To investigate the interaction between FVIII-AnxA5 fusion proteins and phosphatidylserine (PS) on RBCs, we induced PS exposure on RBCs. A total of $0.5x10^6$ RBCs were incubated with phorbol 12-myristate 13-acetate (PMA) at a final concentration of 6 µM. The incubation was performed in 100 µl of buffer A (20 mM HEPES pH 7.4, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 1 mM $CaCl_2$, 1% glucose supplemented with 0.5% HSA) for 30 minutes at 37°C. AnxA5 and FVIII-AnxA5 constructs were pre-labelled with Alexa Fluor 555 and Alexa Fluor 647, respectively, following the manufacturer's protocol. Protein concentrations and dye-to-protein ratios (DOL) were determined spectrophotometrically according to the kit instructions. Upon PMA incubation, RBCs were washed twice with buffer A and incubated with either pre-labelled AnxA5 and FVIII-AnxA5 fusion proteins in a concentration range from 0.01 to 100 nM in buffer B (20 mM HEPES pH 7.4, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 2.5 mM $CaCl_2$, 1% glucose supplemented with 0.5% HSA) for 30 min at RT. Cells were washed and analyzed by flow cytometry (BD LSR II Flow Cytometer).

FVIII-AnxA5 fusion proteins loaded on macrophages

Monocytes were isolated from the peripheral blood mononuclear cell fraction by positive selection using CD14 microbeads and a magnetic cell separator. Monocytes were plated at a concentration of $1x10^6$ cells in a total volume of 2 ml in a 6-well plate (Nunc, Roskilde Denmark) in IMDM complete (2 mM L-glutamine, penicillin–streptomycin 10 U/mL, and 10% fetal calf serum), and supplemented with 50 ng/ml M-CSF for 10 days. At day 3 and day 7, half of the medium was refreshed with IMDM supplemented with M-CSF (50 ng/ml). Upon 10 days of culture, $1x10^6$ macrophages were washed and incubated with 100 nM of each of the four AnxA5 fusion proteins overnight in a final volume of 2 ml IMDM complete.

Phagocytosis of PMA treated RBC preloaded by FVIII-AnxA5 fusion by macrophages

Monocytes were isolated and macrophages were differentiated as previously mentioned. Upon 10 days of culture, RBCs were isolated, treated with PMA, and incubated with AnxA5 and FVIII-AnxA5 constructs as previously described. In total $1x10^6$ macrophages were washed and incubated with FVIII-AnxA5 pre-loaded RBCs overnight in a final volume of 2 ml IMDM complete. The ratio of macrophages and red blood cells was 1:100.

Purification of HLA-DR presented peptides on macrophages

FVIII-AnxA5 and FVIII-AnxA5-RBCs-loaded monocyte derived macrophages were harvested and resuspended in 500 µL of lysis buffer (10 mM Tris-HCl pH 8.0, 0.25% octyl-β-D-glucopyranoside, 1% sodium deoxycholate and 10 mM EDTA) for 30 min at 4°C. Lysates were then centrifuged at 20,000 x g for 15 min at 4°C and supernatants were incubated with 300 µL of Sepharose beads containing 2 mg/ml of anti-HLA-DR monoclonal antibody L243 as described previously (48). Following overnight incubation at 4°C, L243-containing Sepharose beads were washed twice with lysis buffer, and five times with 10 mM Tris-HCl pH 8.0. Bound MHCII molecules were eluted by incubation with 10% acetic acid for 10 min at room temperature. Eluates were collected and heated for 15 min at 70°C to dissociate peptide/MHCII complexes.

Mass spectrometry

Samples were desalted using Empore C18 STAGE tips which were prepared in-house. STAGE tips were equilibrated with 100% acetonitrile and subsequently washed with 1% formic acid. Samples were loaded on Empore C18 STAGE tips and washed once with 1% formic acid and once with 1% formic acid supplemented with 5% acetonitrile. Peptides were eluted from Empore C18 STAGE tips with 60 µL 1% formic acid supplemented with 30% acetonitrile and concentrated to a final volume of 5 µL using vacuum centrifugation. Eluted peptides were separated using columns (New Objective type FS360-75-8-N-5-C20, Inc., Woburn, MA, USA) filled with 1.9 µm C18 particles (Dr.Maisch, Ammerbuch-Entringen, Germany) at a flow rate of 300 nL/min, HPLC Buffer A was composed of 0.1% formic acid in water and buffer B of 0.1% formic acid, 80% acetonitrile. Peptides were loaded for 17 min at 300 nL/min at 5% buffer B, equilibrated for 5 minutes at 5% buffer B (17–22 min) and eluted by increasing buffer B from 5-10% (22–27 min), 10-28% (27–70 min) and 28-85% (70–84 min) followed by a 6 minute wash to 95% and in 1 minute ramped to 5%, follow by a 4 min equilibration at 5%. Column eluate was sprayed directly into the Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific Inc., Bremen, Germany) using a nano electrospray source with a spray voltage of 2.15 kV. Survey scans of peptide precursors from 300 to 1600 m/z were performed at 120K resolution (at 200 m/z) in profile mode with a AGC target of 250% and a maximum injection time of 50 ms. Only precursors with charge state 2–7 were sampled for MS2. The dynamic exclusion duration was set to 20 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on and an intensity threshold of 2e4 was set. The instrument was run in top speed mode with 3 s cycles. Tandem mass spectrometry (MS/MS) was performed using a quadrupole mass analyzer for ion isolation and

higher-energy collisional dissociation (HCD) for fragmentation. The isolation window was set to 0.7 Da, and the normalized collision energy for HCD was 30. Fragment ions were analyzed in the Orbitrap at a resolution of 60k in centroid mode with a mass range of 200–1400 m/z, AGC target of 300% and a maximum injection time of 118 ms. All data were acquired with SII for Xcalibur software.

Data analysis flow cytometry and mass spectrometry

Flow cytometry data were analyzed with FlowJo (BD Biosciences). Peptides were identified using Proteome Discoverer 2.2 (Thermo Scientific, Bremen, Germany). Mass spectrometry raw data (Xcalibur format) were analyzed using both Proteome Discoverer 2.2 (Thermo Scientific, Bremen, Germany) and the FragPipe pipeline (v20.0) incorporating MSFragger (v3.8). For both platforms, the following search parameters were applied: precursor mass tolerance of ±10 ppm, fragment mass tolerance of 0.02 Da, peptide mass range of 3000–7000 Da, peptide length between 6 and 50 amino acids, and a false discovery rate (FDR) threshold of 0.05. In Proteome Discoverer, data were searched against the UniProtKB *H. sapiens* reviewed database (downloaded 2023), supplemented with FVIII-AnxA5 fusion protein sequences. Only peptides with medium or high confidence were retained and grouped by donor. In FragPipe, searches were performed against the human SwissProt database (release 2021.22.04), supplemented with decoy sequences (total 40,887 entries, 50% decoys). Digestion was set to non-specific to allow detection of peptides within the defined mass and length constraints. Peptide quantification was performed using IonQuant (v1.9.8) under default parameters, with a 10 ppm mass tolerance and match-between-runs disabled.

Results

Binding of FVIII-AnxA5 fusion proteins to PS

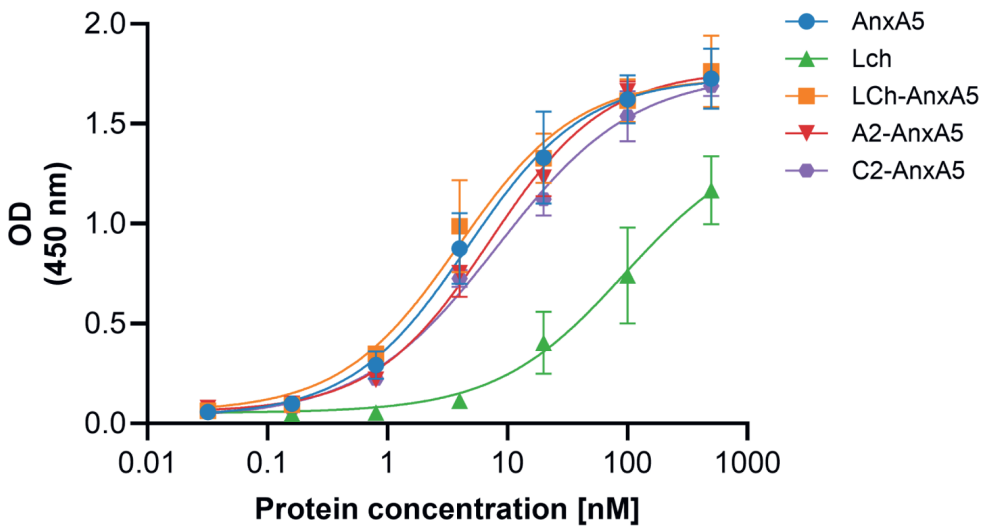
To enable specific targeting of phosphatidylserine (PS) exposed on apoptotic cells, we generated a series of FVIII-AnxA5 fusion proteins. The design incorporated different FVIII domains, including the highly immunogenic A2, C1, and C2 domains, fused to Annexin A5, and the corresponding amino acid sequences are presented in Supplementary Figure 1 (49). To evaluate the PS-targeting capability of the designed FVIII-AnxA5 fusion proteins, plates coated with PS were incubated with increasing concentrations of the three fusion constructs, alongside controls including AnxA5 alone and the FVIII light chain composed of the A3-C1-C2 domains of FVIII. As shown in Figure 1, all fusion proteins demonstrated a clear, sigmoidal dose-dependent binding to PS, exhibiting binding profiles similar to that of AnxA5. The FVIII light chain exhibited reduced binding to PS, consistent with the absence of the AnxA5; however, residual binding was still observed due to the PS-binding properties of the C2 domain (50, 51). Half maximal binding of LCh-AnxA5, A2-AnxA5 and C2-AnxA5 fusion proteins was observed at 40 times lower concentrations when compared to FVIII light chain. The corresponding EC50 values are reported in Figure 1B. For AnxA5 and the FVIII–AnxA5 fusion proteins, EC50 ranged from 3.92 to 8.32. In contrast, the FVIII light chain without Annexin A5 exhibited a substantially higher EC50 of 94.60 nM. The difference between AnxA5 containing proteins and FVIII LCh without Annexin A5 is consistent with published affinities values (52, 53). These findings show that FVIII-AnxA5 fusion proteins bind with higher affinity to PS when compared to FVIII. Overall, our data demonstrate that the FVIII-AnxA5 fusion proteins strongly bind to PS allowing for exploring their use to selectively target FVIII domains to PS exposing apoptotic cells.

FVIII-AnxA5 fusion proteins interaction with PS-exposed red blood cells

To evaluate the binding of FVIII–AnxA5 fusion proteins to PS-exposed red blood cells (RBCs), we performed flow cytometric analysis using RBCs isolated from multiple healthy donors. In untreated samples, RBCs were gated based on their characteristic forward and side scatter profiles (Figure 2A). Upon treatment with PMA, an additional population of RBC-derived microvesicles (MVs) emerged, as evidenced by altered forward and side scatter properties (Figure 2B). Intact RBCs and RBC-derived MVs were gated as separate populations, and the binding of fluorescently labeled FVIII-AnxA5 fusion proteins was assessed. To account for background fluorescence, a cut-off for FVIII-AnxA5-AF647 positivity was established using PMA-treated RBCs that were not incubated with the fusion protein. Events with fluorescence intensity exceeding this threshold were

considered positive for binding of FVIII-AnxA5 fusion proteins. As a positive control, we first assessed the binding of AnxA5 alone to confirm PS exposure on RBC-derived MVs. Minimal fluorescence was detected on intact RBCs, indicating negligible PS exposure (Figure 2C). In contrast, a strong dose-dependent binding was observed for the RBC-derived MVs (Figure 2D). A similar binding pattern was observed for the LCh–AnxA5, A2–AnxA5, and C2–AnxA5 fusion proteins, all of which showed low binding to intact RBCs and robust binding to RBC-derived MVs (Figure 2D). Notably, the FVIII light chain (LCh) alone also showed low but detectable binding to RBC-derived MVs, consistent with its binding to immobilized PS, albeit to a lesser extent when compared to the FVIII-AnxA5 fusion proteins (Figure 2C, D). These results demonstrate that the FVIII–AnxA5 fusion proteins preferentially interact with RBC-derived MVs that expose PS.

A



B

Protein	EC50 (nM)
AnxA5	4.64±0.90
LCh-AnxA5	3.95±0.89
Lch	94.60±65.30
A2-AnxA5	7.12±0.81
C2-AnxA5	8.32±1.20

Figure 1. Dose-response binding of FVIII-AnxA5 fusion proteins to phosphatidylserine (PS). Polysorp microtiter plates were coated with PS and incubated with increasing concentrations (nM) of five different proteins: AnxA5 alone, LCh FVIII, and three FVIII-AnxA5 fusion constructs (LCh- AnxA5, A2- AnxA5, C2- AnxA5). Binding was detected using anti-V5-HRP antibody, and optical density (OD) was measured at 450 nm. All proteins exhibited dose-dependent binding to PS with similar affinities, except for LCh FVIII, which showed reduced binding consistent with the absence of Annexin A5. The binding of LCh FVIII to PS is mediated by the C2 domain. Panel **(A)** shows the binding curves, and panel **(B)** displays the corresponding EC50 values, calculated by fitting the data to a 4 parameters sigmoidal dose response model.

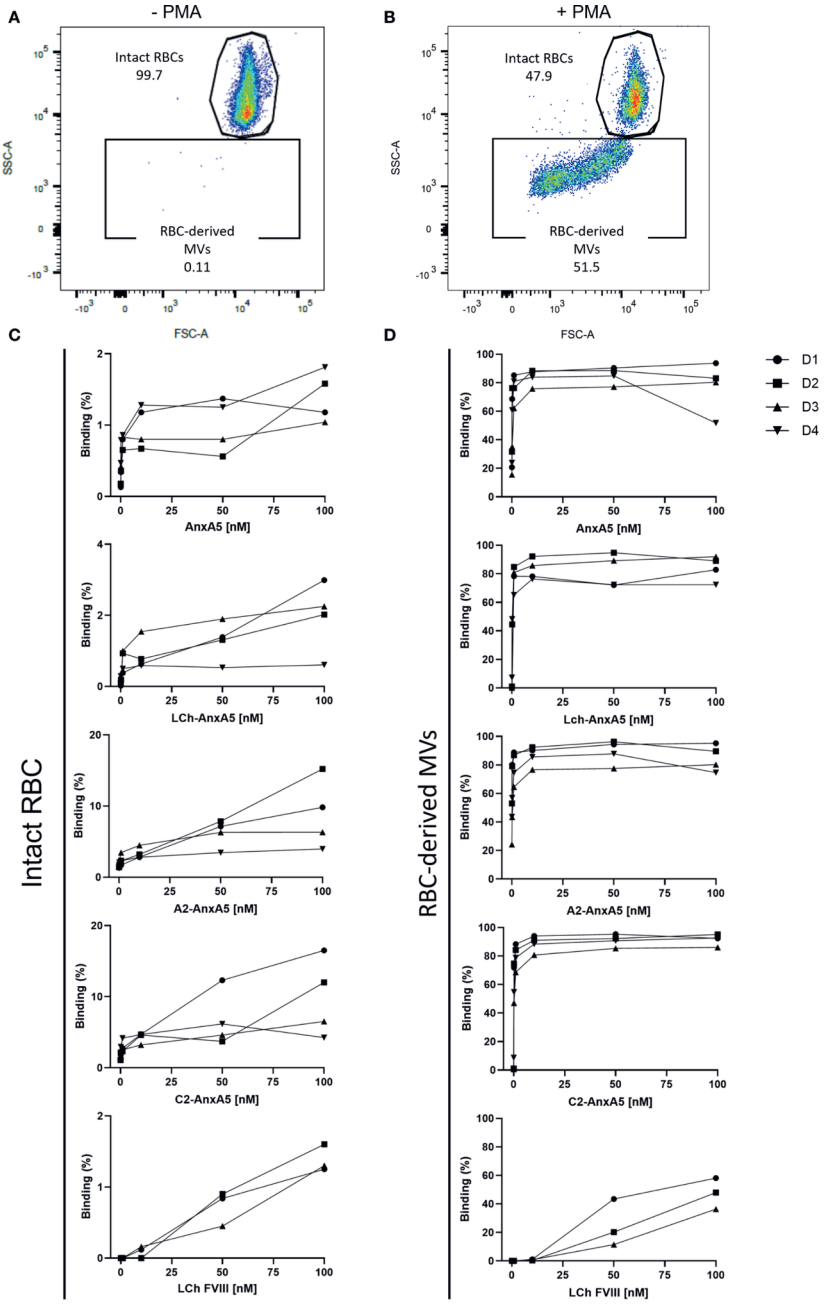


Figure 2. FVIII-AnxA5 fusion proteins interaction with PS exposed red blood cells. The interaction of FVIII-AnxA5 fusion proteins with RBCs was evaluated using flow cytometry. Untreated RBCs were gated according to the forward (FSC) and side (SSC) scatter

parameters (A). Upon treatment with PMA, RBC-derived microvesicles (MVs) with a smaller size emerged (B). The binding of AnxA5 only, LCh- AnxA5, A2- AnxA5, C2- AnxA5, and LCh- AnxA5 to intact (C) and RBC-derived microvesicles (MVs) (D) was expressed as percentage of cells positive to the fusion protein signal. AnxA5 and all the FVIII- AnxA5 fusion proteins bound to vesiculating RBCs, while their binding to intact RBCs was minimal.

Phagocytosis of PS-exposed red blood cells by macrophages

The clearance of aged RBCs is mediated by red pulp macrophages in the spleen (54). To model this process *in vitro*, monocyte-derived macrophages were incubated with AnxA5-loaded RBCs at macrophage:RBC ratios of 1:20, 1:50, and 1:100 for 0, 1, 4, and 24 hours (Figure3). RBCs were first labeled with the DiD membrane dye and subsequently treated with PMA, generating a mixed population of DiD-labelled intact RBCs and RBC-derived MVs. Confocal microscopy was used to visualize uptake of the AnxA5-labeled RBC components by macrophages. At time zero, as expected, no internalization of DiD-labeled RBC components or AnxA5 fusion proteins was observed. By 1 hour, DiD signal became detectable within macrophages, indicating the onset of phagocytosis of RBC components. No consistent differences in uptake were observed for different macrophage:RBC ratios, so a ratio of 1:100 was used for all subsequent experiments. Internalization of DiD-labeled RBC components increased over time, with more macrophages exhibiting DiD signal at 4 and 24 hours when compared to earlier time points. The pattern of internalization varied among individual macrophages. Notably, at the 1 hour time point, AnxA5-AF555 was detected in a larger number of macrophages when compared to DiD-labelled RBC components (Figure3). Subsets of macrophages appeared to exclusively internalize AnxA5-AF555, whereas other macrophages contained primarily DiD-labelled RBC components. This difference may reflect distinct uptake kinetics or a possible dissociation of AnxA5 from RBC-derived MVs prior internalization, potentially induced by the relatively low calcium concentration in the IMDM medium used during co-incubation (~1.5 mM $CaCl_2$). For comparison, AnxA5 binding to RBC-derived microvesicles increases progressively with calcium, becoming detectable at 1 mM and reaching maximal levels around 5 mM (Supplementary Figure 2).

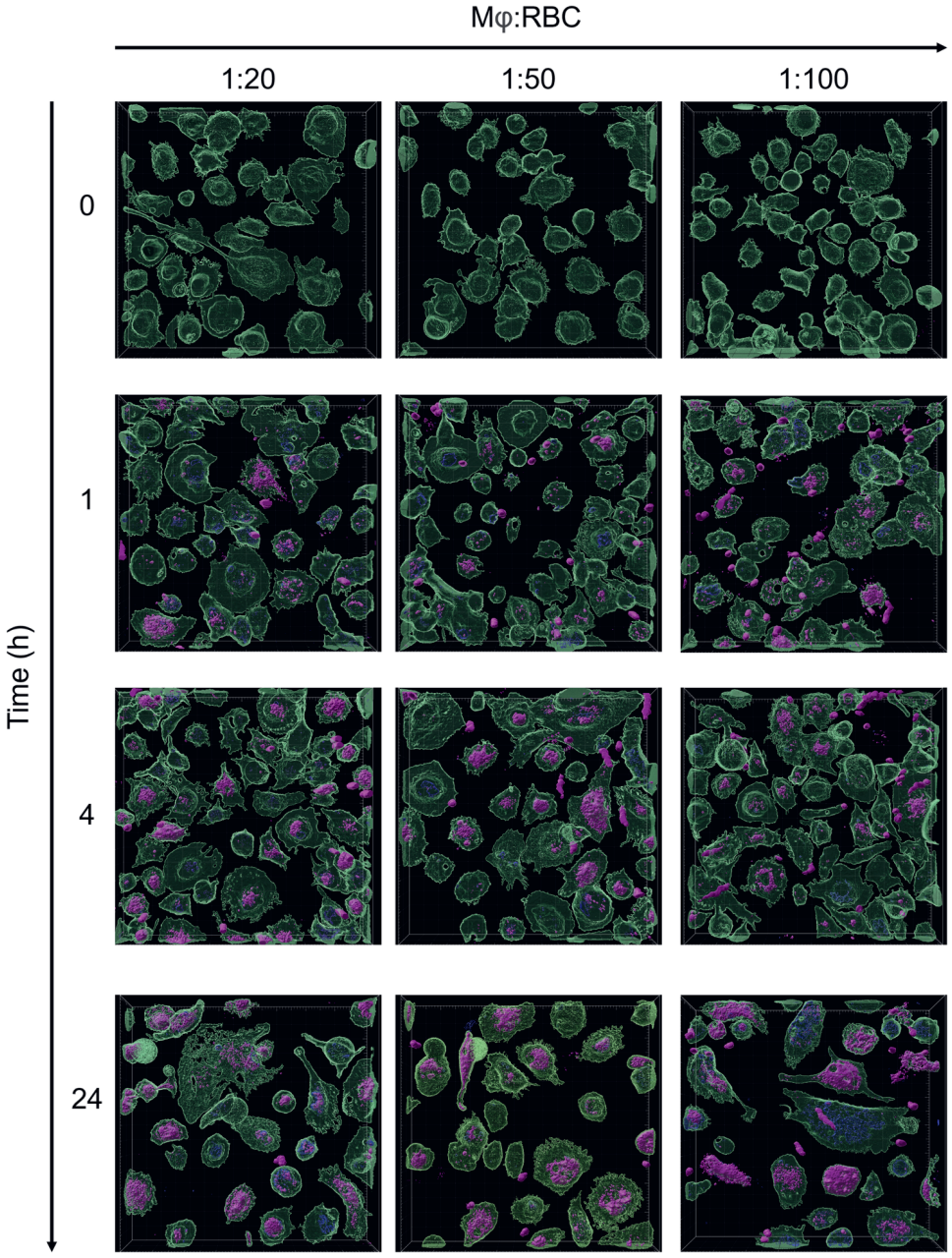


Figure 3. Phagocytosis of PS-exposing red blood cells by macrophages. RBCs were stained with Vibrand DiD (purple). Upon treatment with PMA, PS-exposing RBCs were loaded with AF555 labelled AnxA5 (blue). Vibrant DiO-labeled monocytes-derived macrophages (green) were incubated with AnxA5 loaded RBCs at Mϕ:RBC ratios of 1:20, 1:50 and 1:100

for 0, 1, 4 and 24 hours. After removing unbound RBCs, cells were fixed with 4% paraformaldehyde (PFA) and analyzed using the Zeiss LSM 980 with Airyscan 2. The uptake of RBCs with Mϕ was minimal at time point zero, with increasing binding and phagocytosis at the following time points. After 24 hours incubation, macrophages population containing AnxA5-AF555 and/or DiD-labelled RBC-components were observed at the Mϕ:RBC ratio 1:100.

FVIII-AnxA5 derived peptides presented on HLA-DR

To evaluate the potential of FVIII–AnxA5 fusion proteins to promote antigen presentation, monocyte-derived macrophages from two donors were incubated with purified FVIII-AnxA5 fusion proteins. The experimental conditions included macrophages alone, macrophages incubated with AnxA5, LCh–AnxA5, as well as A2–AnxA5 and C2–AnxA5 fusion proteins; factor VIII light chain (LCh) was included as a control. Upon overnight incubation with 100 nM of the different fusion proteins, macrophages were lysed and HLA-DR peptides complexes were purified using L243 Sepharose. Peptides were eluted from HLA-DR molecules and analyzed by mass spectrometry. Mass spectrometry data were processed using Proteome Discoverer which allowed for identification and quantification of peptides presented by macrophages. The number of identified proteins, peptides, and unique peptides per condition is summarized in the bar plot shown in Figure 4A and detailed in Supplementary Table 1A. Antigen processing results in multiple MHC class II binding peptides with overlapping sequences, due to progressive trimming at their N- and C-termini (55). This processing step generates peptide with a different length which all possess the core sequence stably bound within the MHC binding groove. Across both donors, an average of 1639 ± 304 unique peptides per condition was identified, indicating relatively low inter-donor variability. Importantly, peptide counts were consistent across conditions within each donor, reflecting robust and reproducible antigen presentation. We next examined whether peptides derived from the fusion proteins could be detected (Figure 4B; Supplementary Table 1A). Interestingly, AnxA5-derived peptides were already identified in unstimulated macrophages, likely reflecting the processing of endogenous AnxA5. Upon addition of exogenous AnxA5, the number of AnxA5-derived peptides increased substantially in both donors, indicating that the detected peptides originated from exogenously added AnxA5. When macrophages were incubated with LCh alone, the number of AnxA5-derived peptides was similar to the unstimulated condition, as expected. However, FVIII-derived peptides were detected, demonstrating effective presentation of LCh domain derived peptides on HLA-DR (Figure 4B; Supplementary Table 1A).

Focusing on the fusion proteins containing FVIII domains, AnxA5-derived peptides were consistently detected at similar levels across the LCh–AnxA5, A2–AnxA5, and C2–AnxA5 conditions in both donors, reflecting efficient processing of the AnxA5 domain within these fusion constructs. This consistency allowed us to concentrate on the detection and variability of FVIII-derived peptides (Figure 4B; Supplementary Table 1A). In macrophages stimulated with fusion proteins containing FVIII domains, detection of FVIII-derived peptides varied by domain and donor. For LCh–AnxA5, fusion to AnxA5 enhanced presentation of LCh-derived peptides compared to LCh alone. Similarly, stimulation with A2–AnxA5 led to consistent detection of peptides derived from the A2 domain. For C2–AnxA5, donor-specific differences emerged: donor 1 presented both C2 domain and AnxA5-derived peptides, whereas donor 2 predominantly displayed AnxA5 peptides with no detectable C2 domain derived peptides (Figure 4B; Supplementary Table 1A). This pattern corresponds to the reduced presentation of C2 domain derived peptides observed in donor 2 under the LCh–AnxA5 condition. Although HLA typing data were unavailable, these findings likely reflect individual variability in HLA class II-mediated antigen processing and presentation of FVIII-derived peptides. The dataset was re-analyzed using FragPipe to confirm the findings obtained using Proteome Discoverer. This re-analysis resulted in the identification of a higher number of proteins and an average of 2139 ± 388 unique peptides per condition (Supplementary Table 1A, Supplementary Figure 3A). Importantly, the number and identity of FVIII–AnxA5–derived peptides detected per condition and per donor were consistent with those identified using Proteome Discoverer (Supplementary Table 1A, Supplementary Figure 3B). The peptide sequences identified for each fusion protein, condition, and donor in both analyses are detailed in Supplementary Table 2 (AnxA5), Supplementary Table 3 (LCh–AnxA5), Supplementary Table 4 (A2–AnxA5), and Supplementary Table 5 (C2–AnxA5). These results demonstrate that FVIII–AnxA5 fusion proteins are effectively processed and presented by macrophages.

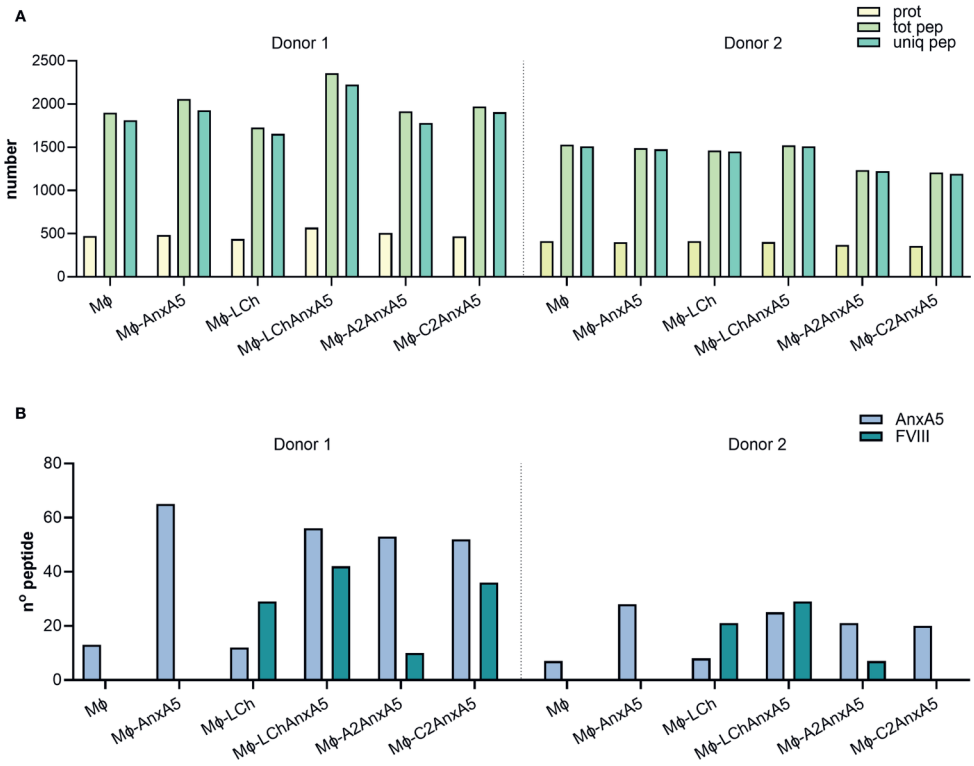


Figure 4. FVIII-Anx5 derived peptides presented on HLA-DR. To evaluate whether FVIII–Anx5 fusion proteins are processed and presented by macrophages, monocyte-derived macrophages from two donors were incubated with the following conditions: untreated (MO), Annexin A5 (MO+Anx5), FVIII light chain (MO+LCh), or FVIII–Anx5 fusion proteins (MO+LChAnx5, MO+A2Anx5, MO+C2Anx5), in the absence of RBCs. Data for the two donors are shown separately, divided by a dashed line. (A) Barplot showing the total number of proteins (prot, pale yellow-green), total peptides (tot pep, light green), and unique peptides (uniq pep, medium aquamarine) identified by mass spectrometry for each condition. (B) Barplot showing the number of peptides specifically derived from Annexin A5 (Anx5, light steel blue) and FVIII (teal blue). Peptide identification was performed using Proteome Discoverer.

Peptide presentation on macrophages incubated with FVIII-AnxA5 loaded RBCs

Building on our analysis of macrophages incubated directly with fusion proteins, we next evaluated antigen presentation following phagocytosis of RBCs and RBC-derived MVs. After uptake, macrophages process RBCs and RBC-derived MVs,

finally resulting in loading of RBC-derived peptides onto MHC class II. Experimental conditions included macrophages alone, macrophages incubated with PMA stimulated RBCs, and macrophages incubated with PMA stimulated RBCs pre-loaded with AnxA5, LCh–AnxA5, A2–AnxA5, or C2–AnxA5 fusion proteins. Following overnight incubation, peptides were eluted from HLA-DR molecules and analyzed by mass spectrometry. Data were processed using Proteome Discoverer. The number of identified proteins, peptides, and unique peptides per condition is summarized in Figure 5A and detailed in Supplementary Table 1B. Data from three donors allowed assessment of inter-donor variability. Across all donors, an average of 1462 ± 414 unique peptides were detected. Hemoglobin-derived peptides were identified even in conditions without added RBCs, likely due to hemolysis during PBMC isolation and subsequent uptake of lysed RBCs by monocytes. Importantly, pulsing of macrophages with RBCs resulted in significantly higher number of hemoglobin derived peptide presented on MHC class II (Figure 5B; Supplementary Table 1B). AnxA5-derived peptides were also detected in control conditions without the addition of FVIII-AnxA5 fusion proteins, consistent with prior findings (Figure 5C; Supplementary Table 1B). Addition of FVIII–AnxA5 fusion proteins resulted in AnxA5-derived peptide presentation at levels similar to control conditions lacking FVIII-AnxA5 fusion proteins. Detection of FVIII domain peptides varied among donors and fusion constructs: donor 3 presented one peptide each from LCh–AnxA5 and A2–AnxA5; donor 4 had no detectable FVIII peptides; donor 5 showed two peptides from A2–AnxA5 (Figure 5C; Supplementary Table 1B). To further evaluate these findings, we applied FragPipe to the dataset. This approach revealed a broader proteome coverage, identifying more proteins and an average of 2,059 ± 527 unique peptides per condition, as shown in Supplementary Figure 4A and Supplementary Table 1B. Despite the higher number of peptides identified, the number of hemoglobin- and AnxA5-derived peptides remained similar to that identified with Proteome Discoverer (Supplementary Figures 4B, C). Notably, FVIII-derived peptides were not detected in any condition, with the exception of a single LCh-derived peptide observed in donor 3. Peptide sequences for both analyses are provided in Supplementary Tables 2-5, where FVIII-derived peptides previously reported as immunogenic are underlined. These results demonstrate that macrophages efficiently process and present RBC-derived peptides. However, FVIII–AnxA5 peptide presentation is markedly less efficient when delivered via RBCs compared to direct loading of fusion proteins onto macrophages.

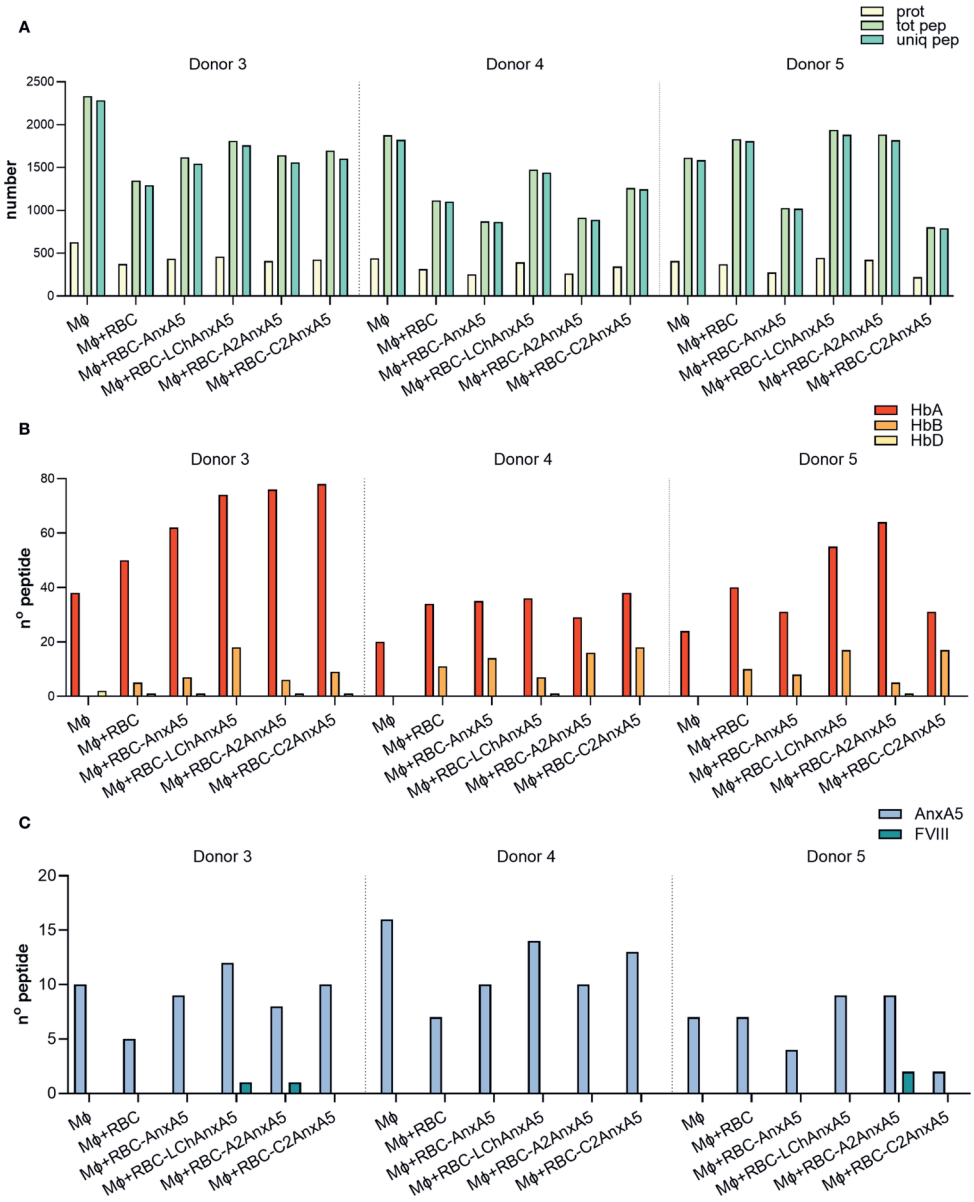


Figure 5. FVIII-AnxA5 loaded RBCs derived peptides presented on HLA-DR. To assess antigen presentation in the context of FVIII–AnxA5–loaded RBCs, monocyte-derived macrophages (Mϕ) from three donors were incubated overnight with medium only, untreated RBCs or RBCs pre-loaded with Annexin A5 (AnxA5), LCh– AnxA5, A2– AnxA5, or C2– AnxA5 fusion proteins. Data are presented separately for two donors, divided by a dashed line. (A) Barplot showing the total number of proteins (prot, pale yellow-green),

total peptides (tot pep, light green), and unique peptides (uniq pep, medium aquamarine) identified by mass spectrometry for each condition. (B) Barplot showing the number of peptides derived from hemoglobin alpha (HbA, pale yellow), beta (HbB, orange-yellow), and delta (HbD, vivid red-orange) chains. (C) Barplot showing the number of peptides specifically derived from Annexin A5 (Anx5, light steel blue) and FVIII (teal blue). Peptide identification was performed using Proteome Discoverer.

Discussion

The development of inhibitors remains a major complication in FVIII replacement therapy for patients with hemophilia A (5). While non-factor replacement therapies such as emicizumab have recently emerged, FVIII administration remains indispensable in certain clinical situations such as acute bleeding or surgical interventions (16). This underscores the continued need for strategies aimed at modulating the immune response to FVIII and promoting antigen-specific tolerance. To this end, we designed fusion proteins combining the highly immunogenic A2, C1, and C2 domains of FVIII with Annexin A5 (AnxA5), a protein with high affinity for phosphatidylserine (PS), resulting in the constructs A2–AnxA5, C2–AnxA5, and A3–C1–C2 light chain–AnxA5 (LCh–AnxA5) (49, 56). PS is externalized on the surface of apoptotic cells, which are typically cleared by phagocytes in a non-inflammatory manner (57). It is well-established that the binding of AnxA5 to PS is calcium-dependent; elegant studies by Andree et al. shown that at 1 mM Ca2+ significant amount of AnxA5 can bind to phospholipid surfaces containing 5% of PS (53). In agreement with this, we showed that AnxA5 can bind to RBC-derived MVs in medium containing 1 mM Ca2+ (Supplementary Figure 2). Binding of the FVIII-AnxA5 fusion proteins to biological PS-containing membranes in vivo would be highly dependent on local Ca2+ concentrations. Therefore, we expect that FVIII-AnxA5 fusion proteins will not fully cover PS-containing membranes on apoptotic cells.

The FVIII-AnxA5 fusion proteins generated in this study bind highly efficient to immobilized PS. We compared the efficacy of PS binding of FVIII-AnxA5 fusion proteins with that of isolated FVIII light chain (LCh). FVIII LCh binds PS through its carboxy-terminal C1 and C2 domains (50, 51, 58). Our data show that FVIII-AnxA5 fusion proteins bind more efficiently to PS when compared to FVIII LCh. Based on this observation, we expect that FVIII-AnxA5 fusion proteins will readily bind to PS containing cellular membranes in vivo. We further demonstrated that fusion proteins are efficiently taken up by macrophages. Mass spectrometry analysis confirmed that macrophages process and present peptides derived from both FVIII and Annexin A5, suggesting successful antigen delivery into the MHC class II pathway. This finding is particularly relevant given that PS-mediated clearance is

primarily executed by tolerogenic macrophage subsets, such as red pulp macrophages (RPMs) in the spleen and Kupffer cells (KCs) in the liver (59). These macrophage populations specialize in the non-inflammatory clearance of apoptotic and aged cells, and they are known to promote immune tolerance through secretion of anti-inflammatory cytokines (IL-10, TGF-β) and the induction of regulatory T cells (Tregs) (35, 42). By fusing FVIII to Annexin A5, we label apoptotic cargo and aim to shift FVIII recognition away from immunogenic pathways, such as marginal zone B-cell/cDC-mediated presentation, toward tolerogenic routes dominated by RPMs and KCs (23).

Supporting this hypothesis, previous studies have demonstrated that PS-containing liposomes loaded with recombinant human acid alpha-glucosidase and FVIII can promote tolerance (60, 61). This tolerogenic effect of PS is antigen-specific and involves increased secretion of TGF- β by antigen presenting cells and promotes expansion of regulatory T cells (60, 61). The core domain of AnxA5 appears responsible for tolerogenic effects on dendritic cells, inhibiting pro-inflammatory responses and costimulatory molecule expression (62). Likewise, binding of nanoparticles coated with the AnxA1-core domain to dendritic cells induced antigen-specific immunosuppression by promoting an anergy-like state in CD4+ T cells (63). Literature data suggest that AnxA5 itself plays a multifaceted role in modulating immune responses to dying cells. It has been postulated that the binding of AnxA5 to PS exposed on apoptotic and necrotic cells may potentially interfere with their immunosuppressive effects (64). AnxA5 knockout mice show reduced immune reactions against allogeneic necrotic cells and increased anti-inflammatory responses of macrophages, suggesting that endogenous AnxA5 promotes inflammation (65). AnxA5 can also function as an immune checkpoint inhibitor by blocking PS-mediated immunosuppression in the tumor microenvironment, enhancing anti-tumor immunity (66). Together these findings indicate that AnxA5 has context-dependent effects on immunotolerance, potentially acting as both pro-inflammatory and immunosuppressive mediators depending on the specific cellular environment and experimental conditions. Our findings suggest that AnxA5-chimeras may have potential therapeutic applications in modulating immune responses to protein-based therapeutics.

Interestingly, our data show that FVIII-AnxA5 fusion proteins were efficiently processed and presented on MHC class II. Also, RBCs and RBC-derived MVs were efficiently processed by macrophages resulting in presentation of RBC-derived peptides on MHC class II. RBCs loaded with FVIII-AnxA5 fusion proteins were also efficiently internalized by macrophages. Under this experimental condition, a very limited number of FVIII-AnxA5 derived peptides were presented on MHC class II.

We hypothesize that the absence of FVIII-derived peptides in the FVIII-AnxA5 loaded RBCs is due to limited surface binding of FVIII-AnxA5 fusion proteins to PS-containing RBC membranes. Alternatively, FVIII-AnxA5 derived peptides may not efficiently compete with abundantly MHC class II presented endogenous or RBC-derived peptides. Follow-up studies are needed to further optimize RBC mediated targeting of FVIII-AnxA5 fusion proteins generated in this study. Notably, several of the FVIII-derived peptides identified correspond to epitopes previously reported as immunogenic, highlighting the potential of FVIII-AnxA5 fusion proteins to ultimately promote antigen-specific tolerance toward clinically relevant FVIII epitopes (67–77).

In conclusion, our studies confirm efficient uptake of FVIII-AnxA5 fusion proteins and MHC class II peptide presentation by macrophages. Our approach provides a potential basis for the design on a platform to mitigate FVIII immunogenicity. Future studies will be needed to clarify whether FVIII–AnxA5–loaded macrophages induce tolerogenic T-cell responses, such as anergy or regulatory T-cell expansion, or instead trigger immune activation, and subsequently validate these immune-modulatory effects in vivo. Together, our findings provide an avenue for the development of a PS-targeted antigen delivery system for the induction of tolerance in hemophilia A, and potentially also other antibody mediated disorders

Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD068424 [1] Perez-Riverol Y, Bandla C, Kundu DJ, Kamatchinathan S, Baia J, Hewapathirana S, John NS, Prakash A, Walzer M, Wang S, Vizcaíno JA. The PRIDE database at 20 years: 2025 update. Nucleic Acids Res. 2025 Jan 6;53(D1):D543-D553. doi: 10.1093/nar/gkae1011

Ethics statement

The studies involving humans were approved by Sanquin Ethical Advisory Board. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in the study were obtained from healthy volunteers. Blood was drawn from healthy volunteers in accordance with Dutch regulations and following approval from Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

MM: Writing – original draft, Writing – review & editing, Conceptualization, Investigation. ML: Writing – review & editing, Conceptualization, Investigation. CZ: Writing – review & editing, Methodology. RB: Writing – review & editing. CR: Writing – review & editing. KF: Writing – review & editing. AH: Writing – review & editing. MB: Writing – review & editing. JV: Writing – review & editing, Writing – original draft, Conceptualization, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 4

Supplementary Data

AnxA5:

MAQVLRGTVTDFPGFDERADAETLRKAMKGLGTDEESILTLLTSRSNAQRQEISAAFKTLFGRDLLDDLKSELTGKFEKLIVALMKPSRLYDAYELKHA
LKGAGTNEKVLTEIIASRTPEELRAIKQVYEEEYGSSLEDDVVGDTSGYYQRMLVVLLQANRDPDAGIDEAQVEQDAQALFQAGELKWGTDEEKFITI
FGTRSVSHLRKVFDKYMTISGFQIEETIDRETSGNLEQLLLAVVKSIRSIPAYLAETLYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKEFRKNFATSLYS
MIKGDTSGDYKKALLLLCGEDDARGHPFEGKPIPNPLLGLDSTRTGWSHPQFEKGGGSGGGSGGSAWSHPQFEK

LCh:

METDTLLLWVLLLWVPGSTGDEITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQ
SGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQ
HHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKEN
YRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGM
STLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDG
KKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPS
KARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLD
PPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLYARGHPFEGKPIPNPLLGLDSTRTGWSHPQFEKGGGSGGGSGGSAWSHPQFEK

A2- AnxA5:

SVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQAS
RPYNIYPHGITDVRPLYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLIGPLLICYKESVDQRGNQIMSD
KRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLEDPEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTL
TLFPFSGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAIEPAGGGGSGGGGSGGGGSMAQVLRG
TVTDFPGFDERADAETLRKAMKGLGTDEESILTLLTSRSNAQRQEISAAFKTLFGRDLLDDLKSELTGKFEKLIVALMKPSRLYDAYELKHALKGAGTNE
KVLTEIIASRTPEELRAIKQVYEEEYGSSLEDDVVGDTSGYYQRMLVVLLQANRDPDAGIDEAQVEQDAQALFQAGELKWGTDEEKFITIFGTRSVSH
LRKVFDKYMTISGFQIEETIDRETSGNLEQLLLAVVKSIRSIPAYLAETLYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKEFRKNFATSLYSMIKGDTSGD
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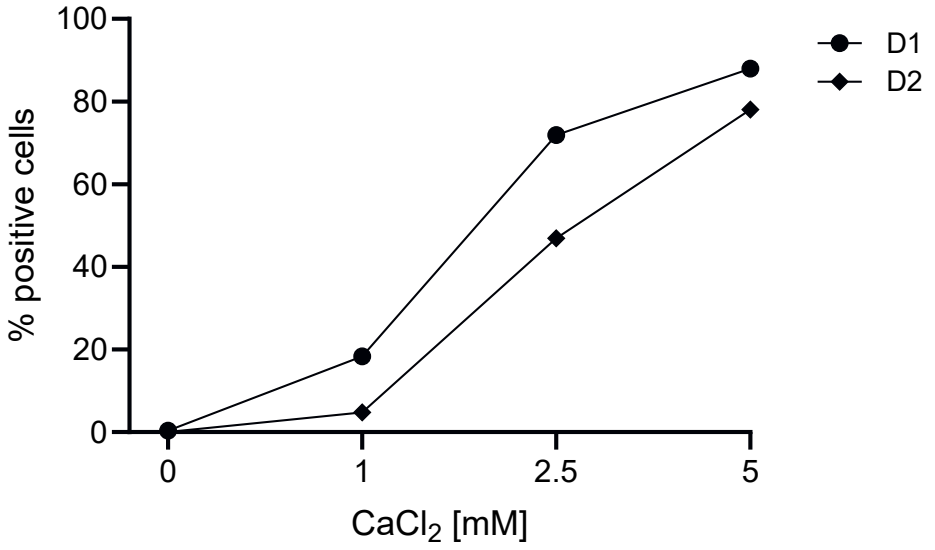
C2- AnxA5:

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EIIASRTPEELRAIKQVYEEEYGSSLEDDVVGDTSGYYQRMLVVLLQANRDPDAGIDEAQVEQDAQALFQAGELKWGTDEEKFITIFGTRSVSHLRKV
FDKYMTISGFQIEETIDRETSGNLEQLLLAVVKSIRSIPAYLAETLYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKEFRKNFATSLYSMIKGDTSGDYKK
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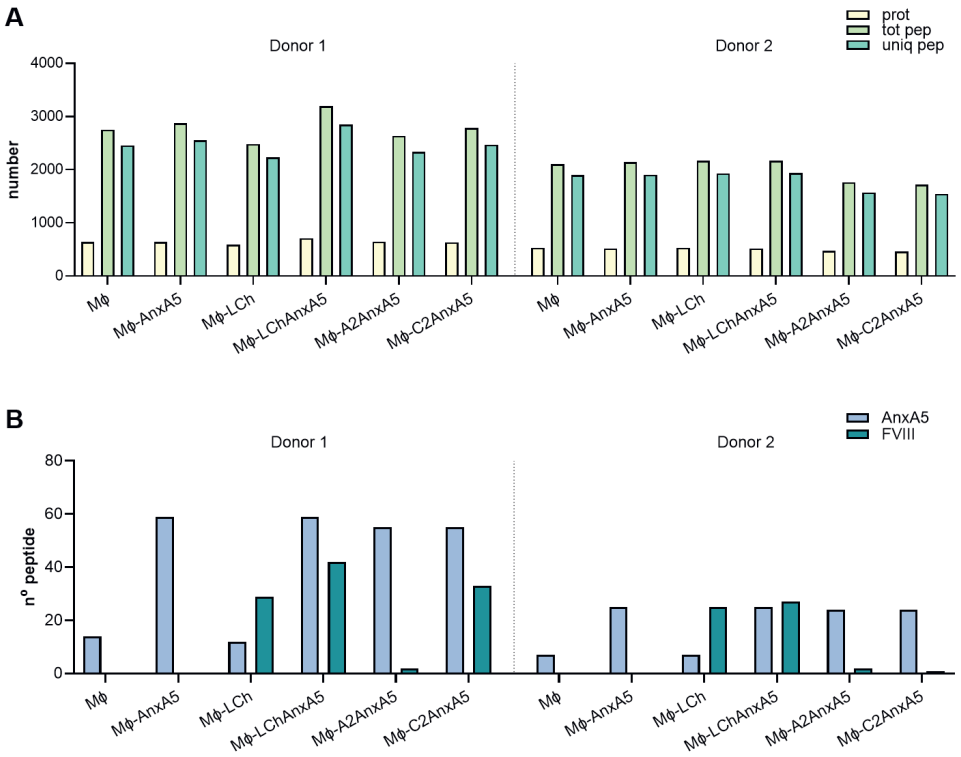
LCh- AnxA5:

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SGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQ
HHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKEN
YRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGM
STLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDG
KKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPS
KARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLD
PPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLYGGGGSGGGGSGGGGSMAQVLRGTVTDFPGFDERADAETLRKAMKGLGTDEESILTLLTSRS
NAQRQEISAAFKTLFGRDLLDDLKSELTGKFEKLIVALMKPSRLYDAYELKHALKGAGTNEKVLTEIIASRTPEELRAIKQVYEEEYGSSLEDDVVGDTSG
YYQRMLVVLLQANRDPDAGIDEAQVEQDAQALFQAGELKWGTDEEKFITIFGTRSVSHLRKVFDKYMTISGFQIEETIDRETSGNLEQLLLAVVKSIR
SIPAYLAETLYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKEFRKNFATSLYSMIKGDTSGDYKKALLLLCGEDDARGHPFEGKPIPNPLLGLDSTRTGW
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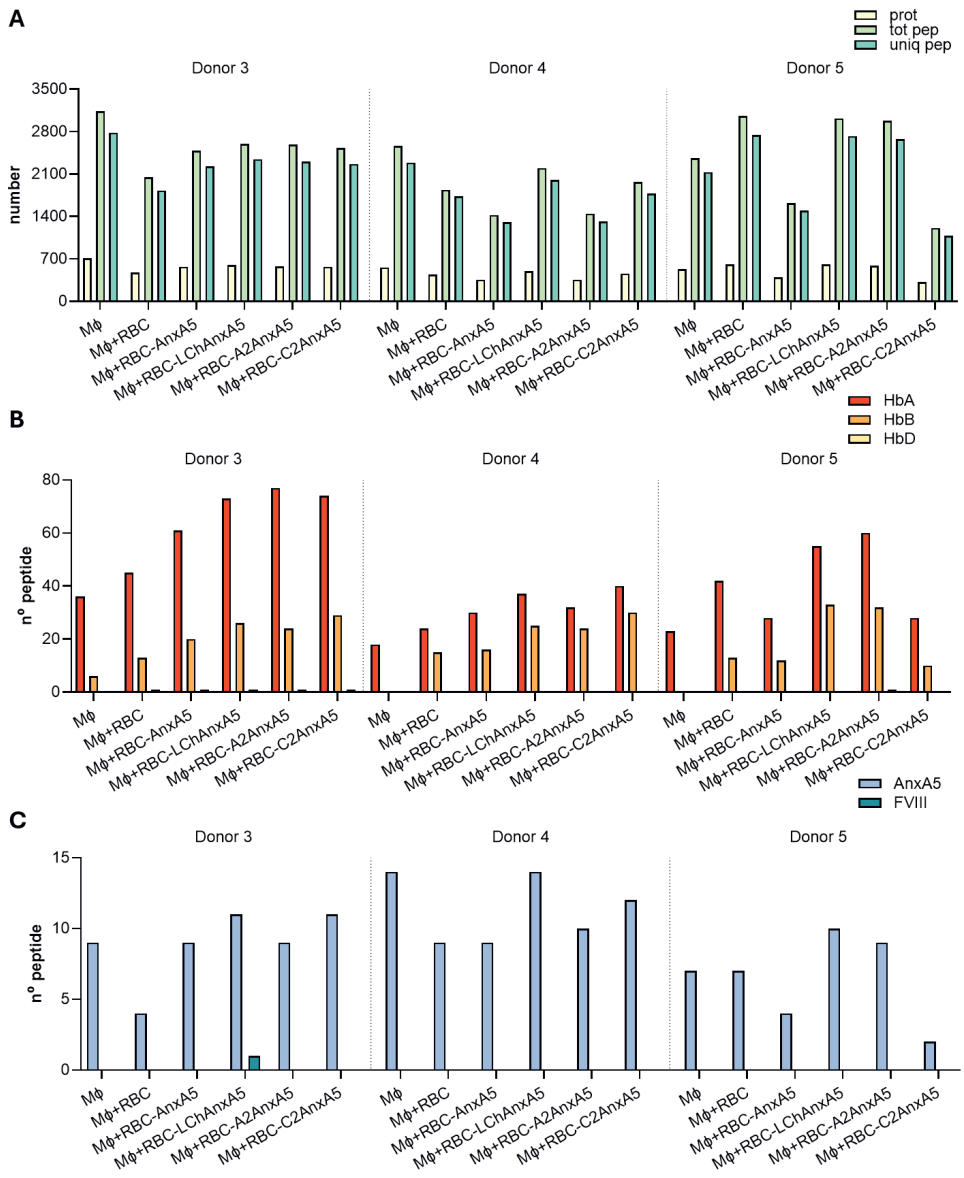
Supplementary Figure 1. Amino acid sequences of the designed FVIII-AnxA5 fusion proteins. The A2, C2, and light chain (LCh) domains of FVIII (blue) were fused to annexin A5 (AnxA5, green) via a flexible linker (orange). A C-terminal V5 tag and Twin-Strep tag (black) were introduced for detection and purification purposes.



Supplementary Figure 2. Calcium-dependent binding of AnxA5 fusion proteins to RBC-derived microvesicles. The percentage of RBC-derived microvesicles bound by AnxA5 increases with $CaCl_2$ concentration for both donors (D1 and D2), with detectable binding from 1 mM and a progressive rise up to 5 mM, consistent with calcium-dependent interaction dynamics.



Supplementary Figure 3: **FVIII-AnxA5 derived peptides presented on HLA-DR identified using FragPipe.** To further characterize FVIII-AnxA5 presentation, HLA-DR–associated peptides previously identified using Proteome Discoverer in figure 4 were reanalyzed using FragPipe. Data from the two donors are separated by a dashed line. (A) Barplot showing the total number of proteins (prot, pale yellow-green), total peptides (tot pep, light green), and unique peptides (uniq pep, medium aquamarine) identified by mass spectrometry for each condition. (B) Barplot showing the number of peptides specifically derived from Annexin A5 (Anx5, light steel blue) and FVIII (teal blue).



Supplementary Figure 4: FVIII-Anx5 loaded RBCs derived peptides presented on HLA-DR analyzed using FragPipe. To further characterize antigen presentation in the context of FVIII–Anx5–loaded RBCs, HLA-DR–associated peptides previously identified using Proteome Discoverer in figure 5 were reanalyzed using FragPipe. Monocyte-derived macrophages from three donors were incubated overnight with medium only, untreated

RBCs, or RBCs pre-loaded with Annexin A5 (Anx5), LCh–Anx5, A2–Anx5, or C2–Anx5 fusion proteins. Data are shown separately for two donors, separated by a dashed line. (A) Barplot showing the total number of proteins (prot, pale yellow-green), total peptides (tot pep, light green), and unique peptides (uniq pep, medium aquamarine) identified by mass spectrometry for each condition. (B) Barplot showing the number of peptides derived from hemoglobin alpha (HbA, pale yellow), beta (HbB, orange-yellow), and delta (HbD, vivid red-orange) chains. (C) Barplot showing the number of peptides specifically derived from Annexin A5 (Anx5, light steel blue) and FVIII (teal blue).

A

Donor	Sample	Proteome discoverer							FragPipe						
		prot	tot pep	uniq pep	AnxA5 pep	FVIII pep	Tag pep		prot	tot pep	uniq pep	AnxA5 pep	FVIII pep	Tag pep	
1	MO only	474	1898	1812	13	0			637	2753	2456	14	0		
	MO+AnxA5	484	2058	1927	65	0	1		638	2871	2553	59	0		
	MO+LCh	439	1728	1655	12	29	1		587	2480	2230	12	29	1	
	MO+LChAnxA5	570	2358	2226	56	42	1		710	3199	2846	59	42	1	
	MO+A2AnxA5	508	1916	1780	53	10			644	2639	2339	55	2		
	MO+C2AnxA5	470	1970	1906	52	36			634	2784	2469	55	33		
2	MO only	413	1531	1509	7	0			530	2106	1895	7	0		
	MO+AnxA5	402	1491	1477	28	0	1		520	2142	1904	25	0		
	MO+LCh	413	1464	1450	8	21	1		530	2164	1929	7	25	1	
	MO+LChAnxA5	403	1521	1511	25	29	2		522	2168	1935	25	27	1	
	MO+A2AnxA5	370	1235	1223	21	7			470	1760	1570	24	2		
	MO+C2AnxA5	358	1208	1190	20	0			463	1719	1544	24	1		

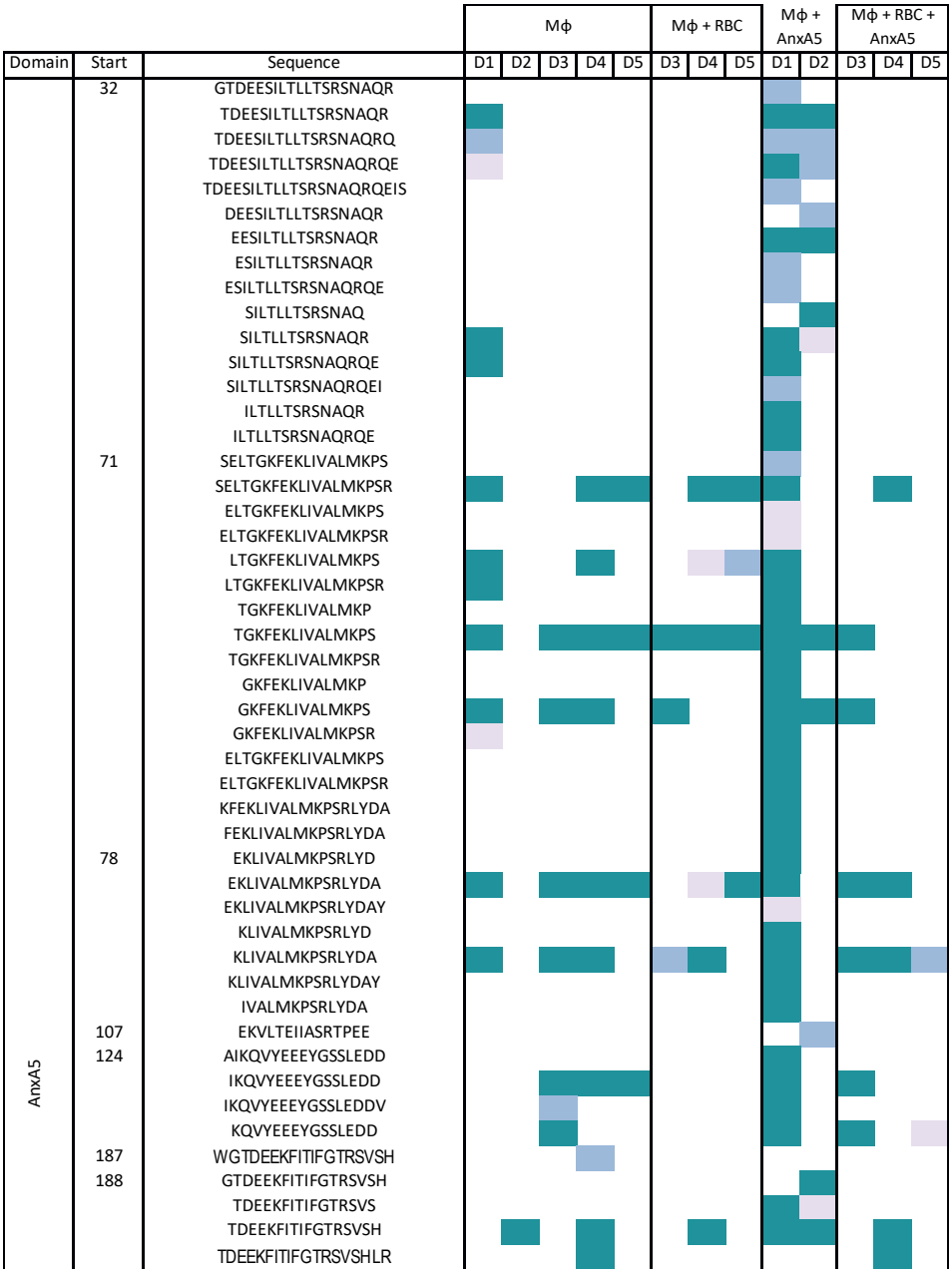
B

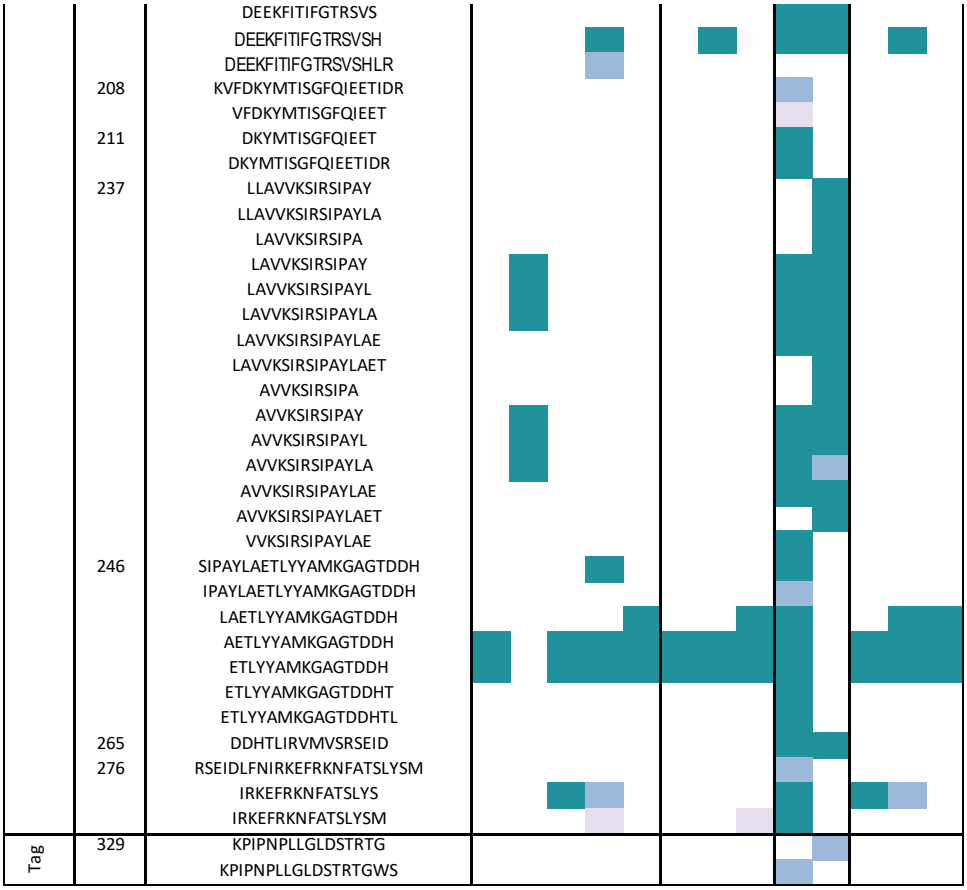
Donor	Sample	Proteome discoverer								FragPipe								
		prot	tot pep	uniq pep	HbA pep	HbB pep	HbD pep	AnxA5 pep	FVIII pep	Tag pep	prot	tot pep	uniq pep	HbA pep	HbB pep	HbD pep	AnxA5 pep	FVIII pep	Tag pep
3	MO	628	2335	2284	38	2	10	0	0	0	714	3137	2782	36	6	0	9	0	0
	MO+RBC	373	1346	1294	50	5	1	5	0	0	475	2046	1826	45	13	1	4	0	0
	MO+RBC+AnxA5	437	1619	1545	62	7	1	9	0	0	572	2483	2230	61	20	1	9	0	0
	MO+RBC+LChAnxA5	461	1811	1759	74	18	0	12	1	0	600	2599	2347	73	26	1	11	1	0
	MO+RBC+A2AnxA5	409	1645	1560	76	6	1	8	1	0	580	2589	2309	77	24	1	9	0	0
	MO+RBC+C2AnxA5	426	1698	1603	78	9	1	10	0	0	570	2527	2267	74	29	1	11	0	0
4	MO	440	1878	1823	20	0	0	16	0	0	562	2564	2292	18	0	0	14	0	0
	MO+RBC	316	1118	1101	34	11	0	7	0	0	444	1839	1732	24	15	0	9	0	0
	MO+RBC+AnxA5	253	872	863	35	14	0	10	0	0	358	1424	1306	30	16	0	9	0	0
	MO+RBC+LChAnxA5	394	1475	1442	36	7	1	14	0	0	500	2201	2003	37	25	0	14	0	0
	MO+RBC+A2AnxA5	264	913	888	29	16	0	10	0	0	357	1448	1321	32	24	0	10	0	0
	MO+RBC+C2AnxA5	345	1261	1246	38	18	0	13	0	0	459	1966	1780	40	30	0	12	0	0
5	MO	409	1614	1587	24	0	0	7	0	0	533	2363	2133	23	0	0	7	0	0
	MO+RBC	372	1833	1809	40	10	0	7	0	0	608	3061	2743	42	13	0	7	0	0
	MO+RBC+AnxA5	275	1028	1020	31	8	0	4	0	0	397	1623	1499	28	12	0	4	0	0
	MO+RBC+LChAnxA5	447	1938	1883	55	17	0	9	0	0	610	3016	2726	55	33	0	10	0	0
	MO+RBC+A2AnxA5	424	1885	1817	64	5	1	9	2	0	589	2978	2677	60	32	1	9	0	0
	MO+RBC+C2AnxA5	222	803	791	31	17	0	2	0	0	319	1211	1080	28	10	0	2	0	0

Supplementary Table 1. Peptides presentation on HLA-DR. Monocyte-derived macrophages from five donors were incubated under different conditions to assess HLA-DR–restricted antigen presentation. Macrophages from Donors 1 and 2 were treated with medium only, Annexin A5 (Anx5), FVIII light chain (LCh), or FVIII–Anx5 fusion proteins (LCh–Anx5, A2–Anx5, and C2–Anx5). Macrophages from Donors 3, 4, and 5 were treated with medium only, untreated RBCs, or RBCs pre-loaded with Anx5, LCh–Anx5, A2–Anx5, or C2–Anx5 fusion proteins.

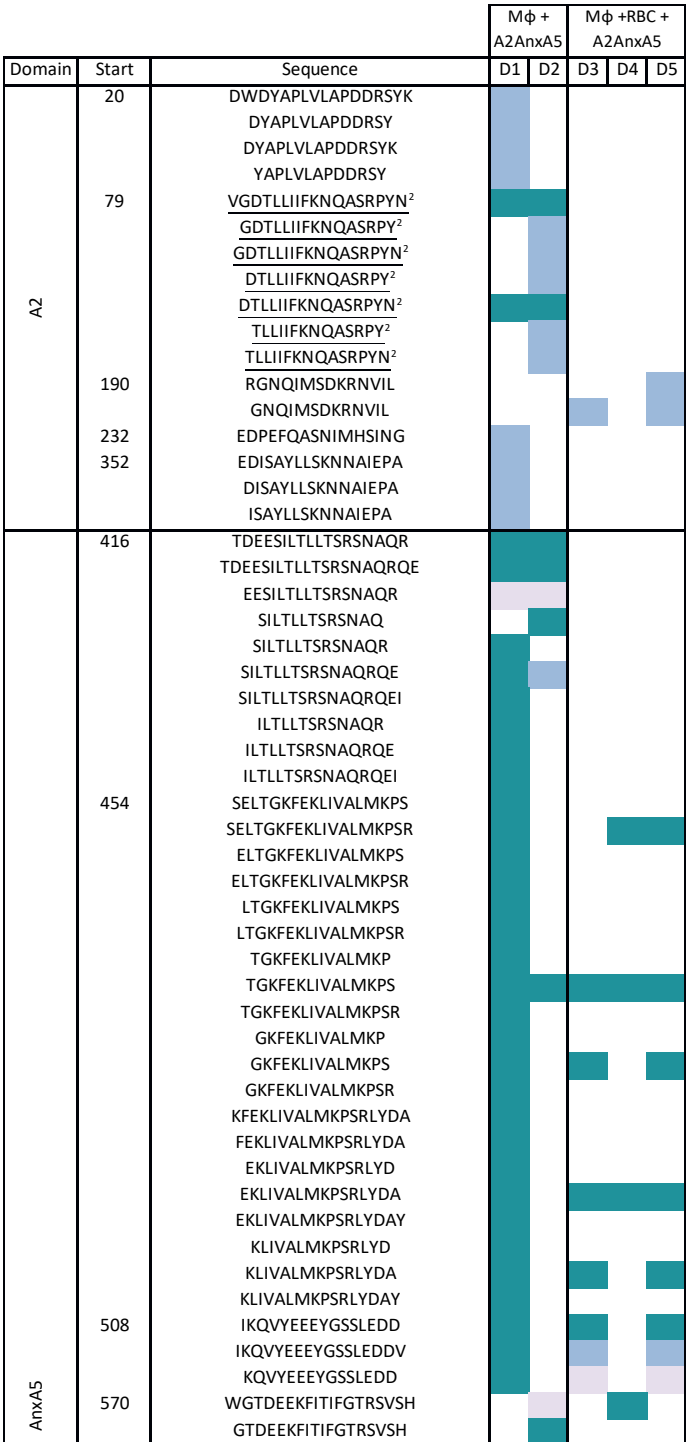
Table A summarizes data from Donors 1 and 2, including the number of proteins (prot), total peptides (tot pep), unique peptides (uniq pep), Annexin A5 peptides (Anx5 pep), and FVIII peptides (FVIII pep).

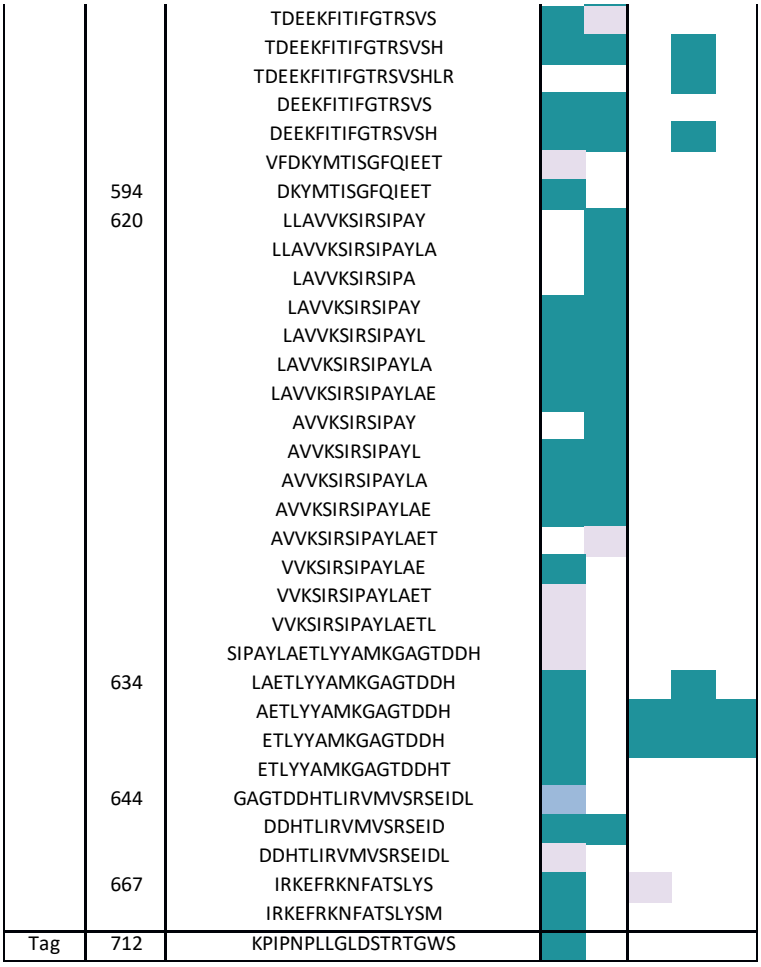
Table B summarizes data from Donors 3, 4, and 5, including proteins (prot), total peptides (tot pep), unique peptides (uniq pep), hemoglobin alpha, beta, and delta peptides (HbA pep, HbB pep, HbD pep), Annexin A5 peptides (Anx5 pep), and FVIII peptides (FVIII pep). For both tables, results from two independent analysis pipelines are shown side-by-side: left, Proteome Discoverer; right, FragPipe.


Mϕ
Mϕ + RBC
Mϕ + AnxA5
Mϕ + RBC + AnxA5
Domain
Start
Sequence
D1 D2 D3 D4 D5 D3 D4 D5 D1 D2 D3 D4 D5
AnxA5
32 GTDEESILTLLTSRSNAQR
TDEESILTLLTSRSNAQR
TDEESILTLLTSRSNAQRQ
TDEESILTLLTSRSNAQRQE
TDEESILTLLTSRSNAQRQEIS
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TDEEKFITIFGTRSVSHLR

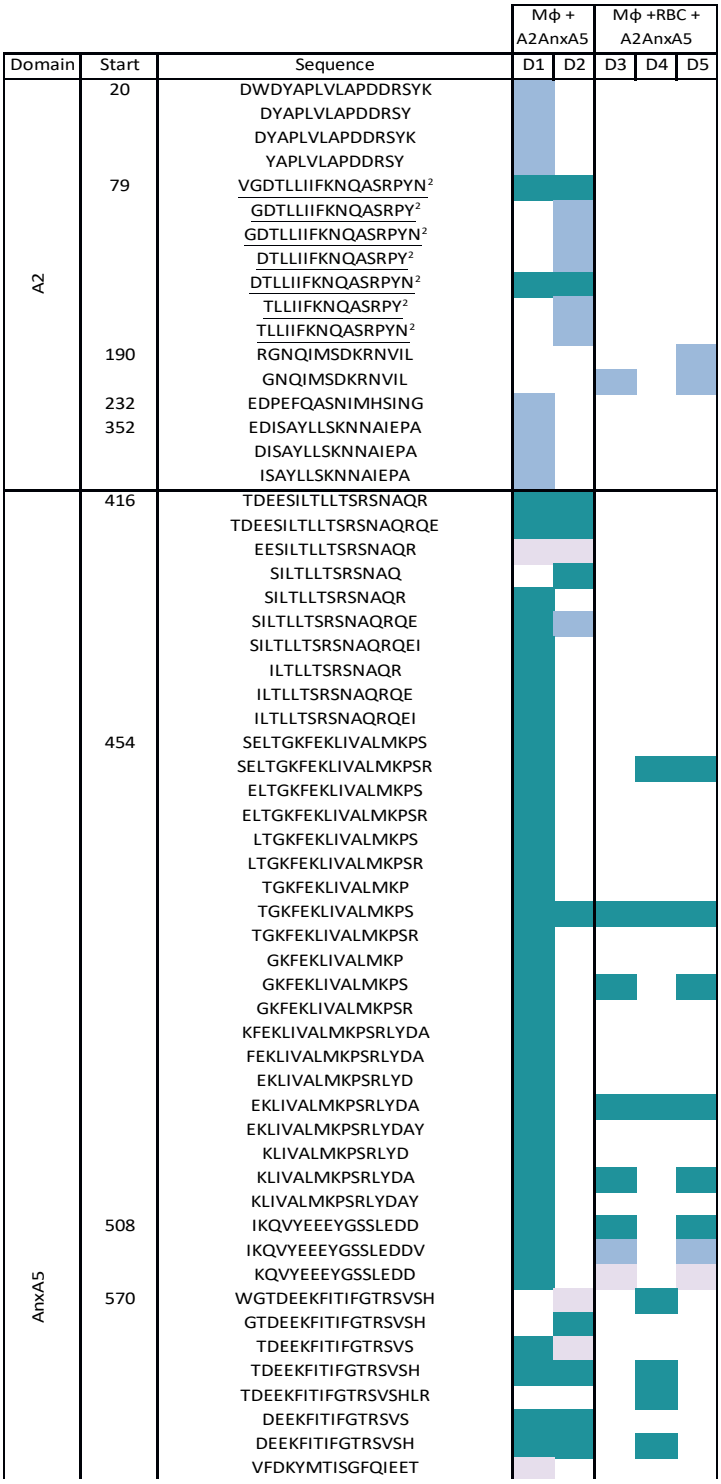


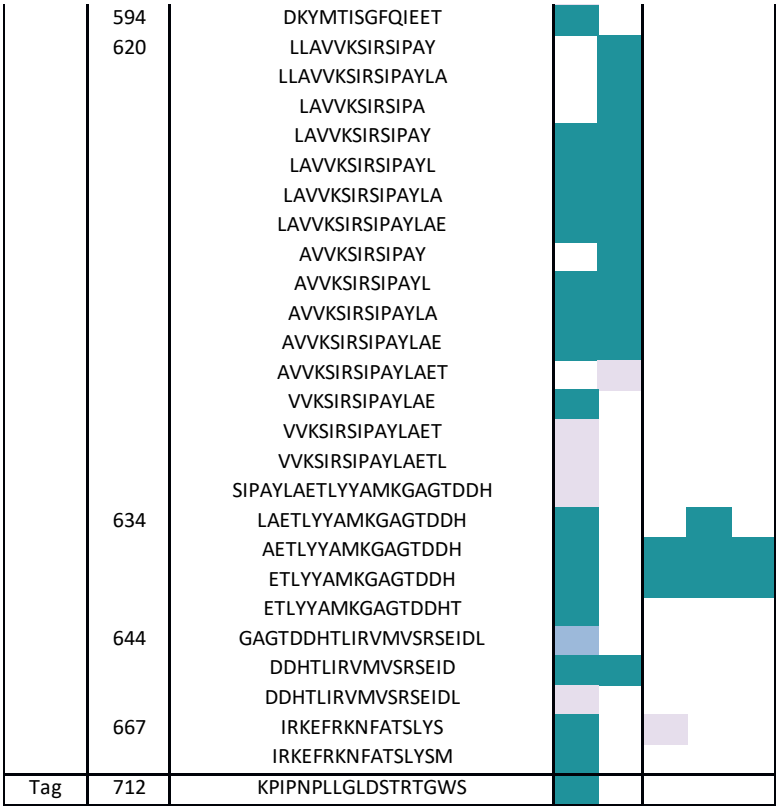
Supplementary Table 3. LCh-Annexin A5–derived peptides presented on HLA-DR. LCh-Annexin A5 (AnxA5)–derived peptides presented on HLA-DR molecules were identified across different donors (D1–D5) and experimental conditions: macrophages with LCh only (Mϕ+LCh), LCh-AnxA5 (Mϕ+LCh-AnxA5), or RBCs pre-loaded with LCh-AnxA5 (Mϕ+RBC+LCh-AnxA5). For each peptide, the corresponding domain, starting position, and amino acid sequence are reported. Peptides identified for a specific condition and donor are shown in dusty blue if detected only with Proteome Discoverer, in pale lilac if detected only with FragPipe, and in deep cyan if detected with both tools. Immunogenic FVIII peptides are underlined (1–11).


Mϕ + A2AnxA5
Mϕ +RBC + A2AnxA5
Domain
Start
Sequence
D1
D2
D3
D4
D5
A2
AnxA5
20 DWDYAPLVLAPDDRSYK
DYAPLVLAPDDRSY
DYAPLVLAPDDRSYK
YAPLVLAPDDRSY
79 VGDTLLIIFKNQASRPYN[2]
GDTLLIIFKNQASRPY[2]
GDTLLIIFKNQASRPYN[2]
DTLLIIFKNQASRPY[2]
DTLLIIFKNQASRPYN[2]
TLLIIFKNQASRPY[2]
TLLIIFKNQASRPYN[2]
190 RGNQIMSDKRNVIL
GNQIMSDKRNVIL
232 EDPEFQASNIMHSING
352 EDISAYLLSKNNAIEPA
DISAYLLSKNNAIEPA
ISAYLLSKNNAIEPA
416 TDEESILTLLTSRSNAQR
TDEESILTLLTSRSNAQRQE
EESILTLLTSRSNAQR
SILTLLTSRSNAQ
SILTLLTSRSNAQR
SILTLLTSRSNAQRQE
SILTLLTSRSNAQRQEI
ILTLLTSRSNAQR
ILTLLTSRSNAQRQE
ILTLLTSRSNAQRQEI
454 SELTGKFEKLIVALMKPS
SELTGKFEKLIVALMKPSR
ELTGKFEKLIVALMKPS
ELTGKFEKLIVALMKPSR
LTGKFEKLIVALMKPS
LTGKFEKLIVALMKPSR
TGKFEKLIVALMKP
TGKFEKLIVALMKPS
TGKFEKLIVALMKPSR
GKFEKLIVALMKP
GKFEKLIVALMKPS
GKFEKLIVALMKPSR
KFEKLIVALMKPSRLYDA
FEKLIVALMKPSRLYDA
EKLIVALMKPSRLYD
EKLIVALMKPSRLYDA
EKLIVALMKPSRLYDAY
KLIVALMKPSRLYD
KLIVALMKPSRLYDA
KLIVALMKPSRLYDAY
508 IKQVYEEEYGSSLEDD
IKQVYEEEYGSSLEDDV
KQVYEEEYGSSLEDD
570 WGTDEEKFITIFGTRSVSH
GTDEEKFITIFGTRSVSH



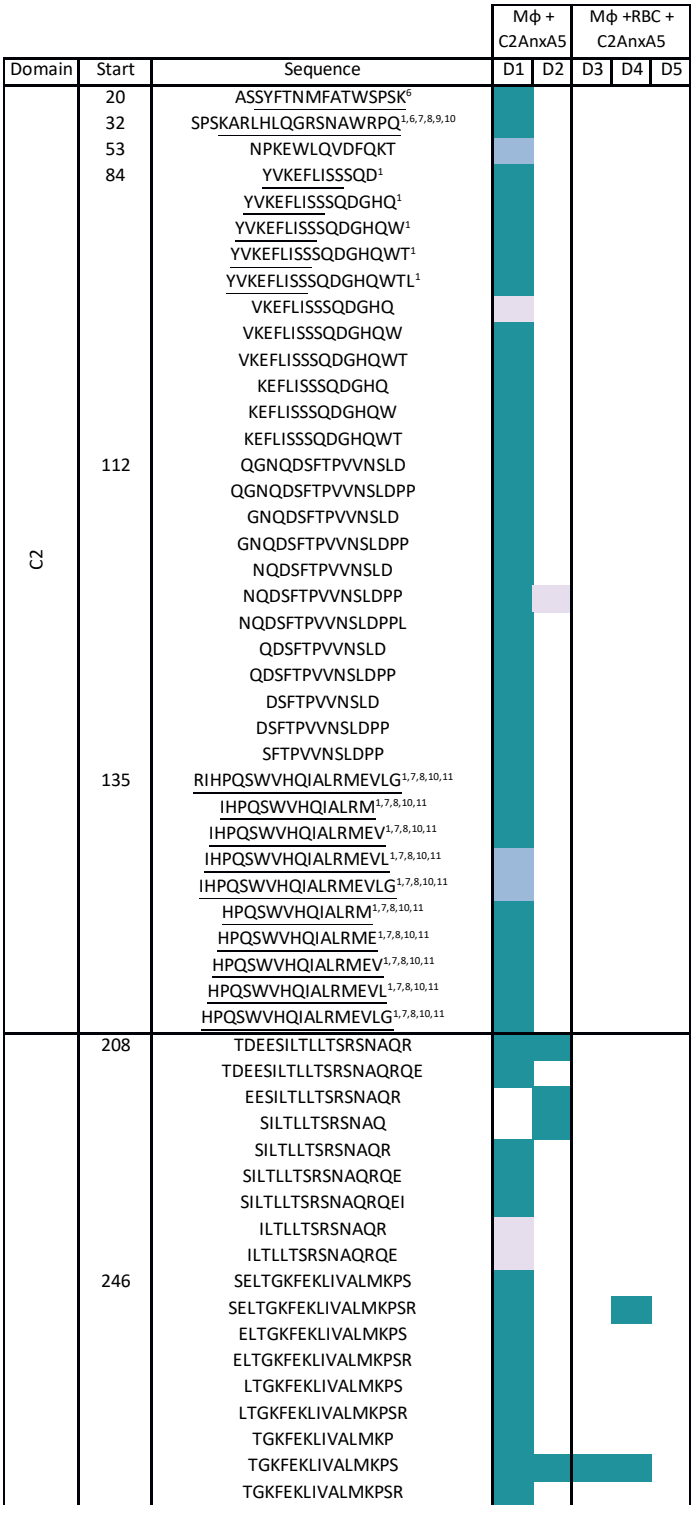
Supplementary Table 4. A2-Annexin A5–derived peptides presented on HLA-DR. A2-Annexin A5 (AnxA5)–derived peptides presented on HLA-DR molecules were identified across different donors (D1–D5) and experimental conditions: macrophages with A2-AnxA5 (Mϕ+A2-AnxA5) or RBCs pre-loaded with A2-AnxA5 (Mϕ+RBC+A2-AnxA5). For each peptide, the corresponding domain, starting position, and amino acid sequence are reported. Peptides identified for a specific condition and donor are shown in dusty blue if detected only with Proteome Discoverer, in pale lilac if detected only with FragPipe, and in deep cyan if detected with both tools. Immunogenic FVIII peptides are underlined (2).

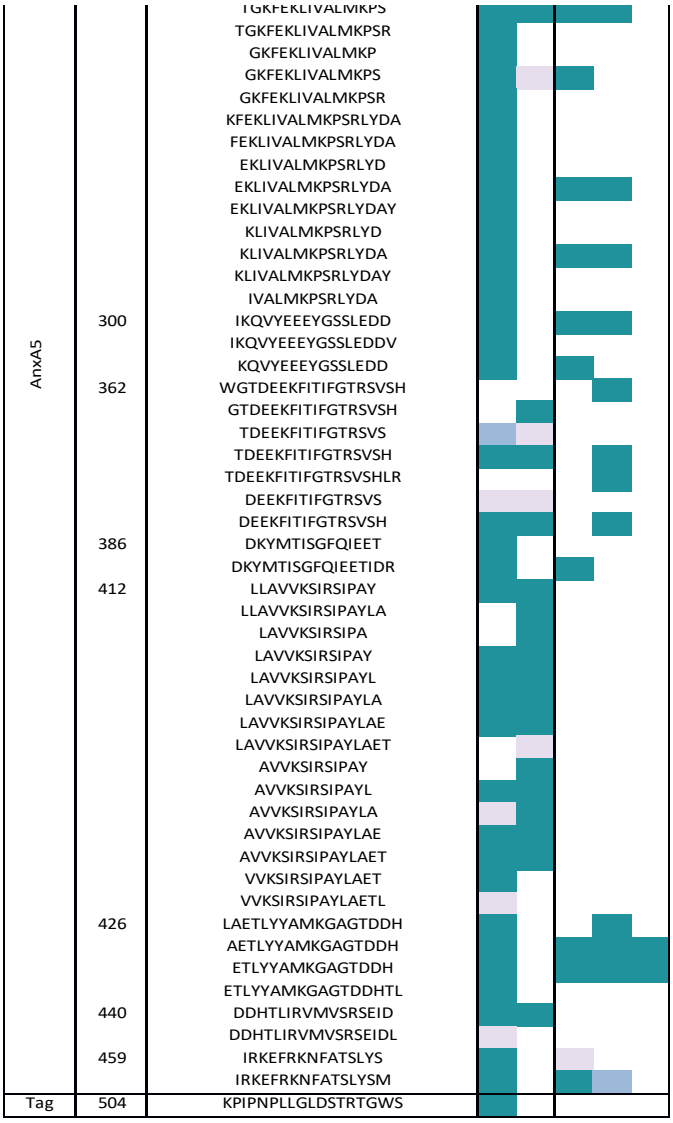

Mϕ + A2AnxA5
Mϕ +RBC + A2AnxA5
Domain
Start
Sequence
D1
D2
D3
D4
D5
A2
AnxA5
20 DWDYAPLVLAPDDRSYK
DYAPLVLAPDDRSY
DYAPLVLAPDDRSYK
YAPLVLAPDDRSY
79 VGDTLLIIFKNQASRPYN2
GDTLLIIFKNQASRPY2
GDTLLIIFKNQASRPYN2
DTLLIIFKNQASRPY2
DTLLIIFKNQASRPYN2
TLLIIFKNQASRPY2
TLLIIFKNQASRPYN2
190 RGNQIMSDKRNVIL
GNQIMSDKRNVIL
232 EDPEFQASNIMHSING
352 EDISAYLLSKNNAIEPA
DISAYLLSKNNAIEPA
ISAYLLSKNNAIEPA
416 TDEESILTLLTSRSNAQR
TDEESILTLLTSRSNAQRQE
EESILTLLTSRSNAQR
SILTLLTSRSNAQ
SILTLLTSRSNAQR
SILTLLTSRSNAQRQE
SILTLLTSRSNAQRQEI
ILTLLTSRSNAQR
ILTLLTSRSNAQRQE
ILTLLTSRSNAQRQEI
454 SELTGKFEKLIVALMKPS
SELTGKFEKLIVALMKPSR
ELTGKFEKLIVALMKPS
ELTGKFEKLIVALMKPSR
LTGKFEKLIVALMKPS
LTGKFEKLIVALMKPSR
TGKFEKLIVALMKP
TGKFEKLIVALMKPS
TGKFEKLIVALMKPSR
GKFEKLIVALMKP
GKFEKLIVALMKPS
GKFEKLIVALMKPSR
KFEKLIVALMKPSRLYDA
FEKLIVALMKPSRLYDA
EKLIVALMKPSRLYD
EKLIVALMKPSRLYDA
EKLIVALMKPSRLYDAY
KLIVALMKPSRLYD
KLIVALMKPSRLYDA
KLIVALMKPSRLYDAY
508 IKQVYEEEYGSSLEDD
IKQVYEEEYGSSLEDDV
KQVYEEEYGSSLEDD
570 WGTDEEKFITIFGTRSVSH
GTDEEKFITIFGTRSVSH
TDEEKFITIFGTRSVS
TDEEKFITIFGTRSVSH
TDEEKFITIFGTRSVSHLR
DEEKFITIFGTRSVS
DEEKFITIFGTRSVSH
VFDKYMTISGFQIEET



	594	DKYMTISGFQIEET
	620	LLAVVKSIRSIPAY
		LLAVVKSIRSIPAYLA
		LAVVKSIRSIPA
		LAVVKSIRSIPAY
		LAVVKSIRSIPAYL
		LAVVKSIRSIPAYLA
		LAVVKSIRSIPAYLAE
		AVVKSIRSIPAY
		AVVKSIRSIPAYL
		AVVKSIRSIPAYLA
		AVVKSIRSIPAYLAE
		AVVKSIRSIPAYLAET
		VVKSIRSIPAYLAE
		VVKSIRSIPAYLAET
		VVKSIRSIPAYLAETL
		SIPAYLAETLYYAMKGAGTDDH
	634	LAETLYYAMKGAGTDDH
		AETLYYAMKGAGTDDH
		ETLYYAMKGAGTDDH
		ETLYYAMKGAGTDDHT
	644	GAGTDDHTLIRVMVSRSEIDL
		DDHTLIRVMVSRSEID
		DDHTLIRVMVSRSEIDL
	667	IRKEFRKNFATSLYS
		IRKEFRKNFATSLYSM
Tag	712	KPIPNPLLGLDSTRTGWS

Supplementary Table 4. A2-Annexin A5–derived peptides presented on HLA-DR. A2-Annexin A5 (AnxA5)–derived peptides presented on HLA-DR molecules were identified across different donors (D1–D5) and experimental conditions: macrophages with A2-AnxA5 (Mϕ+A2-AnxA5) or RBCs pre-loaded with A2-AnxA5 (Mϕ+RBC+A2-AnxA5). For each peptide, the corresponding domain, starting position, and amino acid sequence are reported. Peptides identified for a specific condition and donor are shown in dusty blue if detected only with Proteome Discoverer, in pale lilac if detected only with FragPipe, and in deep cyan if detected with both tools. Immunogenic FVIII peptides are underlined (2).


Mϕ + C2AnxA5
Mϕ +RBC + C2AnxA5
Domain
Start
Sequence
D1
D2
D3
D4
D5
C2
20 ASSYFTNMFATWSPSK[6]
32 SPSKARLHLQGRSNAWRPQ[1,6,7,8,9,10]
53 NPKEWLQVDFQKT
84 YVKEFLISSSQD[1]
YVKEFLISSSQDGHQ[1]
YVKEFLISSSQDGHQW[1]
YVKEFLISSSQDGHQWT[1]
YVKEFLISSSQDGHQWTL[1]
VKEFLISSSQDGHQ
VKEFLISSSQDGHQW
VKEFLISSSQDGHQWT
KEFLISSSQDGHQ
KEFLISSSQDGHQW
KEFLISSSQDGHQWT
112 QGNQDSFTPVVNSLD
QGNQDSFTPVVNSLDPP
GNQDSFTPVVNSLD
GNQDSFTPVVNSLDPP
NQDSFTPVVNSLD
NQDSFTPVVNSLDPP
NQDSFTPVVNSLDPPL
QDSFTPVVNSLD
QDSFTPVVNSLDPP
DSFTPVVNSLD
DSFTPVVNSLDPP
SFTPVVNSLDPP
135 RIHPQSWVHQIALRMEVLG[1,7,8,10,11]
IHPQSWVHQIALRM[1,7,8,10,11]
IHPQSWVHQIALRMEV[1,7,8,10,11]
IHPQSWVHQIALRMEVL[1,7,8,10,11]
IHPQSWVHQIALRMEVLG[1,7,8,10,11]
HPQSWVHQIALRM[1,7,8,10,11]
HPQSWVHQIALRME[1,7,8,10,11]
HPQSWVHQIALRMEV[1,7,8,10,11]
HPQSWVHQIALRMEVL[1,7,8,10,11]
HPQSWVHQIALRMEVLG[1,7,8,10,11]
208 TDEESILTLLTSRSNAQR
TDEESILTLLTSRSNAQRQE
EESILTLLTSRSNAQR
SILTLLTSRSNAQ
SILTLLTSRSNAQR
SILTLLTSRSNAQRQE
SILTLLTSRSNAQRQEI
ILTLLTSRSNAQR
ILTLLTSRSNAQRQE
246 SELTGKFEKLIVALMKPS
SELTGKFEKLIVALMKPSR
ELTGKFEKLIVALMKPS
ELTGKFEKLIVALMKPSR
LTGKFEKLIVALMKPS
LTGKFEKLIVALMKPSR
TGKFEKLIVALMKP
TGKFEKLIVALMKPS
TGKFEKLIVALMKPSR



Supplementary Table 5. C2-Annexin A5–derived peptides presented on HLA-DR. C2-Annexin A5 (AnxA5)–derived peptides presented on HLA-DR molecules were identified across different donors (D1–D5) and experimental conditions: macrophages with C2-AnxA5 (Mϕ+C2-AnxA5) or RBCs pre-loaded with C2-AnxA5 (Mϕ+RBC+C2-AnxA5). For each peptide, the corresponding domain, starting position, and amino acid sequence are reported. Peptides identified for a specific condition and donor are shown in dusty blue if detected only with Proteome Discoverer, in pale lilac if detected only with FragPipe, and in deep cyan if detected with both tools. Immunogenic FVIII peptides are underlined (1–11).

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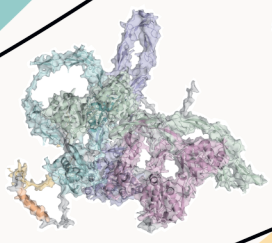
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Chapter 5

Immunopeptidomics of blood coagulation factor IX; a core peptide derived from the protease domain is promiscuously presented on HLA-DR



Michela Leoni, Margot Dierickx, Mariarosaria Miranda, Pieter Langerhorst, Carmen van der Zwaan, Erik Mul, Arie J Hoogendijk, Karin Fijnvandraat and Jan Voorberg

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Abstract

Background: Hemophilia B is an X-linked bleeding disorder that results in a qualitative or quantitative FIX deficiency. A subset of hemophilia B patients treated with FIX replacement therapy develop antibodies against FIX. The development of anti-FIX inhibitors can present with allergic or anaphylactic reactions in some patients. It is currently not known why a subset of hemophilia B patients develop inhibitors. CD4+ T cells recognizing FIX derived peptides on MHC class II on antigen presenting cells are crucial for the development of class switched high affinity anti-FIX antibodies.

Objective: In this study we explore MHC class II presentation of FIX derived peptides by antigen presenting cells.

Methods: We incubated purified FIX with monocyte derived dendritic cells of HLA-typed healthy donors. MHCII-peptide complexes were immunopurified, followed up by peptide elution and mass spectrometry based analysis of eluted peptides.

Results: We analyzed 12 healthy donors, out of them 8 present FIX derived peptides. The majority of FIX peptides were derived from the serine protease domain of FIX. With the exception of 1 donor, all of them present at least one peptide containing the same core sequence “NVIRIIPHHN” (residues 295-306 of FIX).

Conclusions: Taken together, we identified a promiscuously presented peptide derived from the protease domain of FIX. Based on its frequent presentation by multiple donors we speculate that this peptide may be recognized by FIX specific CD4+ T cells that develop in hemophilia B patients with inhibitors. Peptides identified in our study can be used for inhibitor risk stratification and development of novel therapies to induce tolerance.

Key words

Blood Coagulation; Hemophilia B; HLA-DR; Mass Spectrometry; Peptides

Essentials

- Part of hemophilia B patients develop inhibitors, anti-factor IX (FIX) antibodies

- FIX incubated with monocyte-derived dendritic cells; peptides detected by mass spectrometry
- FIX peptides are detected in eight out of twelve donors, mostly from the serine protease domain
- The peptide 295-NVIRIIPHHN-306 likely a CD4+ T cell epitope, was presented in all but 1 donor

Introduction

Hemophilia B is an X-linked bleeding disorder, characterized by genetic defects within the F9 gene that results in either qualitative or quantitative factor IX (FIX) deficiency[177]. The incidence of hemophilia B is 1 in 30000 males; historically hemophilia B patients have been treated with infusion of purified plasma or recombinant FIX to prevent joint bleeds. Depending on the remaining FIX activity patients are classified as severe (<1%), moderate (1%-5%) and mild (5%-40%). The circulatory half-life of FIX ranges from 16-18 hours and in order to maintain therapeutic levels 2-3 weekly infusions of FIX are needed[44]. Recently, extended half-life products composed of PEGylated FIX, FIX-Fc and FIX-albumin fusions with a 3-4 fold prolonged half-life have been developed which allow for one to two weekly infusions of FIX[178]. AAV based gene therapy for FIX has been successfully developed and has now been approved by EMA and FDA for clinical use in patients with hemophilia B[86,87]. Both AAV5 based as well as AAV8 based vectors are being used; the naturally occurring gain-of-function FIX-Padua (R338L) variant has been used to boost expression of functional FIX approximately 5 fold[52]. In contrast to the decline in FVIII expression in gene therapy trials employing AAV5; current data show sustained and very stable levels of FIX in patients treated with both AAV5 and/or AAV8 based gene therapy vectors in which the FIX-Padua variant is expressed under the control of a liver specific promoter [89,179].

FIX is part of the vitamin-K dependent blood coagulation factors that also includes protein C, protein S, prothrombin, factor VII and factor X. FIX is composed of different domains: the Gla domain at the N-terminus contains multiple Ca^{2+} binding ɣ-carboxy glutamic acid residues, 2 EGF like domains, of which the first one contains a Ca^{2+} binding site[53]. The EGF domains are followed by an activation peptide that is released during activation of FIX by factor XIa or the tissue factor/factor VIIa complex[59]. The carboxy terminal serine protease domain of FIX contains a metal ion-binding site as well as the active site composed of a catalytic triad composed of Ser195, His57 and Asp102 (chymotrypsin numbering)[54,61].

Similarly to hemophilia A, patients with hemophilia B can develop antibodies against FIX that interfere with treatment. These so-called FIX inhibitors occur in 1 to 4% of patients with hemophilia B [97,112]. Patients who develop FIX inhibitors tend to develop allergic or anaphylactic reactions prior to or during inhibitor formation for reasons that are incompletely understood. One study suggested that anaphylactic shocks are due to relatively high dosage of FIX used for standard

infusions when compared to FVIII[98]. Additionally, diffusion of FIX in the extracellular space has been suggested to contribute to the risk of anaphylactic shocks and allergic reactions[98]. IgE mediated reactions directed towards FIX have also been proposed as a potential mediator of allergic reactions in patients with hemophilia B[99]. The risk of inhibitors is strictly associated with the severity of hemophilia and with the type of FIX mutations[113,180]. In the case of missense, splice site or promoter mutations, inhibitors develop in 0.4% of patients, for nonsense and frameshift mutations inhibitors develop in 5% of patients[94,181]. Importantly, the risk of inhibitor in hemophilia B patients with large deletions extended beyond 50 base pairs is relatively high amounting to 27%[94].

FIX inhibitors are not as well characterized as the ones for hemophilia A but previous studies show that the epitopes recognized by inhibitory antibodies localize to the Gla and the serine protease domains of FIX[113]. The EGF domains and the activation peptide do not seem to contain any binding sites for inhibitory antibodies[113]. Subclass analysis revealed that FIX inhibitors are primarily composed of IgG1 and IgG4; in some patients anti-FIX antibodies of subclass IgE have been described which may be linked to anaphylactic reactions and allergic responses in patients with hemophilia B[182].

Currently, there is limited information on the processing and presentation of FIX derived peptides by antigen presenting cells. A study by Saini and co-workers reported on genetic determinants of immunogenicity to FIX in 5 hemophilia B patients with inhibitors. In silico MHC class II peptide binding studies suggested that the ability of MHC class II alleles to present FIX derived peptides was linked to the efficacy of immune tolerance induction in a brother pair with hemophilia B[183].

In this study, we explored the repertoire of FIX derived peptides that is presented on HLA-DR upon pulsing of monocyte derived dendritic cells with FIX. The dendritic cells used were at the immature stage when pulsed with FIX in order to allow for optimal endocytosis of FIX and subsequentially maturated with LPS for optimal peptide presentation on MHC class II[184–186]. Our findings indicate that a peptide derived from the protease domain is promiscuously presented on multiple HLA-DR alleles.

Methods

Reagents

Ficoll-Paque™ PLUS (Cytiva), anti-CD14 magnetic MicroBeads (Milteny Biotec), CellGenix GMP DC Medium (Sartorius), 1% penicillin/streptomycin solution: 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma), GM-CSF, IL-4 (PeptroTech), Nonafact (ultrapure, monoclonal antibody-purified factor IX concentrate), Sanquin, Amsterdam[187] , Hoechst 33342 (Invitrogen), Amicon ultra centrifugal filter, 10 kDa MWCO (Millipore), anti-CD209 IgG2a PE, clone FAB1621P (R&D Systems), Alexa Fluor 647antibody labelling kit (Thermo Fisher).

HLA-DR peptide enrichment and sample preparation for mass spectrometry

Cells were detached from plates with scrapers and washed twice with CellGenix medium. The cells were collected and lysed in 1.5 mL of lysis buffer composed of 10 mM Tris-HCl (pH 8.0), 0.25% n-octyl β-D-glucopyranoside (Sigma®), 1% desoxycholate (bioPLUS™) and 10 mM EDTA (UltraPure™ 0.5 M EDTA pH 8.0, Invitrogen™). Lysates were incubated end-over-end for 30 minutes at 4°C. The lysate was cleared by centrifugation at18000 g for 20 minutes at4°C. To isolate the HLA-DR-peptide complexes, the cell lysate was incubated together with CNBr-activated SepFast™ MAG 4HF magnetic beads (BioToolomics) coupled to anti-human HLA-DR antibody L243 (final concentration 2 mg/ml; source InVivoMAb, Bio X Cell) overnight at 4°C with end-over-end rotation. Each sample was divided into triplicate, over 300 µL of L243 conjugated beads. Beads were washed twice with lysis buffer and five times with 10 mM Tris-HCl pH 8.0. Peptides were eluted from the beads using 500 µL 10% Acetic Acid (LC-MS LiChropur®, Merck). The supernatant was collected and incubated for 15 minutes at 70°C and then frozen at -30°C until mass spectrometry analysis.

Mass spectrometry analysis. Mass spectrometry analysis was performed as described previously[188]. Briefly, we loaded samples on Evotip PureTips (Evosep), which were eluted onto an EV1137 Performance column (Evosep), the gradient was set to 15 samples per day (SPD). TimsTOF HT Mass Spectrometer (Bruker Daltonics) was used for acquisition and operated in ddsPASEF mode.

HLA-DR peptide search and identification

The mass-spectrometry file were analyzed using MSFragger (v 3.8) in the FragPipe (v20) pipeline as described previously[188,189]. Peptides and proteins were identified

using the filtered human Swissprot database (released 2021.22.04) with 50% added decoys. The peak precursor mass tolerance was set at -20 to +20 ppm with a fragment mass tolerance of 10 ppm. The peptide mass range was set to 600-3000, the peptide length to 8-30 amino acids and protein digestion was set as non-specific. The allowed modifications were γ-carboxylation of glutamic acids, N-terminal acetylation, methionine oxidation, pyroglutamylation and cysteinylation. Peptides were quantified using IonQuant (v1.9.8) using default settings without allowing match between runs.

ImageStream

FIX (Nonafact) was dissolved in 10 mL of water and concentrated with Amicon spin column, FIX was labelled with AF647 following manufacturer's instructions. iDCs were incubated with 100 nM labelled FIX for 1 hour and stained with membrane staining (CD209) and Hoechst 33342. Images were acquired on an imaging flow cytometer (Amnis ImageStream Imaging Flow Cytometer) as described previously[190].

Results

Immunopeptidomics of FIX by antigen presenting cells

As a first step towards the identification of HLA-DR presented FIX peptides, we monitored the internalization of FIX by antigen presenting cells. We employed iDCs (immature dendritic cells) that were incubated with 100 nM AF647 labelled FIX; uptake was monitored by ImageStream (Figure 1). AF647 labelled FIX was incubated with iDCs for 1 hour at 37°C. Upon this time-point most of the iDCs contained AF647 labelled FIX; representative images obtained are shown in Figure 1. Fixed cells were also stained for CD209 which is highly expressed on the surface of iDCs. Together these findings indicate that FIX is internalized by iDCs.

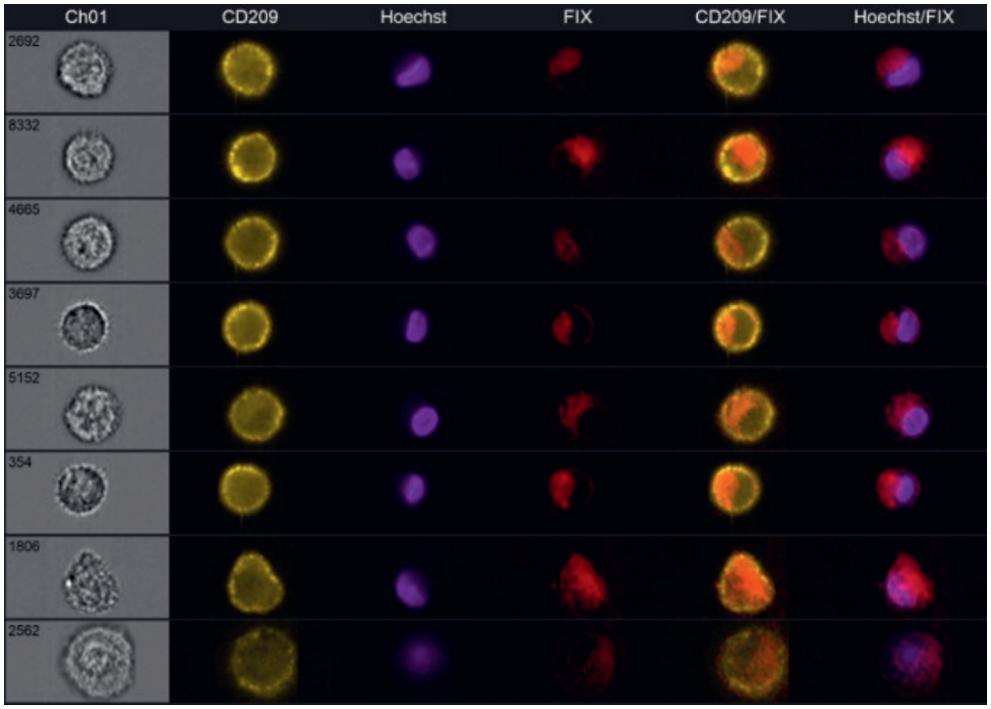


Figure 1. Uptake of FIX by dendritic cells. Internalization of FIX was monitored by ImageStream. Representative images are shown. Brightfield and fluorescence images of iDCs following incubation with 100 nM of AF647 labelled FIX (pseudocolored to red); iDCs are also stained for DC-SIGN (CD209, in yellow); nuclei are stained with Hoechst (violet).

Having shown that FIX is readily endocytosed by antigen presenting cells, we subsequently purified peptide-MHC class II complexes from dendritic cells pulsed

with 100 nM FIX employing monoclonal antibody L243 which specifically binds to HLA-DR. Subsequently, HLA-DR bound peptides were eluted from HLA-DR using acetic acid and analyzed by mass spectrometry. For donor 1 (HLA-DRB1*01/13) the number of peptides eluted from HLA-DR ranged from 2227 to 2488 (Figure 2A and 2B). Peptides presented on HLA-DR were derived from 511-544 unique proteins (Figure 2C). The number of peptides derived from the isotype control immunoprecipitations ranged from 577 to 939 peptides derived from 200-250 unique proteins (Figure 2B and 2C). The length distribution of peptides derived from the isotype control ranged between 8 and 30 residues while the majority of L243 derived peptides having a length of 15-25 amino acid residues (Figure 2D).

A

Sample			Num Peptides	Num Proteins	Num FIX Peptides
CTRL	Isotype control	A	669	253	0
		B	753	259	0
		C	577	205	0
	HLA-DR	A	2488	544	0
		B	2400	535	0
		C	2231	523	0
FIX	Isotype control	A	743	238	0
		B	665	221	0
		C	939	228	0
	HLA-DR	A	2267	535	9
		B	2262	511	8
		C	2227	522	8

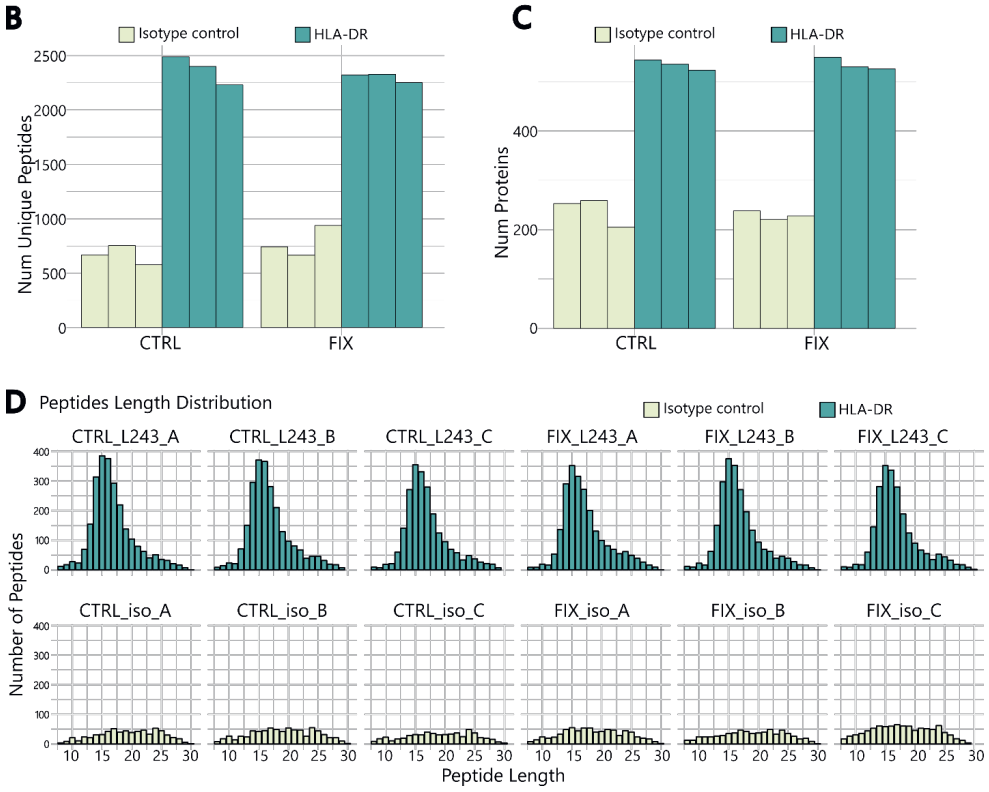
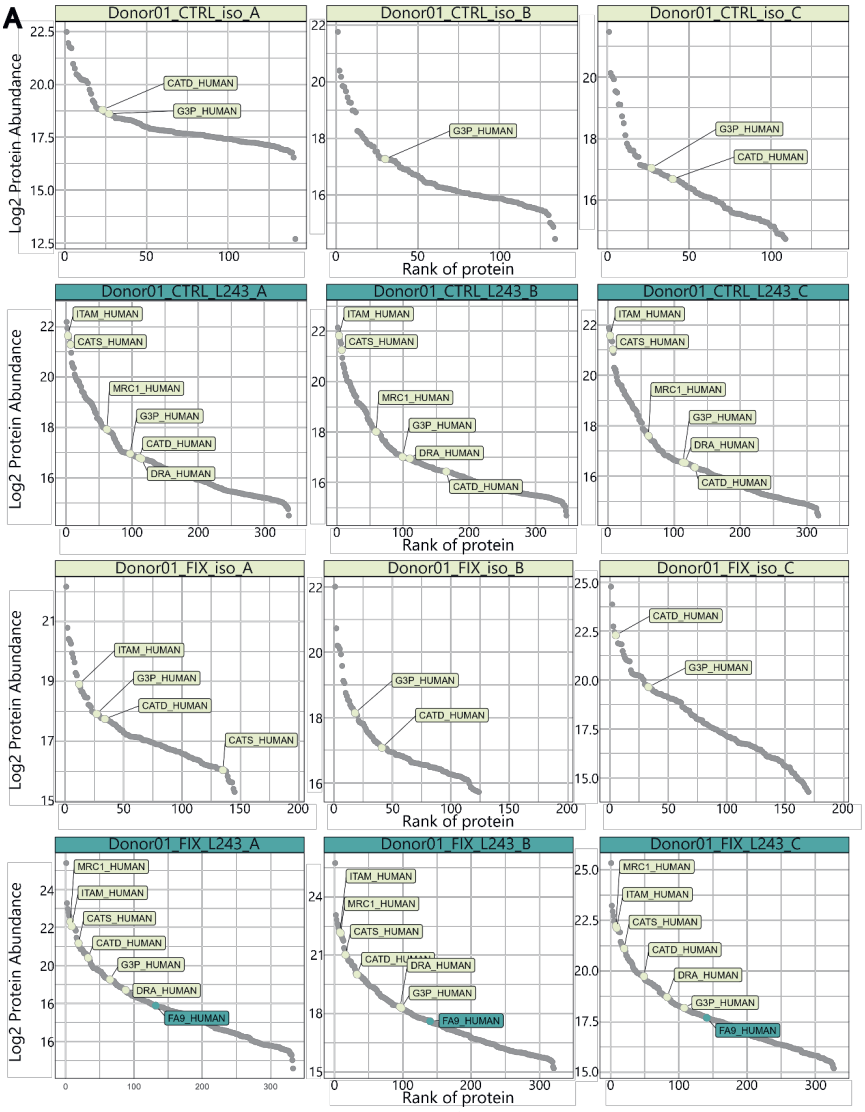


Figure 2. Quantity and length distribution of HLA-DR presented peptides. (A) Number of peptides presented on FIX pulsed and control DCs (CTRL). For both conditions HLA-DR was purified employing monoclonal antibody L243; three independent experiments (A, B and C) were performed for each conditions. As a control for MHC class II specificity an isotype control antibody (Isotype control; C1.18.4 mouse IgG2a was used). Number of peptides, number of proteins and number of FIX derived peptides is indicated for each condition. (B, C) Graphical representation of the number of peptides recovered for each condition. Dark

green bars represent number of peptides and proteins obtained following immunoprecipitations employing HLA-DR antibody L243 where light green bars represent number of peptides and proteins recovered by isotype control antibody. (D) Upper row; length distribution of peptides recovered for control DCs (not pulsed with FIX) and FIX pulsed DCs. Lower row; length distribution of peptides recovered from isotype control treated samples obtained for DCs not pulsed with FIX and DCs pulsed with FIX.

HLA-DR presented peptides were mostly derived from intracellular proteins present in the cytosol, plasma membrane and endolysosomal compartments, this included also peptides derived from the MHC class II itself (Figure 3A). For donor 1 in total 10 peptides derived from human FIX were detected for the three replicates (Figure 2A, Figure 3C). Strikingly, all 10 peptides contained the core sequence NVIRIIPHHNYNA that corresponds to amino acids 295-307 of human FIX (Figure 3C). This indicates that this specific FIX peptide which is derived from the protease domain of FIX is preferentially presented in HLA-DR. The panel of FIX peptides HLA-DR presented peptides contains a number of larger peptides with amino-terminal and carboxy-terminal extensions. This is consistent with the typical “proteolytic signature” of MHC class II presented peptides in which the core-peptide is protected from proteolysis by virtue of its presence in the binding pocket of MHC class II. Amino-terminal and carboxy-terminal extensions of core peptides are subjected to processing by endosomal and lysosomal proteases following their loading on MHC class II[191–195].



B

Protein Identifier	Full Name
CATS_HUMAN	Cathepsin S
CATD_HUMAN	Cathepsin D
DRA_HUMAN	HLA class II histocompatibility antigen, DR alpha chain
FA9_HUMAN	Coagulation factor IX
G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase
ITAM_HUMAN	Integrin alpha-M
MRC1_HUMAN	Macrophage mannose receptor 1

C

Peptide Sequence	Start	End	Peptide Length	Detected (out of 3)
ETEHTEQKRNVIRIIPHHNYNAAIN	286	310	25	2
KRNVIRIIPHHNYNAA	293	308	16	2
NVIRIIPHHNYN	295	306	12	3
NVIRIIPHHNYNA	295	307	13	2
NVIRIIPHHNYNAA	295	308	14	3
NVIRIIPHHNYNAAIN	295	310	16	3
NVIRIIPHHNYNAAINK	295	311	17	3
NVIRIIPHHNYNAAINKYNHDIAL	295	318	24	3
QKRNVIRIIPHHNYN	292	306	15	2
RNVIRIIPHHNYNAA	294	308	15	2

Figure 3. Properties of HLA-DR presented peptides. (A) Protein abundance rank based on log2 transformed LFQ intensities. Names of a subset of proteins presented are indicated in the graph; the full name of the proteins indicated is shown in panel B. Factor

IX is also displayed in the graph of the protein abundance rank; FIX was only detected in cells that were pulsed with FIX and not in control cells. (C) Peptide sequence of FIX derived peptides identified in donor 1. Numbering of residues is according to UniProt entry P00740. The last column shows in how many of the triplicates specific FIX peptides were detected.

Analysis of FIX-presented peptides on MHC class II

To further characterize the repertoire of factor IX derived peptides presented on HLA-DR, we used DCs of 11 additional healthy donors with a known HLA-DRB1 genotype. In agreement with the results obtained for donor 1, the number of peptides detected using the isotype control was much lower compared to that obtained employing L243 conjugated beads (Supplementary Table 1). Similar to donor 1, the length of peptides derived from the isotype control ranged between 8-30 amino acids whereas the HLA-DR presented peptides displayed were primarily between 14-18 amino acid residues (Supplementary Figure 1). As expected, we did not observe any FIX derived peptides in dendritic cells that were not pulsed with FIX (Supplementary Table 1). The total number of HLA-DR-presented peptides observed for the different donors ranged between 1459 and 4619 (Figure 4A and B) which were derived from 381-1028 unique proteins (Figure 4A and C). The number of FIX derived peptides was consistent among the three replicates and ranged from 0 FIX derived peptides for donors 2, 6, 10, 11 and up to 14 FIX derived peptides for donor 8 (Figure 4A). In 8 out of the 12 donors analyzed we detected at least one FIX peptide (Figure 4A).

A

Donor	Replicate	Num Peptides	Num Proteins	Num FIX peptides
1	A	2267	535	9
	B	2262	511	8
	C	2227	522	8
2	A	3414	721	0
	B	3506	758	0
	C	3500	736	0
3	A	1750	416	0
	B	1563	397	1
	C	1459	392	1
4	A	2227	446	0
	B	1884	408	1
	C	1664	381	0
5	A	4619	1008	2
	B	4513	978	4
	C	4062	992	3
6	A	3011	679	0
	B	2636	663	0
	C	3352	774	0
7	A	2786	668	3
	B	2904	684	2
	C	2755	661	2
8	A	2845	713	11
	B	2797	704	14
	C	2707	682	11
9	A	3592	848	2
	B	3584	849	2
	C	3750	855	2
10	A	2871	760	0
	B	2512	677	0
	C	2780	741	0
11	A	3724	946	0
	B	3876	981	0
	C	3743	951	0
12	A	3925	1000	1
	B	4008	1028	1
	C	3868	975	1

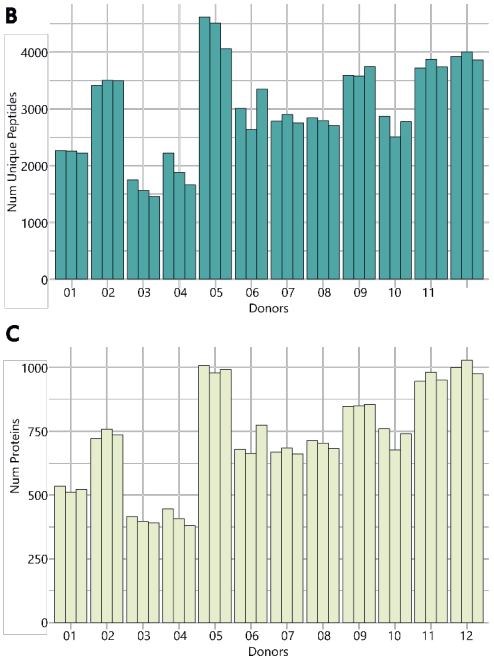


Figure 4. Number of HLA-DR presented peptides and proteins of a cohort of 12 donors. (A) Number of peptides, proteins and FIX derived peptides from a panel of 12 donors. For each donor three replicates were performed. (B, C) Graphical representation of the number of peptides and proteins recovered for each of the 12 donors. Dark green bars represent number of peptides and light green bars represent number of proteins obtained following immunoprecipitations employing HLA-DR antibody L243.

Strikingly, 6 out of 12 donors presented peptides containing core sequence NVIRIIPHHNYN (residues 295-306). Inspection of the HLA-DRB1 genotype (Supplementary Table 2) suggests that peptides containing this core sequence can be presented by multiple HLA-DRB1 alleles. We employed NetMHCIIpan - 4.1[196] for the in silico prediction of FIX derived peptides presented on HLA-DRB1 alleles. Bioinformatic analysis predicted that core sequence 292-QKRNVIRIIPHHNYNAAINK-306 was indeed presented by multiple HLA-DRB1 alleles. Peptides derived from peptide sequence 292-306 were predicted as being weak to strong binder for almost all HLA-DRB1 alleles (Supplementary Figure 2 and 3). The prediction of presented peptides derived from other regions of FIX did not fully align to our experimental data; some of the predicted peptides were not identified in our donors and some of the peptides that we found were not predicted to be strong binders by NetMHCIIpan -4.1 (Supplementary Figure 2).

Donor 12 presented a unique WIVTAAHCVETGVK (residues 261-275) peptide that was not detected in other donors. This peptide has been detected with a cysteinylation (+119.0041 C), this modification can either occur “in vivo” before peptide loading or after the immunoprecipitation of the MHC II-peptide complex during sample preparation[197,198]. Interestingly, donor 8 (DRB1*16/16) presented multiple peptides derived from the FIX protease domain (Figure 4). Apart from the core peptide NVIRIIPHHNYN (residues 295-306), three additional peptides derived from the protease domain of FIX are presented on HLA-DRB1*16 of this specific donor. The data obtained suggests that multiple peptides derived from amino acid region 312-340 can be presented by dendritic cells derived from the HLA-DRB1*16 homozygous donor (Figure 5). In addition, a series of carboxy-terminal trimmed peptides derived from amino acid residues 443-459 are presented by dendritic cells derived of donor 8. For 4 of the12 donors, no FIX peptide presentation was detected. One of these four donors (donor 6) was homozygous for DRB1*04 which may suggest that FIX derived peptides are not efficiently presented by DRB1*04. Despite of the lack of detected FIX derived peptides a large repertoire of peptides derived of endogenous proteins was found to be presented on dendritic cells derived of donor 2, 6, 10 and 11. This finding suggests that the lack of presentation of FIX derived peptides on HLA-DR was not linked to a general defect in peptide presentation in these donors.



Figure 5. Heatmap and sequence characteristics of FIX derived peptides. Heatmap representation is based on LFQ intensity of identified peptides from L243 immunoprecipitates. Peptide abundance is indicated by a green gradient. The sequence of the FIX peptides identified is displayed in the right part of the figure. On the top of the figure the HLA genotype of the 12 donors included in this study is show.

Discussion

The development of inhibitory antibodies towards FIX in patients with hemophilia B is a rare event occurring in 1-5% of patients usually within 50 exposure days[94,199,200]. The risk of development of FIX inhibitors is strongly related to the FIX gene mutation; hemophilia B patients with inhibitors almost exclusively have large FIX deletions and nonsense mutations [94,201]. The frequent occurrence of missense mutations and large deletions suggest that lack of endogenous FIX contributes to the risk of inhibitor development in patients with hemophilia B[97]. Nevertheless, inhibitors only develop in 27-30% of hemophilia B patients with high-risk mutations, suggesting that other genetic and/or treatment related risk factors may contribute to inhibitor development in hemophilia B [94].

In the current study we show that a FIX derived peptide with the core binding sequence NVIRIIPHHNYN (residues 295-306) is presented by multiple donors. In silico prediction employing NetMHCIIpan -4.1 revealed that peptides derived from this region were predicted to bind with moderate to strong affinity to almost all HLA-DRB1 alleles (Supplementary Figure 2 and 3). Based on our data, we propose that CD4+ T cells recognizing this peptide may contribute to inhibitor development in patients with hemophilia B. Interestingly, 295-NVIRIIPHHNYNA-306 containing peptides were not detected in all donors. This provides a potential explanation for FIX inhibitor development in only a subset of patients with large deletions or nonsense mutations. Although a link between MHC class II genotype and inhibitor development in hemophilia B has not been established yet, a large international study on the natural history of hemophilia B (the "B-Natural" study) has recently been initiated which aims to explore a potential relationship between MHC class II genotype and inhibitor development in hemophilia B[202]. It will be of interest to see whether HLA-DR molecules that can present FIX derived peptide containing the 295-NVIRIIPHHNYNA-306 core sequence are more prevalent in hemophilia B patients with inhibitors when compared to patients with high risk mutations without inhibitors.

Donor 8 (DRB1*16/16) presented an unusually rich peptide repertoire from the FIX protease domain. None of the hemophilia B patients with inhibitors reported in a previous study was positive for DRB1*16[183]. Interestingly DRB1*16 has been associated with acquired hemophilia A as well as other autoimmune disorders[203]. Based on the large number of FIX peptides that is presented on DRB1*16 we expect that the presence of the HLA-DRB1*16 allele in hemophilia B patients with

a large deletion in the FIX gene may provoke inhibitor development. Genetic analysis of large cohorts of patients is needed to address whether HLA-DRB1*16 is a risk factor for inhibitor development in hemophilia B patients with large deletions.

We also found a peptide that contains Arg384, the amino acid that is modified in the Padua variant of FIX, commonly referred to as R338L (alternating numbering caused by inclusion of 46 residues derived of the signal and propeptide included in the UniProt numbering used in this study). This may be relevant because FIX Padua is the variant used for gene therapy in hemophilia B. However, based on our analysis, amino acid Arg384 is not part of the core peptide presented by donor 8, as shown in Figure 5. Formally immunopeptidomic studies employing the FIX-Padua would be needed to address potential immunogenicity. So far no FIX inhibitors have been reported in AAV gene therapy trials which employ FIX-Padua[204]. Together this shows that there is currently no evidence of presentation of Leu384 containing peptides (FIX-Padua derived peptides) on MHC class II.

Apart from hemophilia B, inhibitory antibodies may also develop in patients with FVII deficiency upon initiation of replacement therapy[205]. Interestingly, in patients with FVII deficiency inhibitor development is also linked to the large deletions and nonsense mutations in the FVII gene[205]. Activated FVII (FVIIa) has been successfully developed as a bypassing agent for treatment of hemophilia A patients with inhibitors[206]. Bioengineered FVIIa variants with improved catalytic activity have been designed[207]. Upon *in vivo* testing of these bio-engineered variants, anti-drug antibodies developed in a subset of patients[208,209]. The engineered FVIIa variant (designated vatreptocog alfa) contained three amino acid substitutions when compared to wild-type FVIIa (V158D, E296V and M298Q)[210]. Immunopeptidomics revealed that a peptide containing the E296V and M298Q substitution was preferentially presented by dendritic cells of 12 out of 17 donors[210]. Subsequent analysis showed that the modified peptide with amino acid sequence 351-GATAL***V***L***Q***VLNVPR-365 (modified residues in bold/underlined) was capable of inducing proliferation of CD4+ T cells derived from normal donors[208]. These data show that changes in the protease domain of bio-engineered FVIIa allowed for presentation of a neo-epitope on MHC class II which promoted development of an immune responses in recipient of this bio-engineered FVIIa variant[210]. Alignment of the FIX and FVII sequence did not show overlap between the FVII peptide sequence and the 295-NVIRIIPHHNYN-306 FIX core sequence that was identified

in this study. It is interesting to note that both for FVII and FIX presented peptides are derived from the protease domain.

Similar to other vitamin K dependent proteases, FIX is extensively modified by post-translational modifications[211]. Vitamin K dependent coagulation proteases can bind to phospholipids by virtue of their ɣ-carboxyglutamic acid domain (Gla domain) which contains glutamic acid residues that are modified by a vitamin K dependent ɣ-glutamyl carboxylase[212]. The GLA domain of FIX contains 12 ɣ-carboxyglutamic acid residues[211]. Apart form ɣ-carboxylation, residue Asp64 is modified by β-hydroxylation. Residues Tyr155 and Ser158 in the activation peptide of FIX are modified by tyrosine sulfation and phosphorylation, respectively[211]. O-glycosylation sites are present at Thr159, Thr169, Thr172, Thr179, Ser53 and Ser61 and N-glycans are linked to Asn157 and Asn167[211]. Interestingly, posttranslational modifications of FIX are located in the Gla domain, the two EGF domains and the activation peptide; no post-translational modifications have been linked to the serine protease of FIX. In our data-processing we took into account potential post translational modifications such as ɣ-carboxylation and β-hydroxylation of presented peptides using the FragPipe software used for the analysis of the presented peptide. The detection of ɣ-carboxylated peptides is challenging due to spontaneous loss of CO_2 during mass fragmentation[213]. In addition, application of polarity switching positive and negative ionization modes has been recommended for optimal detection of ɣ-carboxylated peptides[213]. Implementation of this specific protocol for the detection of ɣ-carboxylated peptides can potentially extend the findings documented in the current study [49,50]. Potentially, also due to their inherent heterogeneity, O-linked glycan containing peptides derived of the EGF1 and AP domain may have been missed by the data-processing applied in this study [214,215]. In this respect it is interesting to note that O-linked glycan containing peptides have been shown to be presented on MHC class I[192].

Several methods for mass spectrometry based identification of MHC class II presented peptides have been described in the literature[192,216]. Mild acid elution based methods utilize living cells to directly elute peptides from surface expressed MHC class I provides an elegant approach for the isolation of MHC class I presented peptides[195,217]. Immunopeptidomics of MHC class II involves purification of MHC class II-peptide complexes employing monoclonal antibodies directed towards HLA-DR, HLA-DQ or HLA-DP[216]. In this and previous studies we have successfully used L243 to explore HLA-DR presented peptide repertoires of human monocyte derived dendritic cells pulsed with blood coagulation FVIII and ADAMTS13[218,219].

MSFragger based analysis of the resulting spectra consistently yielded 2000-5000 different MHC class II presented peptides which included commonly HLA-DR presented FIX peptides with core sequence NVIRIIPHHNYN. It should be noted that a considerable number of peptides was also recovered from isotype controls (Figure 2), and we hypothesize that these peptides correspond to non-HLA-DR binding peptides that are derived from proteolytic degradation of endogenous cellular proteins. Inspection of the peptide lengths that were recovered from the isotype control did not reveal amino and carboxy-terminal trimming which is a specific feature of MHC class II presented peptides. We are currently optimizing our purification protocol for MHC class II/peptide complexes to further reduce the background within our isotype controls. We did not recover any FIX derived peptides from the isotype controls and therefore we hypothesize that our data on the presentation of FIX derived peptides are not affected by the relatively high background observed in the isotype controls.

Overall, our findings highlight the presence of an immunodominant peptide derived from the protease domain of FIX that can bind to multiple MHC class II alleles. Future studies employing analysis of CD4+ T cell responses should reveal whether FIX peptides containing the core sequence 295-NVIRIIPHHNYN-306 contribute to the development of inhibitors in patients with hemophilia B. Additionally, our results suggest that MHC class II genotype could also be relevant for the risk of inhibitor development in hemophilia B. Potentially, the identification of a promiscuously presented peptide as identified in this study can be exploited for the design of novel peptide based approaches for treatment of inhibitors in patients with hemophilia B.

Author contributions:

ML, MD, MM, KF and JV designed the study. ML performed the experiments. ML and JV wrote the first draft of the manuscript. ML and PL performed the data analysis. PL, MD, AJH and CvdZ helped to develop the immunopeptidomics workflow, performed the final processing of the samples and acquired the MS data. EM assisted with the ImageStream experiments. All authors critically reviewed and approved the manuscript.

Conflict of interest:

K. F. has received unrestricted research grants from CSL Behring, SOBI and Novo Nordisk and consultancy fees from F. Hoffmann-La Roche, Sanofi, SOBI, and Novo Nordisk; all were paid to her institution.

The other authors have no conflicts of interest to declare.

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Data-sharing statement:

Data can be obtained from the authors upon request. The raw mass spectrometry acquisition files and MSFragger search files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository[220] dataset identifier PXD 073718.

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Supplementary Data

Methods

Peripheral blood samples

Blood samples of 50-55 mL were collected from healthy volunteers in accordance with the Dutch regulations and following approval of the Sanquin Ethical Advisory Board and the Declaration of Helsinki.

Dendritic cells differentiation and incubation with FIX

Peripheral blood mononuclear cells (PBMCs) were isolated from 50-55 mL blood by density gradient centrifugation using Ficoll-Paque™ PLUS. From this fraction monocytes were isolated using anti-CD14 magnetic MicroBeads. Typical yields for monocytes obtained using this protocol ranged from 14-21 x 10^6 monocytes per 50-55 mL of blood. Monocytes were plated at a density of 1x10^6 cells per mL in CellGenix GMP DC Medium with supplemented with 1% penicillin/streptomycin solution and differentiated to dendritic cells (immature dendritic cells, iDCs using 1000 IU/mL human GM-CSF and 800 IU/mL human IL-4. After 5 days of differentiation, iDCs were detached and counted. Cells were replated at a density of 1,5 to 2 x 10^6 cells/mL in CellGenix GMP DC Medium supplemented with 1% penicillin/streptomycin , 1000 IU/mL human GM-CSF and 800 IU/mL human IL-4. 100 nM of FIX corresponding to 15 IU/mL FIX (Nonafact, Sanquin Plasma Products) was added to the iDCs and after 5 hours LPS (final concentration 1 µg/ml) and HSA (final concentration 1%) were added. To allow phagocytosis, protein processing and peptide exposition dendritic cells were incubated for 24 hours after FIX addition.

HLA-DR prediction

We utilized the software NetMHCIIpan - 4.1 to predict FIX peptides presented by HLA-DR molecules. The in silico predicted FIX peptides binding to the DRB1 alleles of the donors included in this study is shown in Supplemental Figure 2. We also used NetMHCIIpan – 4.1 to predict which DRB1 alleles could potentially bind to the promiscuously presented FIX sequence 292-QKRNVIRIIPHHNYNAAINK-311. For both predictions the selected length of the peptide was 15 aa.

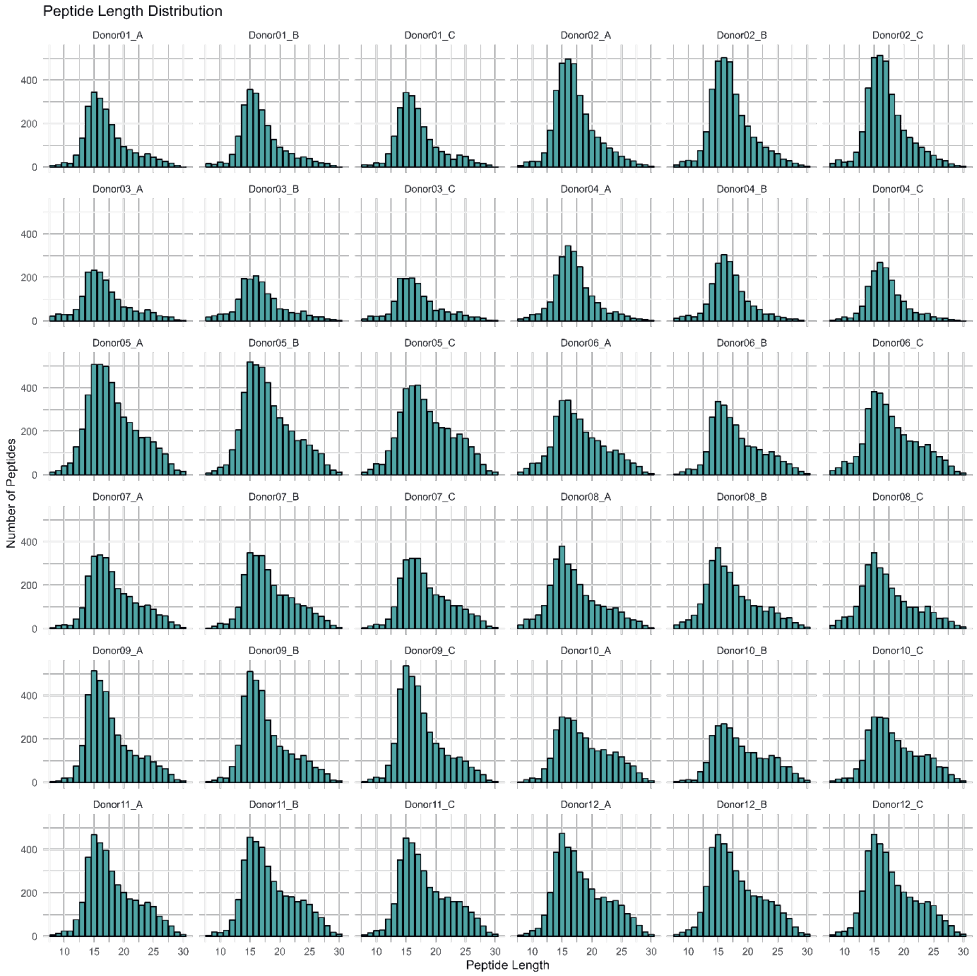
Sample	Condition	Ab used	Replicate	Num Peptides	Num Proteins	Num FIX peptides
Donor02	CTRL	iso	A	606	254	0
			B	1080	303	0
			C	810	264	0
		L243	A	2903	689	0
			B	2994	693	0
			C	2912	662	0
	FIX	iso	A	708	261	0
			B	664	240	0
			C	553	224	0
Donor03	FIX	iso	A	800	255	0
			B	492	195	0
			C	576	202	0
Donor04	FIX	iso	A	200	104	0
			B	552	174	0
			C	791	169	0
Donor05	CTRL	L243	A	3596	879	0
			B	3680	898	0
			C	3413	850	0
Donor06	CTRL	L243	A	4183	987	0
			B	4061	951	0
			C	3641	876	0
Donor07	CTRL	L243	A	3534	804	0
			B	2846	674	0
			C	2960	723	0
Donor08	CTRL	L243	A	2213	619	0
			B	2173	605	0
			C	2328	641	0
Donor09	CTRL	L243	A	3752	893	0
			B	3704	851	0
			C	4390	996	0
Donor10	CTRL	L243	A	3625	894	0
			B	6289	1174	0
			C	3469	846	0
Donor11	CTRL	L243	A	2566	690	0
			B	2773	735	0
			C	2664	721	0
Donor12	CTRL	L243	A	2804	773	0
			B	1720	591	0
			C	3432	841	0

Supplementary Table 1. Description of HLA-DR presented peptides of a cohort of 12 donors. Details on number of peptides recovered after immunoprecipitations employing HLA-DR antibody L243 from FIX pulsated DCs of 12 healthy donors. The number of

peptides, number of proteins and number of FIX derived peptides is indicated for each donor in triplicates labelled A, B and C.

Donor	DRB1	
1	01	13
2	01	03
3	01	07
4	01	13
5	01	13
6	01	13
7	04	11
8	16	16
9	03	04
10	03	04
11	01	04
12	01	07

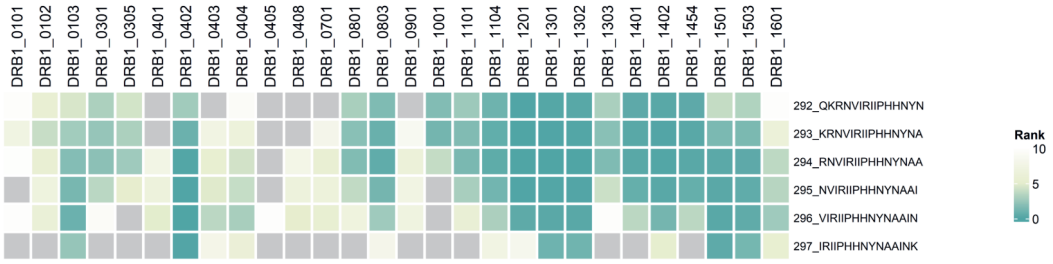
Supplementary Table 2. HLA-DRB1 alleles of the donors. Description of the HLA-DRB1 alleles of the 12 donors used in the study



Supplementary Figure 1. Length distribution of HLA-DR peptides. Graphical representation of length distribution of peptides recovered after immunoprecipitations employing HLA-DR antibody L243 from FIX pulsed DCs of 12 healthy donors.


DRB1 0101
DRB1 0102
DRB1 0103
DRB1 0301
DRB1 0305
DRB1 0701
DRB1 1101
DRB1 1104
DRB1 1301
DRB1 1302
DRB1 1303
DRB1 1601
2_QRVNMIMAESPGLIT
3_RVNMIMAESPGLITI
28_CTVFLDHENANKILN
29_TVFLDHENANKILNR
30_VFLDHENANKILNRP
35_ENANKILNRPKRYNS
36_NANKILNRPKRYNSG
37_ANKILNRPKRYNSGK
38_NKILNRPKRYNSGKL
72_EEAREVFENTERTTE
73_EAREVFENTERTTEF
74_AREVFENTERTTEFW
75_REVFENTERTTEFWK
76_EVFENTERTTEFWKQ
129_ELDVTCNIKNGRCEQ
155_CSCTEGYRLAENQKS
156_SCTEGYRLAENQKSC
157_CTEGYRLAENQKSCE
158_TEGYRLAENQKSCEP
159_EGYRLAENQKSCEPA
178_CGRVSVSQTSKLTRA
179_GRVSVSQTSKLTRAE
180_RVSVSQTSKLTRAET
181_VSVSQTSKLTRAETV
183_VSQTSKLTRAETVFP
184_SQTSKLTRAETVFPD
196_FPDVDYVNSTEAETI
197_PDVDYVNSTEAETIL
198_DVDYVNSTEAETILD
199_VDYVNSTEAETILDN
200_DYVNSTEAETILDNI
216_QSTQSFNDFTRVVGG
217_STQSFNDFTRVVGGE
218_TQSFNDFTRVVGGED
219_QSFNDFTRVVGGEDA
220_SFNDFTRVVGGEDAK
221_FNDFTRVVGGEDAKP
222_NDFTRVVGGEDAKPG
235_PGQFPWQVVLNGKVD
236_GQFPWQVVLNGKVDA
237_QFPWQVVLNGKVDAF
255_SIVNEKWIVTAAHCV
256_IVNEKWIVTAAHCVE
257_VNEKWIVTAAHCVET
258_NEKWIVTAAHCVETG
259_EKWIVTAAHCVETGV
270_ETGVKITVVAGEHNI
271_TGVKITVVAGEHNIE
272_GVKITVVAGEHNIEE
273_VKITVVAGEHNIEET
274_KITVVAGEHNIEETE
291_EQKRNVIRIIPHHNY
292_QKRNVIRIIPHHNYN
293_KRNVIRIIPHHNYNA
294_RNVIRIIPHHNYNAA
295_NVIRIIPHHNYNAAI
296_VIRIIPHHNYNAAIN
297_IRIIPHHNYNAAINK
298_RIIPHHNYNAAINKY
300_IPHHNYNAAINKYNH
301_PHHNYNAAINKYNHD
302_HHNYNAAINKYNHDI
307_AAINKYNHDIALLEL
308_AINKYNHDIALLELD
309_INKYNHDIALLELDE
310_NKYNHDIALLELDEP
314_HDIALLELDEPLVLN
315_DIALLELDEPLVLNS
316_IALLELDEPLVLNSY
317_ALLELDEPLVLNSYV
340_EYTNIFLKFGSGYVS
341_YTNIFLKFGSGYVSG
342_TNIFLKFGSGYVSGW
343_NIFLKFGSGYVSGWG
349_GSGYVSGWGRVFHKG
350_SGYVSGWGRVFHKGR
354_SGWGRVFHKGRSALV
355_GWGRVFHKGRSALVL
356_WGRVFHKGRSALVLQ
357_GRVFHKGRSALVLQY
358_RVFHKGRSALVLQYL
364_RSALVLQYLRVPLVD
365_SALVLQYLRVPLVDR
366_ALVLQYLRVPLVDRA
367_LVLQYLRVPLVDRAT
373_RVPLVDRATCLRSTK
435_CAMKGKYGIYTKVSR
436_AMKGKYGIYTKVSRY
437_MKGKYGIYTKVSRYV
438_KGKYGIYTKVSRYVN
439_GKYGIYTKVSRYVNW
440_KYGIYTKVSRYVNWI
441_YGIYTKVSRYVNWIK
442_GIYTKVSRYVNWIKE
445_TKVSRYVNWIKEKTK
446_KVSRYVNWIKEKTKL
447_VSRYVNWIKEKTKLT
Rank
10
5
0

Supplementary Figure 2. Heatmap of predicted FIX peptide presented on HLA-DRB1 employing NetMHCIIpan – 4.1. Predicted affinity of peptides is expressed as a percentage rank score. Only peptides with a rank score below 10% are displayed. Peptides with a rank score below 5% are considered weak binders and peptides with a rank score lower than 1% are considered strong binders.

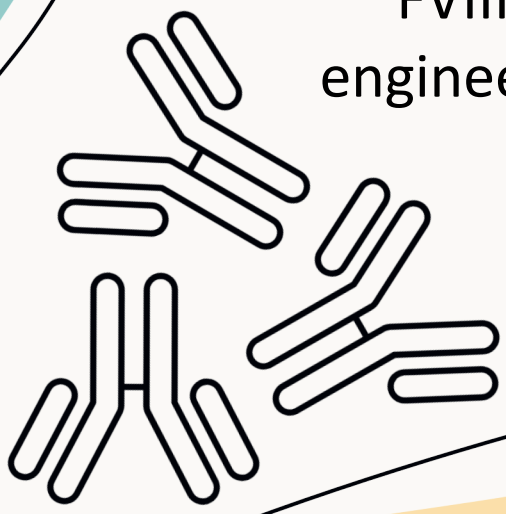


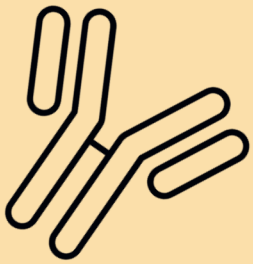
Supplementary Figure 3. HLA-DRB1 alleles predicted to bind with high affinity to peptide 292-QKRNVIRIIPHHNYNAAINK-311. The predicted affinity of different HLA-DRB1 alleles for the peptide are presented as a color code based on the percentage rank score.



Chapter 6

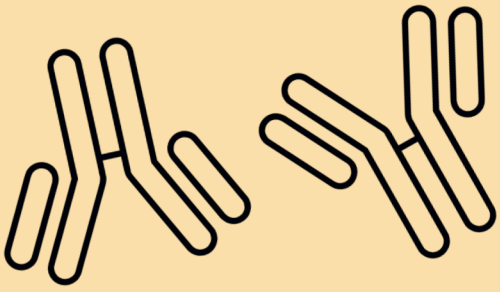
Affinity of human FcγR receptors for FVIII-Fc and an Fc domain engineered human anti-FVIII antibody





Ilja Oomen[†], Mariarosaria Miranda[†], **Michela Leoni**[†], Paul Kaijen, Arthur Bentlage, Gestur Vidarsson, Sietse Q Nagelkerke, Taco W. Kuijpers, Karin Fijnvandraat, Jan Voorberg*, Samantha Gouw*

Manuscript in preparation



Summary

IgG Fc domain fusions have been widely used to improve the circulating half-life of biologicals. Fusion of the Fc domain of human IgG1 to FVIII improves in vivo half-life of circulating FVIII. Here, we studied the affinity of FVIII-Fc to different classes of activating and inhibitory FcγRs. Our results show that FVIII-Fc binds with high affinity to the activating receptor FcγRI and with modest affinity to the activating receptors FcγRIIA (131H), FcγRIIA (131R), FcγRIIIa (158F) and FcγRIIIa (158V). Limited binding to the activating receptors FcγRIIIA (158F, 158V, NA1, NA2) and the inhibitory receptor FcγRIIB was observed. In parallel, we evaluated the FcγR binding properties of wild type and an Fc domain engineered EL14, a human anti-FVIII antibody isolated from B cells of a patient with acquired hemophilia. The affinity of wild type EL14 IgG1 of the different human FcγRs was similar to that of FVIII-Fc. Modification of three residues in the Fc region of EL14 (P329G, L234A, L235A; designated EL14-PGLALA) completely abolished binding to all FcγRs. Fc domain modified EL14-EGPHPA (containing amino acid substitutions E233D/G237D/P238D/H268D/P271G/A330R) was found to bind with high affinity to the activating FcγRI and inhibitor FcγRIIB. Low affinity binding to FcγRIIA (131R) was observed; no binding to FcγRIIIA, FcγRIIIA and FcγRIIB was detected. Targeting of the inhibitory FcγRIIB has been proposed as a means to modulate pathogenic immune response. Based on our results we propose that introduction of an FcγRIIB targeting moiety within FVIII-Fc or co-administration of FVIII and EL14-EGPHPA complexes may be explored as a potential option to prevent or more efficiently eradicate FVIII inhibitors that develop in patients with hemophilia A.

Introduction

Hemophilia A is a hereditary bleeding disorder caused by a deficiency in clotting factor VIII (FVIII), resulting in spontaneous joint bleeds and debilitating joint damage[1]. For decades, FVIII replacement therapy has been the cornerstone of prophylactic treatment for patients with hemophilia A[2]. Standard half-life (SHL) recombinant FVIII (rFVIII) products, such as Advate®, have a FVIII half-life of about 12 hours[3]. To reduce the frequency of intravenous injections, recombinant FVIII with extended half-lives (EHLs) have been designed. One strategy involves fusing rFVIII to the crystallizable fragment Fc region of IgG1 (Elocta®)[4]. The Fc domain enabled binding to the neonatal Fc receptor (FcRn), which is responsible for protecting IgG from degradation and facilitates its recycling[5]. Introduction of Fc domain in FVIII has been shown to result in a half-life extension of 1.4-1.6 fold ranging from 14 to 19 hours when compared to unmodified FVIII[5,6]. Further enhancement of half-life of FVIII-Fc was accomplished by engineering efanesoctocog alfa (BIVV001), a recombinant FVIII (rFVIII) molecule, which does not bind to endogenous von Willebrand factor (VWF)[7]. Together, these structural modifications result in a 3- to 4-fold longer half-life compared to non-modified rFVIII products[8].

A significant challenge of FVIII replacement therapy is the development of neutralizing antibodies, or inhibitors, which occur in about one-third of patients with severe hemophilia A—typically within the first 50 exposure days[9,10]. These high-affinity IgG antibodies interfere with FVIII's procoagulant function and are thought to arise from a combination of genetic predisposition and treatment-related factors, including the type of FVIII product and intensity of exposure[11,12]. Immune tolerance induction (ITI) therapy is the only treatment option to eradicate inhibitors, consisting of frequent infusions of high doses FVIII concentrates[1]. Eradication of the inhibitor is a desirable treatment goal because it allows FVIII treatment in case of bleeding or surgery. However, the disadvantage of ITI is that it is very demanding and burdensome for patients, and in addition, ITI is associated with very high health care costs[13]. Unfortunately, ITI fails in about 30% of patients[14]. The exact mechanism underlying ITI is still unknown, but it has been hypothesized that successful ITI depends on the elimination of FVIII-specific B-memory and FVIII-specific CD4+ T-memory cells and induction of regulatory T cells (T_{reg})[15–17]. Recent findings suggest that this process may be mediated through PD-L1/PD-1 signaling, where PD-L1^{+} Tregs induce apoptosis of FVIII-specific B cells, thereby contributing to immune tolerance[18].

FcγRs are glycoproteins primarily expressed on myeloid cells, which bind the Fc region of IgG to regulate humoral and cellular immunity[20]. They are broadly

classified as activating (FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa) or inhibitory (FcγRIIb), with signaling driven by immunoreceptor tyrosine-based activation or inhibition motifs (ITAMs/ITIMs)[21]. Polymorphisms in their extracellular domains significantly impact their function. In FcγRIIa, histidine (H) at position 131 (His131, now known as His166) enhances the affinity of the receptor for IgG2 when compared to arginine (R) (Arg131, now known as Arg166)[22]. Eckhardt et al. reported that hemophilia A patients with the HH genotype of the *FCGR2A* polymorphism have a more than 3-fold increased risk of inhibitors development compared with patients with the RR genotype[23]. Similarly, FcγRIIIa shows higher affinity for IgG1 and IgG3 when valine occupies position 158 (Val158, now known as Val176) rather than phenylalanine (Phe158, now known as Phe176)[22]. The *FCGR3B* gene encodes FcγRIIIb, with three polymorphic haplotypes—NA1, NA2, and SH—defined by six SNPs. These haplotypes influence neutrophil function, with NA1 showing higher phagocytic activity than NA2 and SH, while SH uniquely features a p.Ala78Asp substitution, potentially altering protein structure[22]. Copy number variations (CNVs) in *FCGR3B* have been linked to several autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis (RA)[24,25]. However, CNV of FCGR3A, FCGR3B or FCGR2C was not associated with inhibitor development in patients with severe hemophilia A[23]. The Ile232Thr substitution in FcγRIIb diminishes inhibitory signaling, and FcγRIIc expression depends on the *FCGR2C*-open reading frame (ORF) haplotype[26].

It has been established that antibodies in complex with antigen can modulate the immune response to the same antigen[27]. In a humanized mouse model for systemic lupus erythematosus Horton et al. showed that crosslinking of the inhibitory FcγRIIb receptor and the B-cell receptor suppresses B-cell activation[28]. Additionally, Werwitzke et al. reported that FcγRIIb is a crucial regulator of FVIII-specific B cells and is required for differentiation of B cells into antibody-secreting cells, as demonstrated in a hemophilia A mouse model[29]. Hartholt et al. showed that multiple Fc receptors are involved in the internalization of FVIII-IC in bone marrow derived dendritic cells (BMDC)[30]. These findings together suggest the involvement of the FcγRs receptor in the modulation of the immune response in hemophilia A.

In this study we determined the affinity of FVIII-Fc to activating and inhibitory FcγRs. In parallel, we evaluated the FcγR binding properties of wild type and an Fc domain engineered human anti-FVIII antibody designated EL-14 that was isolated from B cells of a patient with acquired hemophilia. Based on the results we discuss potential strategies to employ Fc region engineered FVIII-Fc and anti-FVIII antibodies to improve current protocols for inducing tolerance. To this end, we generated FVIII immune complexes composed of either FVIII or FVIII-Fc in

combination with one of the two variants of EL-14, a previously characterized human monoclonal anti-FVIII antibody[31]. The EL-14 variants carried distinct Fc region modifications: one harboring the LALAPG mutation, which abrogates the binding to the FcγRs, and the other incorporating the EGPHPA mutation, which preferentially bind the inhibitory FcγRIIb[32,33]. By elucidating these interactions, this work aims to provide mechanistic insights and identify potential strategies to mitigate inhibitor development in hemophilia A.

Materials and Methods

Full-length recombinant FVIII (Advate®) was obtained from Takeda Pharmaceuticals (Tokyo, Japan). B-domain deleted recombinant FVIII with an Fc fusion protein (Elocta®) was obtained from Sobi (Stockholm, Sweden).

Variants of the human monoclonal antibody EL14, an IgG1 antibody with specificity for the C2 domain of FVIII (EL14 IgG1), were generated for this study. To abrogate binding to FcγRs, an EL14 IgG1 variant was engineered with three amino acid substitutions (L234A/L235A/P329G) in the Fc region (EL14-LALAPG IgG1), previously described as LALAPG variant by Wilkinson et al[33]. To enhance binding to the inhibitory FcγRIIb, a second EL14 IgG1 variant was produced incorporating the P238D mutation along with five amino acid substitutions (E233D/G237D/H268D/P271G/A330R) in the Fc region (EL14-EGPHPA IgG1), described as V12 variant by Mimoto et al[21,32]. EL14-LALAPG and EL14-EGPHPA human monoclonal antibodies were expressed in Expi293 cells using the ExpiFectamine 293 transfection kit (Thermo Fisher Scientific Inc., Waltham, USA). Both EL14-LALAPG and EL14-EGPHPA were purified by protein A Sepharose 4B (Thermo Fisher Scientific Inc., Waltham, USA).

Binding kinetics of EL14, EL14-LALAPG and EL14-EGPHPA IgG1 antibodies and rFVIIIFc to FcγRs

Affinity measurements to human Fcγ receptors were performed using an IBIS MX96 (IBIS Technologies) device and a Continuous Flow Microspotter (Wasatch Microfluidics). Two different setups were used, depending on the tag attached to the FcγR; either biotin or his-tagged. For affinity measurements of biotinylated FcγRs (hFcγRIIa-131H, hFcγRIIa-131R, hFcγRIIb, hFcγRIIIa-158V, hFcγRIIIa-158F, hFcγRIIIb-NA1, hFcγRIIIb-NA2) an array of 4 or 5 concentrations of a 3-fold dilution series of C-terminally biotinylated human FcγRs was captured simultaneously on a SensEye G-streptavidin sensor (Ssens; cat#1-09-04-006) in PBS at pH 7.4 supplemented with 0.075% (v/v) Tween-80 (PBST). The start concentration differed depending on the Fcγ receptor (100 nM for hFcγRIIIb-NA1, 30 nM for hFcγRIIIa-158F and hFcγRIIIb-NA2, and 10 nM for hFcγRIIa-131H, hFcγRIIa-131R, hFcγRIIb and hFcγRIIIa-158V). Next, a 2-fold dilution series of the samples was injected ranging from 0.49 nM to 1000 nM in PBST for the antibodies and 6.04 nM to 773 nM for rFVIII-Fc. Regeneration of the sensor surface between the cycles was performed by injection of 10 mM Glycine-HCl pH 2.4. For affinity measurements of the C-terminally his-tagged human FcγRI, 5 concentrations of a 3-fold dilutions

series of C-terminally biotinylated anti-His mIgG1 (GenScript; cat#A00613) was captured on a SensEye G-streptavidin sensor (Ssens; Cat#1-09-04-006) in PBST. Subsequently, 20 nM human FcγRI was captured and antibodies were titrated from 0.41 nM to 100 nM, and rFVIII-Fc was titrated from 0.32 nM to 77 nM, in a 3-fold dilution series in PBST. Regeneration of the sensor surface between the cycles was performed by injection of 10 mM Glycine-HCl pH 2.1. Affinity (KD) calculations were performed using an equilibrium analysis, interpolating to an Rmax of either 500 RU for antibodies or 733 RU rFVIII-Fc. A 1:1 Langmuir binding model was used for fitting, assuming equilibrium to be reached after 360s of IgG containing analyte injections, as described previously[36]. Analysis and calculations were performed using Scrubber Software Version 2 (BioLogic Software), Excel (Microsoft) and Graphpad Prism (Prism).

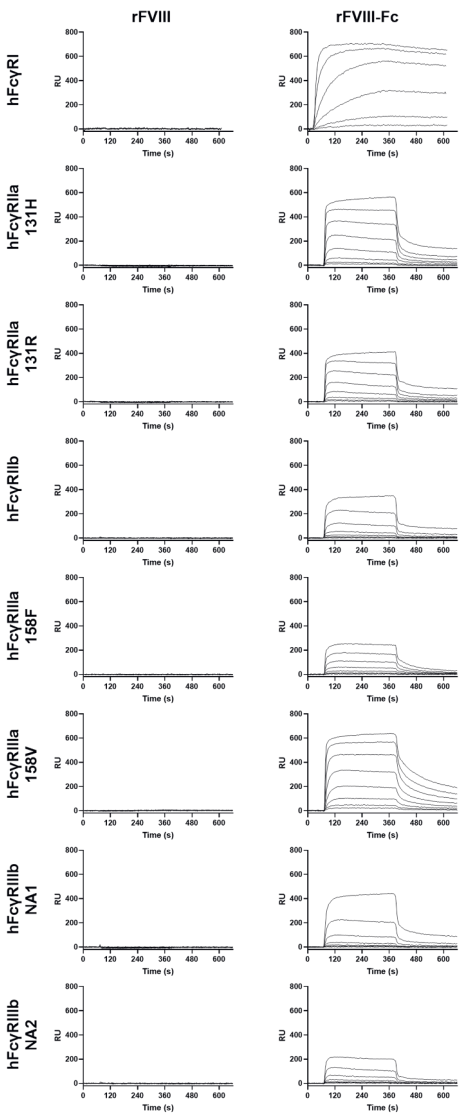
Results

Binding affinity of EL14, EL14-LALAPG and EL14-EGPHPA IgG1 antibodies to FcγRs

In this study, we engineered two EL14 IgG1 variants: EL14-LALAPG, containing 3 Fc-region mutations to abrogate FcγR binding; and EL14-EGPHPA, incorporating six Fc mutations designed to enhance binding affinity specifically for the inhibitory FcγRIIb[21,32,33]. Binding affinity of EL14 IgG1, EL14-LALAPG and EL14-EGPHPA IgG1 antibodies to all FcγRs were assessed using surface plasmon resonance (SPR). Representative sensograms are shown in **Figure 2A**, and equilibrium dissociation constants (K_D) derived from the fitted curves are summarized in **Figure 2B**. The wildtype EL-14 IgG1 displayed moderate to high binding to all tested FcγRs, with the strongest affinity observed for hFcγRI (2.5 ± 0.1 nM). Binding to hFcγRIIa (131H and 131R) and hFcγRIIIa (158F and 158V) was also detectable, with affinities ranging from 111 ± 2 nM (hFcγRIIIa 158V) to 370 ± 16 nM (hFcγRIIIa 158F). No binding was detected to hFcγRIIb or hFcγRIIIb alleles. The EL-14 EGPHPA variant exhibited a markedly increased affinity for hFcγRI (8.7 ± 0.9 nM) compared to EL-14 IgG1. Notably, this variant also gained binding capability to hFcγRIIb (12 ± 0.7 nM) and showed a reduced binding to hFcγRIIa 131R (122 ± 5 nM). Binding to other FcγRs was not detected or not assessed. As expected, the EL-14 LALAPG variant, engineered to abrogate FcγR interactions, showed no detectable binding (n.a.) to any of the FcγRs tested, confirming the effectiveness of Fc region silencing.

Representative sensograms are shown in **Figure 1** and K_D derived from the fitted curves are summarized in **Supplementary Figure 1**. SPR experiments demonstrated that rFVIIIFc binds several human FcγR subtypes, including FcγRI, FcγRIIa (131H and 131R variants), and FcγRIIIa (158F and 158V), with varying affinities. No binding was detected to FcγRIIb or FcγRIIIb.

A

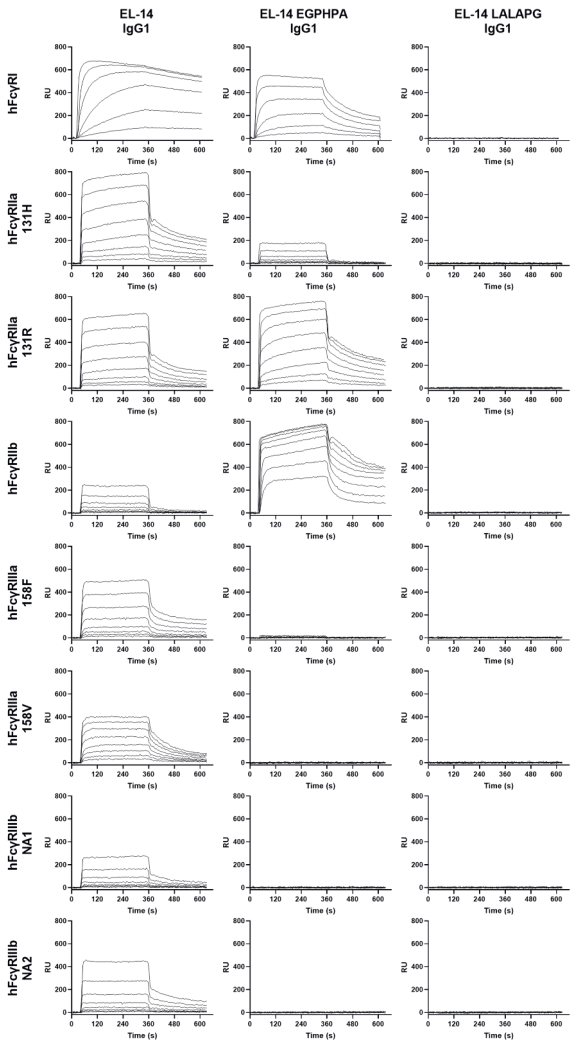


B

FcγR subtype	rFVIII	rFVIII-Fc
hFcγRI	n.d.	5,2 ± 0,1
hFcγRIIa 131H	n.d.	343 ± 1,7
hFcγRIIa 131R	n.d.	440 ± 2,4
hFcγRIIb	n.d.	n.c.
hFcγRIIIa 158F	n.d.	833 ± 20
hFcγRIIIa 158V	n.d.	228 ± 0,81
hFcγRIIIb NA1	n.d.	n.c.
hFcγRIIIb NA2	n.d.	n.c.

Figure 1. Binding kinetics of rFVIIIFc to human Fcγ receptors as measured by surface plasmon resonance (SPR). Human Fcγ receptors (FcγRI, FcγRIIa-131H, FcγRIIa-131R, FcγRIIb, FcγRIIIa 158F, FcγRIIIa-158V, FcγRIIIb-NA1, and FcγRIIIb-NA2) were immobilized on a streptavidin-coated 14 sensor chip (via biotinylation or anti-His antibody capture), and binding interactions with injected rFVIIIFc were monitored using SPR. A) Sensograms show real-time association and dissociation phases at rFVIIIFc concentrations ranging from 6.04 to 773 nM. Binding affinities were calculated using a 1:1 Langmuir binding model at equilibrium. B) Mean equilibrium dissociation constants (KD) are shown in the accompanying table and presented as mean ± standard deviation (nM) from three independent measurements. 'n.a.' denotes no detectable binding under the tested conditions.

A



B

FcγR subtype	EL-14 IgG1	EL-14 EGPHPA IgG1	EL-14 LALAPG IgG1
hFcγRI	2.5 ± 0.1	8.7 ± 0.9	n.a.
hFcγRIIa 131H	246 ± 15	n.c.	n.a.
hFcγRIIa 131R	329 ± 19	122 ± 5	n.a.
hFcγRIIb	n.c.	12 ± 0.7	n.a.
hFcγRIIIa 158F	370 ± 16	n.a.	n.a.
hFcγRIIIa 158V	111 ± 2	n.a.	n.a.
hFcγRIIIb NA1	n.c.	n.a.	n.a.
hFcγRIIIb NA2	n.c.	n.a.	n.a.

Figure 2. Binding kinetics of EL14 IgG1, EL14-EGPHPA IgG1, and EL14-LALAPG IgG1 to human Fcγ receptors as measured by surface plasmon resonance (SPR). Human Fcγ receptors (FcγRI, FcγRIIa-131H, FcγRIIa-131R, FcγRIIb, FcγRIIIa-158F, FcγRIIIa-158V, FcγRIIIb-NA1, and FcγRIIIb-NA2) were immobilized on a streptavidin-coated sensor chip (via biotinylation or anti-His antibody capture), and binding interactions with injected EL14-IgG1, EL14-EGPHPA IgG1, and EL14-LALAPG IgG1 were monitored using SPR. **A**) Sensograms show real-time association and dissociation at various concentrations (ranging from 0.49 to 1000 nM). Binding affinities were calculated using a 1:1 Langmuir binding model at equilibrium. **B**) Mean equilibrium dissociation constants (KD) are shown in the accompanying table and presented as mean ± standard deviation (nM) from three independent measurements. 'n.a.' denotes no detectable binding under the tested conditions.

Discussion

Replacement therapy with recombinant factor VIII (rFVIII) has long been the cornerstone of hemophilia A management, aiming to prevent and control bleeding episodes. The development of extended half-life products, such as rFVIIIFc and efanesoctocog alfa, represents a significant advancement by reducing infusion frequency[6]. Beyond its interaction with the FcRn, rFVIIIFc also engages Fcγ receptors (FcγR) expressed on various immune cells, including dendritic cells and macrophages[5,20]. These interactions may influence the immunogenicity of Fc-fusion therapeutics, potentially by modulating antigen uptake and presentation by dendritic cells[38,39]. Interestingly, rFVIIIFc has also been proposed to accelerate immune tolerance induction (ITI) and influence $CD4^+$ T cell responses through these mechanisms, although clinical data supporting this hypothesis remain limited[40].

A critical challenge in hemophilia A treatment remains the development of anti-FVIII inhibitory antibodies[41]. Upon FVIII infusion, circulating FVIII immune complexes (FVIII-ICs) form in patients with inhibitors. Reipert et al. reported distinct antibody signatures appearing prior to FVIII inhibitor development[42]. They showed that non-neutralizing antibodies (NNAs) against FVIII emerge before clinically relevant neutralizing inhibitors. These NNAs may form immune complexes with FVIII, potentially influencing the immune response and modulating inhibitor development[42]. The development of FVIII-IC may also influence the outcome of ITI in patients with inhibitors. These ICs play a pivotal role in immune regulation, as their engagement with FcγR, particularly FcγRIIb, can modulate immune responses[38]. FcγRIIb is essential for maintaining immune tolerance, as it regulates antigen uptake and suppresses pro-inflammatory signaling in dendritic cells[43,44]. In murine models, FVIII-ICs engaging FcγRIIb

enhance the internalization of these complexes by bone marrow-derived dendritic cells (BMDCs), suggesting a potential pathway for immune regulation[30]. However, murine DC cultures may be predisposed to tolerance due to their immature phenotype and the absence of inflammatory cues, which should be considered when interpreting these findings.

The mechanisms by which FVIII-ICs interact with dendritic cells, and the implications of these interactions for immune modulation, remain incompletely understood. To address this knowledge gap, we with engineered variants of the human FVIII-specific monoclonal antibody EL14. Specifically, we designed EL14-LALAPG, an IgG1 variant lacking FcγR binding capacity, and EL14-EGPHPA, an IgG1 variant with enhanced affinity for FcγRIIb and FcγRIIc. Based on this, we speculate that targeting FcγRIIb on antigen presenting cells may potentially be used to prevent inhibitor development in hemophilia A patients.

The recombinant FVIII sources utilized in this study was rFVIIIFc (Elocta®). While our experimental data were limited to this specific product, we anticipate that binding properties of BIVV001, a next generation FVIII-Fc variant containing the D'D3 domain of VWF as well as XTEN polypeptides will be similar.

In conclusion, our results suggest that it may be worthwhile to explore a modulating role of FcγRIIb in the development of FVIII inhibitors. Fusion of the EGPHPA containing Fc domain to protein therapeutics such as FVIII may result in decreased immunogenicity due to selective targeting to the inhibitory FcγRIIb.

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Authorship contributions

JV and SCG designed study; IO, ML, MM performed experiments; GV provided constructs for FcγRIIb-enhanced binding antibodies; AB performed the SPR binding experiments; SQN, GV, TWK, JV and SCG supervised data analysis; JV, KF and SCG secured funding for this study; IO, MM, ML, AB, JV and SCG wrote the manuscript; all authors critically reviewed manuscript; all authors approved the final version of the manuscript.

Conflicts of Interest

IO was supported by an unrestricted research grant from Sobi, EAHAD, CSL Behring (Heimburger Award) and Grifols (Martin Villar research grant). SCG received unrestricted research funding from Sobi, EAHAD, CSL Behring (Heimburger Award), and the Netherlands Organization for Scientific Research (NWO). KF has received unrestricted research grants from CSL Behring, Sobi, and Novo Nordisk and consultancy fees from Sobi, Novo Nordisk, and Roche (all fees to the institution). JV is listed as an inventor of a patent application on ADAMTS13. Other authors stated that they had no interests which might be perceived as posing a conflict or bias.

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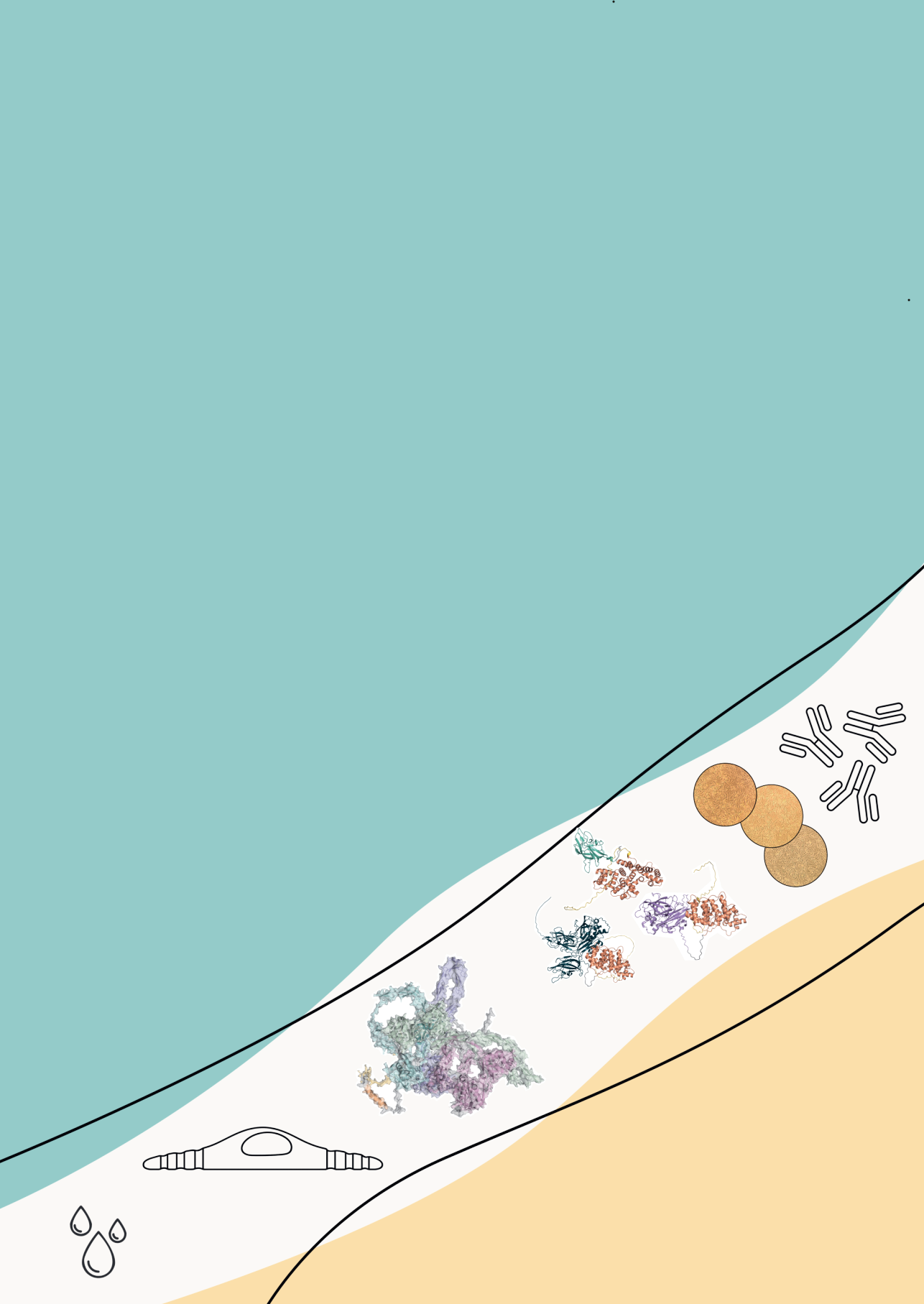
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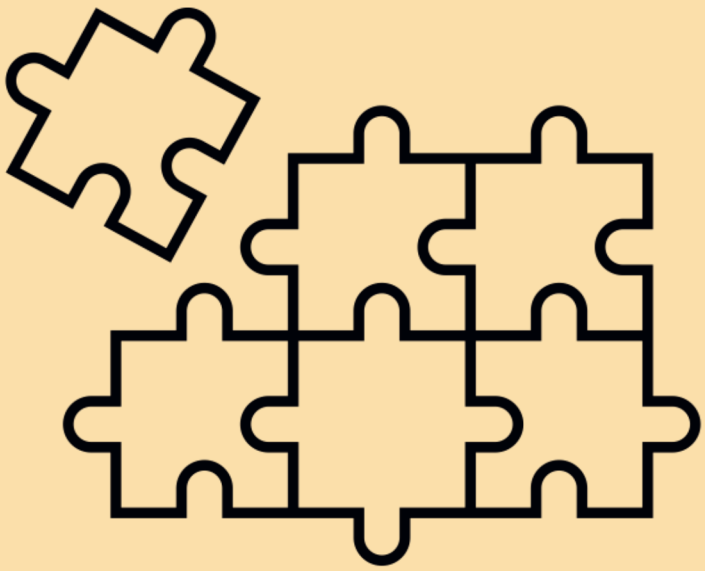
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Chapter 7
Discussion



General Discussion

Many novel treatment options have become available for hemophilia A and B patients in the last decades, and this has resulted in a greatly improved quality of life. The use of non-factor replacement therapy has revolutionized the treatment for hemophilia A. Breakthrough bleeds in these patients are still treated with FVIII, and therefore inhibitor development against infused FVIII remains a potential side-effect. For hemophilia B, extended half-life products have been developed. Inhibitor development in hemophilia B is relatively rare but, currently, incompletely understood. Gene therapy for hemophilia A and B has been developed and provides a potential cure. Uptake of gene therapy of hemophilia A is slow due to concerns with respect to liver toxicity and declining levels of FVIII over time. For hemophilia B, successful clinical protocols have been developed but, due to high costs, the uptake has been slow. Altogether, development of anti-FVIII antibodies in hemophilia A and anti-FIX antibodies in hemophilia B provides a clinically relevant side-effect of treatment. In this thesis we address the challenges of hemophilia patients with anti-drug antibodies and identify potential treatment options for this complication of hemophilia care.

Liver sinusoidal endothelial cells; synthesis and clearance of FVIII

The importance of liver sinusoidal cells (LSECs) in the context of hemophilia A has been only recently appreciated as outlined in detail in **Chapter 2**. LSECs line the hepatic sinusoids and are continuously exposed to nutrients, pathogens and cellular debris present in circulating blood in the liver[261]. Two unique characteristics of their morphology are the lack of basal lamina and the presence of numerous fenestrations of 100-150 nm. These adaptations allow for the passage of small and medium size complexes and particles from the circulation to the Space of Disse[261,262]. The expression profile and functionality of LSECs depends on the localization inside the liver, particularly relative to the lobule. LSEC have been divided into zones depending on their proximity to the portal triad (portal vein, hepatic artery and bile duct) and central vein. If closest to the portal triad, LSECs are categorized as periportal zone LSEC, or Zone 1 LSEC; if closest to the central vein, central zone or Zone 3 LSECs. The cells in between are called midzone or Zone 2 LSECs, often they are grouped together with Zone 3 LSECs[263]. Single cell sequencing has shown that expression profiles of Zone 1 and Zone 2/3 differs significantly and this, most likely, results in functional specialization of Zone 1 and Zone 2/3 localized LSECs[264]. Periportal Zone 1 LSECs express high levels of CD36 and CD31[264]. Zone 2/3 LSECs express higher levels of FVIII[38]. Zone 1 cells are

subjected to high blood flow rate and shear stress that has been shown to negatively regulate FVIII expression in endothelial cells culture; this may be one of the reasons for the lower FVIII expression in Zone 1 when compared to Zone 2/3[38]. Proteins connected with binding and uptake of ligand and immune pathways are enriched in Zone 2/3 LSEC. Some of the proteins enriched on Zone 2/3 LSEC are CD32, STAB1, STAB2, CLEC4G and LYVE-1[157,265].

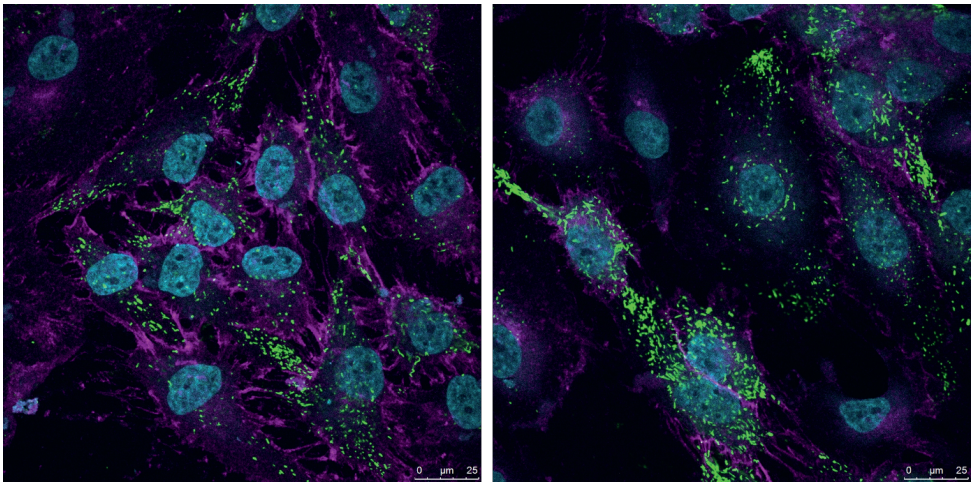


Figure 1. Primary LSECs. Primary liver sinusoidal endothelial cells were fixed and stained for VE-cadherin (magenta) and von Willebrand factor (green). Nuclei are shown in cyan.

Even though originally hepatocytes were believed to be the principal site for FVIII synthesis, it has now been convincingly demonstrated that LSEC are the principal cells involved in FVIII synthesis[36–38]. Most likely, the previous observations on FVIII expression in hepatocytes was due to an incorrect identification or separation of the two cells types[266,267]. Moreover, LSEC transplantation can correct the bleeding phenotype in hemophilia A bleeding model while hepatocytes can not[266,268].

The scavenging ability of LSECs allows them to endocytose a large number of molecules and particles present in hepatic blood that include viruses[269]. LSECs are also involved in the clearance of FVIII from circulation due to the expression of CLEC4M and stabilin-2[269–272]. Another important receptor that is expressed on LSEC is CD32 or FcγRIIb, responsible for the elimination of immune complexes[273]. CD32 mediated endocytosis prevents the deposition of pathogenic immune complexes, thereby limiting inflammation. LSECs expresses both MHC I and MHC II complexes allowing them to interact with both CD8+ and CD4+ T cells[274]. The ability of LSECs to directly interact with T cells, together with the efficient scavenging properties, positions LSECs as a unique class of antigen presenting cells. Under quiescent

conditions, LSECs promote tolerance to non-pathogenic compounds in the hepatic circulation due to expression of high levels of PD-L1[274]. Sensing of pathogens by pattern recognition receptors on LSECs can promote a vigorous response, depending on the antigen load, that results in down-regulation of PD-L1, allowing for the generation of cytotoxic CD8+ T cells targeting viruses[275,276]. LSEC further express GARP, a latent TGF-β binder on their surface, which recruits both endogenously synthesized TGF-β as well as TGF-β present in the liver microenvironment[277]. The interaction of naïve CD4+ T cells with both a specific peptide, presented by MHC II through the T cell receptor, and TGF-β, localized in the area by the GARP protein, drives the cells toward a regulatory T-cell phenotype by inducing expression of Foxp3 which is regarded as the master regulator of Tregs.[277,278]. Co-stimulatory molecules such as CD80 and CD86 promote the generation of mature CD4+ and CD8+ T cells; the lack of such molecules on LSECs helps skew the interaction towards a regulatory phenotype[279]. Furthermore, the metabolic profile of LSECs allows the release of IL-10 that promote the creation of an immunosuppressive microenviroment[280].

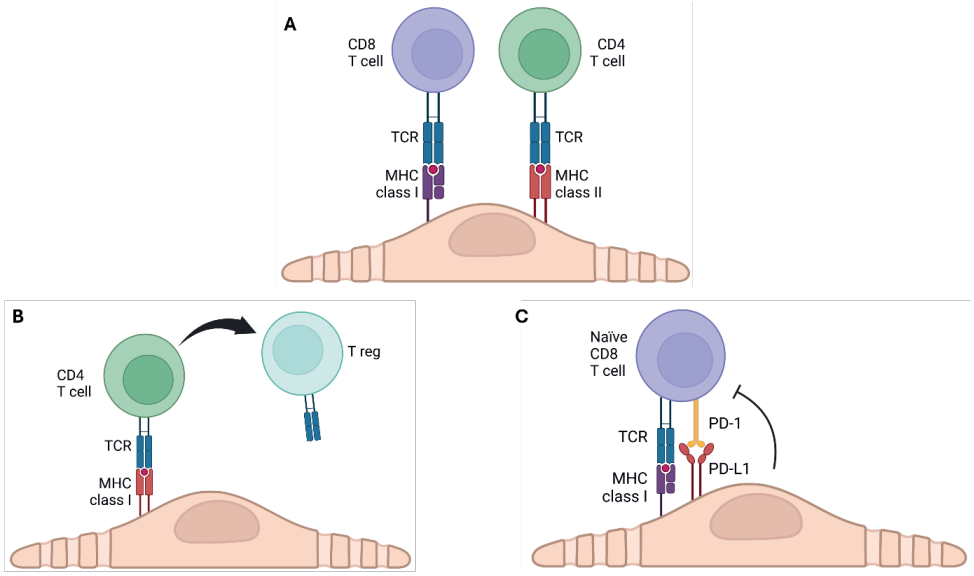


Figure 2. Interaction of LSECs and T cells. LSECs can interact with both CD4+ T cells and CD8+ T cells via respectively MHC class I or MHC II complexes (A). The interaction with CD4+ T cells can results in the development of T reg (B). The high levels of PD-L1 expressed by LSECs results in the inhibition of CD8+ T cells (C).

Altogether, LSECs play a crucial role in FVIII homeostasis. Their ability to synthesize FVIII positions them as an attractive target for gene therapy. Moreover, there is a

clear role for scavenger receptors such as stabilin-2 and CLEC4M, which are highly expressed on LSECs, in the clearance of FVIII from the circulation. The ability to selectively promote Tregs provides a very interesting angle for the development of new therapies that aim to eliminate inhibitors in hemophilia A.

Stabilin-2 and FVIII clearance

In circulation, FVIII is mostly in complex with von Willebrand factor (VWF), just in a small percentage of FVIII circulates not in complex. The FVIII-VWF complex is cleared via multiple pathways in the liver but also in the spleen[281]. Liver and spleen residing macrophages clear the FVIII-VWF complex mainly via the low density protein receptor protein-1 (LRP-1), but the FVIII-VWF complex can also interact with the macrophages receptor SR-A1, the macrophages-galactose-like lectin and Siglec-5[281–285]. In the liver, other than Kupfer cells, also hepatocytes and LSEC contribute to the clearance of the VWF-FVIII complex. In LSEC, three receptors have been reported to bind to the FVIII-VWF complex: SCARA-5, CLEC4M and stabilin-2[145,272]. Apart from LSEC, SCARA-5 is also expressed on splenic sinusoidal endothelial cells [286]. SCARA-5 can bind to FVIII-VWF complex in a VWF dependent manner[272]. CLEC4M, on the surface of LSEC, can bind the FVIII-VWF complex but also VWF alone; binding to CLEC4M is dependent on the presence of mannose ending N-glycans on FVIII and VWF[145].

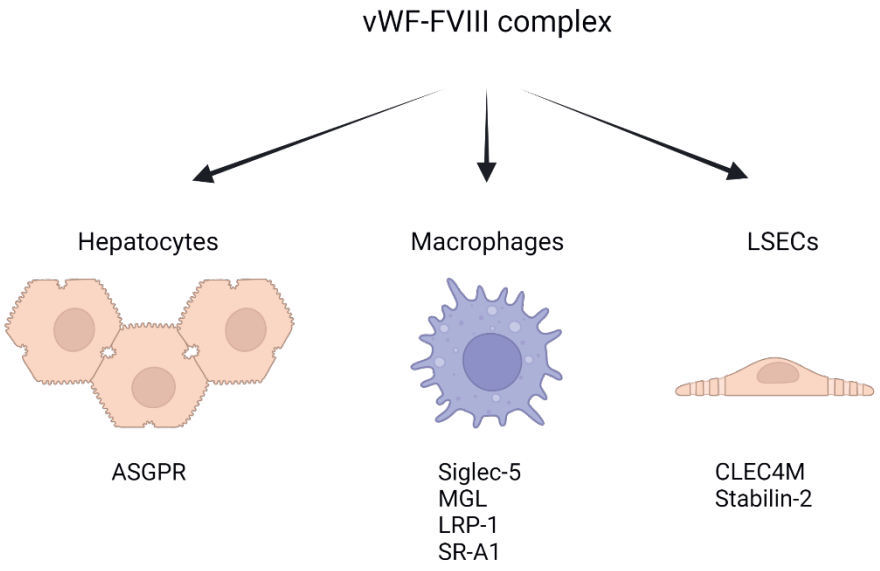


Figure 3. Clearance routes of the VWF-FVIII complex. The VWF-FVIII can be cleared my multiple cells in the body, in the figure are also highlighted the main receptors responsible for binding and endocytosis of the FVIII-VWF complex.

In **Chapter 3** we studied the binding of FVIII to stabilin-2. According to single cells transcriptomic data in mouse, stabilin-2 is principally located on liver endothelial cells, spleen endothelial cells and partly in the testis[287]. Stabilin-2 has been confirmed to be expressed primarily in spleen, liver, lymph node in human and at lower levels in heart, placenta, skeletal muscle, pancreas, thymus, and bone marrow[288]. Stabilin-2 KO mice have been previously used to show that stabilin-2 is involved in the clearance of the FVIII-VWF complex [289]. No *in vitro* binding experiments were reported in that study. In **Chapter 3** we show that stabilin-2 can bind to FVIII *in vitro*. Both surface plasmon resonance as well as solid phase binding assays revealed that FVIII can bind to the extracellular domain of stabilin-2. One of the principal ligands of stabilin-2 is hyaluronic acid from which it also takes one of its first names, HARE, hyaluronic acid receptor for endocytosis[270]. This protein is present in vivo in two different isoforms of 190 kDa and 315 kDa, the first one is the cleaved version of full length stabilin-2. The ratio of the two isoform changes depending on the tissue but in humans it is usually between 1:1 to 3:1[290]. In stable cell lines, however, the ratio changes and goes as high as 10:1 [290]. While stabilin-2 is a large multi-domain protein with a complex structure, one specific domain, the so-called “Link” domain has been shown to contain the binding site

for hyaluronic acid (HA). The Link domain is also present in a number of other receptors which include CD44 and TSG-6[291]. Based on the available three-dimensional structure of CD44 and TSG-6 an homology-based model of the Link domain of stabilin-2 was created[291,292].

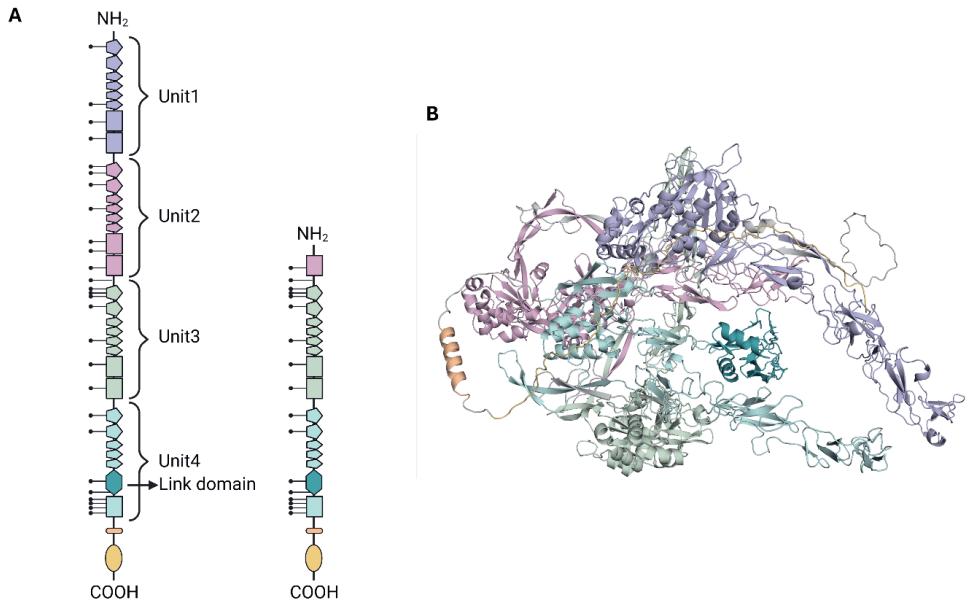


Figure 4. Stabilin-2 structure and domains. The extracellular domain of stabilin-2 is composed of multiple units and is highly glycosylated (black lines plus dot), and can be present on cellular surfaces either as a 315kDa or a 190kDa isoform (A). The structure of stabilin-2 derived from the AlphaFold model is shown using the same color scheme (B).

In **Chapter 3** we showed that the Link domain of stabilin-2 also interacts with FVIII. Based on our data we cannot exclude that, apart from the Link domain, also other domains present on the extracellular domain contribute to the binding of FVIII. We showed that the two N-glycans present in the Link domain are not crucial for the binding of FVIII. We further showed that FVIII does not bind to the Link domain of CD44. Interestingly, hyaluronan, a known ligand for stabilin-2, only partially inhibited binding of FVIII to the extracellular domain of stabilin-2. More pronounced inhibition of binding of FVIII to stabilin-2 was observed upon the addition of increasing concentrations of dextran sulfate. These data indicate that the hyaluronan and FVIII binding sites on stabilin-2 do not overlap. These findings suggest that multiple ligands can bind simultaneously to stabilin-2 which is in agreement with the ability of scavenger and clearance receptors to bind and internalize multiple distinct ligands[293].

Although we identified one of the domains that is involved in binding to FVIII, many questions remain on the specificity of the binding, such as identifying the specific binding sites on both FVIII and stabilin-2. In **Chapter 2**, we did not explore the binding of VWF to stabilin-2. Previous studies have shown that half-life of FVIII-VWF is prolonged in stabilin-2 knock-out mice[289]. Also, immune responses towards the FVIII-VWF complex were strongly reduced in stabilin-2 deficient mice[293]. These data suggest that stabilin-2 mediated uptake of FVIII-VWF contributes to inhibitor development in hemophilia A. Stabilin-2 marks sinusoidal endothelial cells which are also expressed in the spleen and lymph nodes[294]. Further studies are therefore needed to more precisely define the role for stabilin-2 in inhibitor development in hemophilia A.

Using phosphatidylserine as a novel approach for immunomodulation

One of the mechanisms used to remove aging or damaged cells is apoptosis[295]. Using this mechanism cells are eliminated without causing inflammation and it is especially important for cells with a high turnover rate, such as red blood cells (RBCs)[295]. The process of phagocytosis of apoptotic cells is called efferocytosis and can be performed by both phagocytes such as macrophages and dendritic cells[295,296]. RBCs circulate in blood for an average of 120 days and are then degraded, mostly by red pulp macrophages in the spleen but also by the macrophage populations of the liver and bone marrow[297]. RBCs express CD47 on their membrane, this molecule is recognized and impairs phagocytosis of the cells, the inhibitory signal is transduced from the CD47 receptor via the phosphatases SHP1 and SHP2[298]. One of the “eat me” signals is phosphatidylserine (PS). PS is usually present in the inner leaflet of the membrane, in this case instead it is flipped to the outer leaflet and exposed on the surface. This signal can be recognized by phosphatidylserine binding receptors on macrophages[299,300].

In **Chapter 4** we designed novel recombinant proteins that contain different domains of FVIII (C2 , A2 domain and light chain) and AnnexinA5, a protein which binds with high affinity to phosphatidylserine (PS).

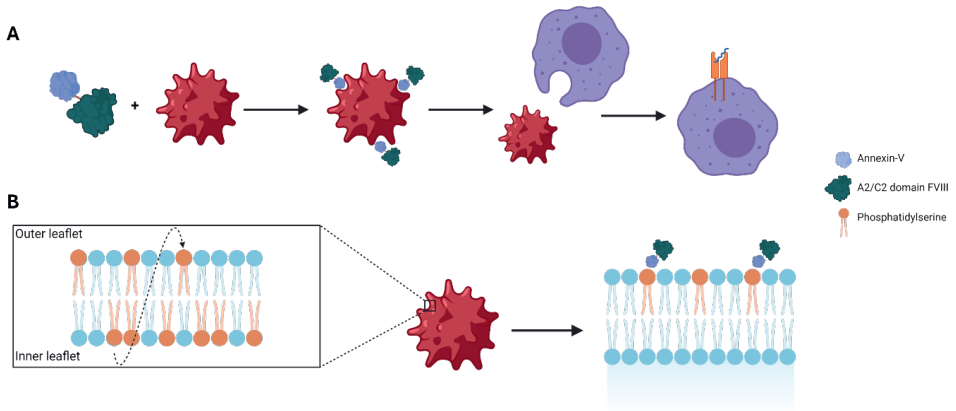


Figure 5. Mode of action of novel FVIII-annexin A5 fusion proteins. When recombinant FVIII-annexin A5 proteins are incubated together with red blood cells, they bind to their surface via phosphatidylserine (PS). The complex is subsequentially endocytosed by macrophages. The fusion proteins are processed and peptides derived from the FVIII moiety are presented on MHC class II complexes (A). During the apoptotic process, PS is translocate from the inner to the outer leaflet of the membrane of red blood cells, enabling the annexin A5 moiety of the fusion proteins to interact with the apoptotic cells (B).

Firstly we confirmed that FVIII-AnxA5 fusion proteins could indeed bind to RBCs. We confirmed by confocal microscopy that FVIII-AnxA5 loaded RBCs were endocytosed by macrophages. Subsequently, we checked the ability of the macrophages to present peptides derived from internalized FVIII-AnxA5 fusion proteins. We were able to identify FVIII peptides that were presented on HLA-DR but the number of FVIII peptides identified was relatively limited. We confirmed that peptides originating from the RBCs are presented on macrophages since we detected of hemoglobin derived peptide in the samples. Direct loading of FVIII-AnxA5 fusion proteins on macrophages resulted in more efficient presentation of FVIII derived peptides on HLA-DR. Further optimization of loading of FVIII-AnxA5 fusion proteins is needed to promote RBC mediated delivery to macrophages. Despite this limitation, our findings provide proof of concept of this novel strategy that uses FVIII loaded RBCs for the induction of tolerance to FVIII in hemophilia patients. Further *in vitro* and *in vivo* studies are needed to evaluate whether this protocol can be used to induce tolerance in hemophilia A. In animal experiments, two potential strategies could be adopted, first *ex-vivo* loading of RBCs with FVIII-AnxA5 fusion proteins can be employed to evaluate whether inhibitor development can be prevented using this strategy in a mouse model of hemophilia A. Alternatively, FVIII-AnxA5 fusion proteins can be directly infused, allowing for a broader therapeutic targeting of PS exposing apoptotic cells. Close monitoring of the biodistribution of infused FVIII-AnxA5 fusion proteins over the different tissues

is needed in this case. Altogether, the results outlined in **Chapter 4** may help to further improve current strategies for tolerance induction in patients with hemophilia.

FIX peptide presentation

One of the unmet needs of hemophilia B is the lack of adequate treatment for patients with inhibitors. Bleeding episodes in hemophilia B patients with inhibitors can potentially be treated with bypassing therapies such as FVII and activated prothrombin complex concentrates. Alternatively, monoclonal antibodies targeting TFPI such as marstacimab and concizumab can be used for treating bleeding episodes in hemophilia B patients with Inhibitors (and also hemophilia A patients with inhibitors)[73,301]. The development of antibodies that interfere with treatment is quite rare in hemophilia B when compared to hemophilia A; anti-FIX antibodies develop in, 1 to 4% of patients with hemophilia B. However, the same group of patients also tends to develop allergic and anaphylactic reactions to FIX[97,112]. The mechanism behind the development of these reactions and their relationship with inhibitor formation is currently not known but may involve development of FIX inhibitors of subclass IgE[98,99].

In **Chapter 5** we applied an MS approach to identify FIX derived peptides that can be presented on MHC class II on antigen presenting cells. In our studies we used monocyte derived dendritic cells of healthy donors. The use of antigen presenting cells derived from healthy donors is based on the assumption that antigen presentation is unlikely to proceed via different mechanisms in healthy donors compared to patients with hemophilia B. Indeed, a study on antigen presentation in the context of hemophilia A did not observe major differences with respect to peptide presentation by healthy donors compared to patients with hemophilia A[302].

One of the first results that we obtained was that only 8 out of 12 donors tested were able to present FIX derived peptides. This was an unexpected finding since all donors were capable of presenting similar amounts of peptides derived of endogenous proteins on MHC class II (see **Chapter 5**). We do not have an explanation as to why 4 out of 12 donors did not present FIX derived peptides on MHC class II. This can potentially be due to competition of peptides derived of endogenous proteins for loading onto MHC class II. It should be noted that in our study we only explored peptides bound to HLA-DR. FIX peptides may interact with other MHC class II alleles such as HLA-DQ or HLA-DP. In addition, not all HLA-DR alleles analyzed in our study may be able to bind FIX derived peptides with high

affinity. To address this issue, we performed an *in silico* prediction of FIX peptides that can be presented on different types of HLA-DRB1 alleles. This analysis revealed quite some differences with respect to the repertoire of FIX derived peptides presented on HLA-DR (see Supplementary Figure 2; Chapter 5). HLA-DRB1*16 was predicted to present FIX peptides derived from multiple regions of FIX. This was confirmed by the large repertoire of FIX derived peptides that was presented on dendritic cells derived from a HLA-DRB1*16 homozygous donor. HLA-DRB1*07 was also predicted to bind to multiple FIX derived peptides, but this was not reflected in our immunopeptidomic studies, which revealed only a limited number of FIX peptides presented on HLA-DRB1*07 expressing dendritic cells. These results highlight the limitations of *in silico* approaches that do not take into account the uptake and (*in vivo*) processing of antigens by lysosomal proteases, but only look at the affinity of peptide sequences for MHC class II. In view of the high number of FIX peptides presented on HLA-DRB1*16, it may be interesting to explore whether this particular MHC class II allele provides a risk factor for the development of FIX inhibitors. An international study, the "B-Natural" study, has recently been initiated and this epidemiological study could potentially uncover this relation[202]. The identification of specific MHC class II alleles as a risk factor could potentially help to further develop personalized treatment options for hemophilia B patients with inhibitors.

The Gla domain contains a large number of post translational modifications, including 12 ɣ-carboxyglutamic acid residues[211,212]. The detection of this modification is challenging due to the loss of CO_2 during peptide fragmentation[213]. The absence of peptides derived from the Gla domain could potentially be due to technical limitations. We show that the peptide core sequence 295-NVIRIIPHHNYNA-306 was presented by multiple donors with different MHC II alleles. We propose that this promiscuously presented peptide contributes to the development of inhibitors. The potential outlook of this work is firstly to verify that this specific peptides are indeed recognized by $CD4^+$ T cells in hemophilia B patients with inhibitors. If this is the case, this specific peptide with core sequence 295-NVIRIIPHHNYN-306 can potentially be utilized for the design of novel peptide-based approaches for the induction of tolerance in hemophilia B patients.

Immunomodulation

Fc gamma receptors (FcγRs) are glycoproteins that can bind the Fc portion of IgG antibodies and regulate cellular and humoral immunity[303]. These receptor are divided in activating (FcγRI, FcγRIIa, FcγRIIIa} and inhibitory (FcγRIIb) receptors.

The cytosolic domain of activating FcγRs contain a so-called immunoreceptor tyrosine-based activation motif (ITAM), whereas the cytoplasmic tail of the inhibitory FcγRIIb receptor contains an immunoreceptor tyrosine-based inhibition motif (ITIM)[304]. The affinity of the different FcγRs for soluble IgG and IgG complexes differs considerably[305]. First- as well as second-generation FVIII-Fc fusion have been successfully developed over the past two decades[66,306,307]. The extended half-life of FVIII-Fc fusion proteins is based on their interaction with the neonatal Fc receptor which allows for endocytosis and subsequent recycling of Fc fusion proteins[308]. Apart from half-life extension it has been proposed that the Fc portion of FVIII-Fc fusion proteins interact with other classes of Fc receptors which could potentially result in modulation of immune responses towards FVIII in patients with hemophilia A[309].

In **Chapter 6** we generated variants of the human monoclonal anti-FVIII antibody EL-14 that were predicted to bind with different affinity to Fc receptors. EL-14 is one of the characterized anti-FVIII antibodies, whose epitope is localized within the C2 domain of FVIII[310]. We designed and constructed a variant, designated EL-14-EGPHPA, containing six amino acid substitutions in its Fc region, that were previously shown to enhance its binding to the inhibitory FcγRIIb receptor[311]. We also generated a variant, designated EL-14-PGLALA, that contained amino acid substitutions that completely prevent its interaction with Fc receptors. Using a large panel of FcγR variants, we assessed the binding of wild type EL-14, EL-14-EGPHPA and EL-14-PGLALA and compared our findings to that of FVIII-Fc. Our study provides the first data on the *in vitro* binding of FVIII-Fc to the total repertoire of human Fc receptors. Overall, our findings indicate that FVIII-Fc and wild type EL-14 IgG bind with similar characteristics to human Fc receptors.

In a recent study by Krishnamoorthy et al. it was reported that lower levels of FVIII inhibitors were observed upon treatment with rFVIII-Fc when compared to rFVIII in a mouse model of hemophilia A[312]. This finding suggests a potential benefit for rFVIII-Fc in reducing the prevalence of FVIII inhibitors when compared to wild type FVIII. Clinical studies in previously untreated patients (PUPs) with hemophilia A revealed an incidence of 31% for FVIII Fc (Elocta)[313]. Similar to other studies, patients with high risk mutations in the FVIII gene were at highest risk of developing inhibitors following treatment with FVIII-Fc[101]. The results of the study indicated that the immunogenicity of FVIII-Fc is similar to that of wild type recombinant FVIII. It was further proposed that FVIII-Fc can potentially accelerate immune tolerance induction (ITI) in hemophilia A patients with inhibitors[314]. Administration of FVIII-Fc may modulate the immune response through the binding of FVIII immune complexes by either B cells and/or different populations

of antigen presenting cells. The interaction of FVIII immune complexes with either activating or inhibitory Fc receptors is crucial in determining whether immune tolerance is maintained or pro-inflammatory signaling is initiated[310]. The results of the verITI-8 study showed that in 12 out of 16 patients immune tolerance (defined by negative inhibitor titer) was achieved in median time of 7.4 weeks[314]. The comparison with a previous study suggested that the success-rate of ITI is similar for FVIII-Fc when compared to recombinant FVIII[118]. Time interval needed to achieve tolerance was suggested to be shorter for FVIII-Fc when compared to historical data of the International Immune Tolerance Study[314].

Based on the results of our *in vitro* studies we propose that administration of EL-14-EGPHPA, which has an increased affinity for FcγRIIb, may be used together with FVIII or FVIII-Fc to efficiently promote interaction of FVIII with the inhibitory FcγRIIb receptor. This may help to more efficiently silence FVIII specific B cells through simultaneous engagement of the (FVIII specific) B cell receptor and FcγRIIb on the surface of B cells. Additionally, FcγRIIb mediated uptake of immune complexes composed of FVIII/FVIII-Fc and EL14-EGPHPA can promote their uptake by LSEC in an FcγRIIb-dependent manner. This process could lead to the generation of FVIII specific Treg cells that may be able to accelerate the induction of peripheral tolerance in hemophilia A patients with inhibitors. Finally, our findings raise the possibility of producing FVIII-Fc fusion proteins containing the “EGPHPA” motif that promotes binding to FcγRIIb. It would be interesting to assess whether such a variant can indeed be used for more efficient restoration of immune tolerance when compared to current FVIII and FVIII-Fc based therapies.

Concluding remarks

The treatment of hemophilia has seen a steep improvement over the past decades but the development and presence of inhibitors has not yet been fully resolved. Nowadays, bypass therapies such as emicizumab are available for hemophilia A patients with inhibitors, but breakthrough bleeds and surgery require the administration of FVIII. Additionally, new replacement products such as BIVV-001, a VWF independent recombinant FVIII-Fc with extended half-life, have successfully been successfully developed[315]. Despite these advances, novel therapeutic strategies to prevent and/or eradicate inhibitor development in hemophilia are urgently needed. The advancement of knowledge and novel strategies for tolerance induction will be key in the next years to further improve “Quality-of-Life” for patients with hemophilia.

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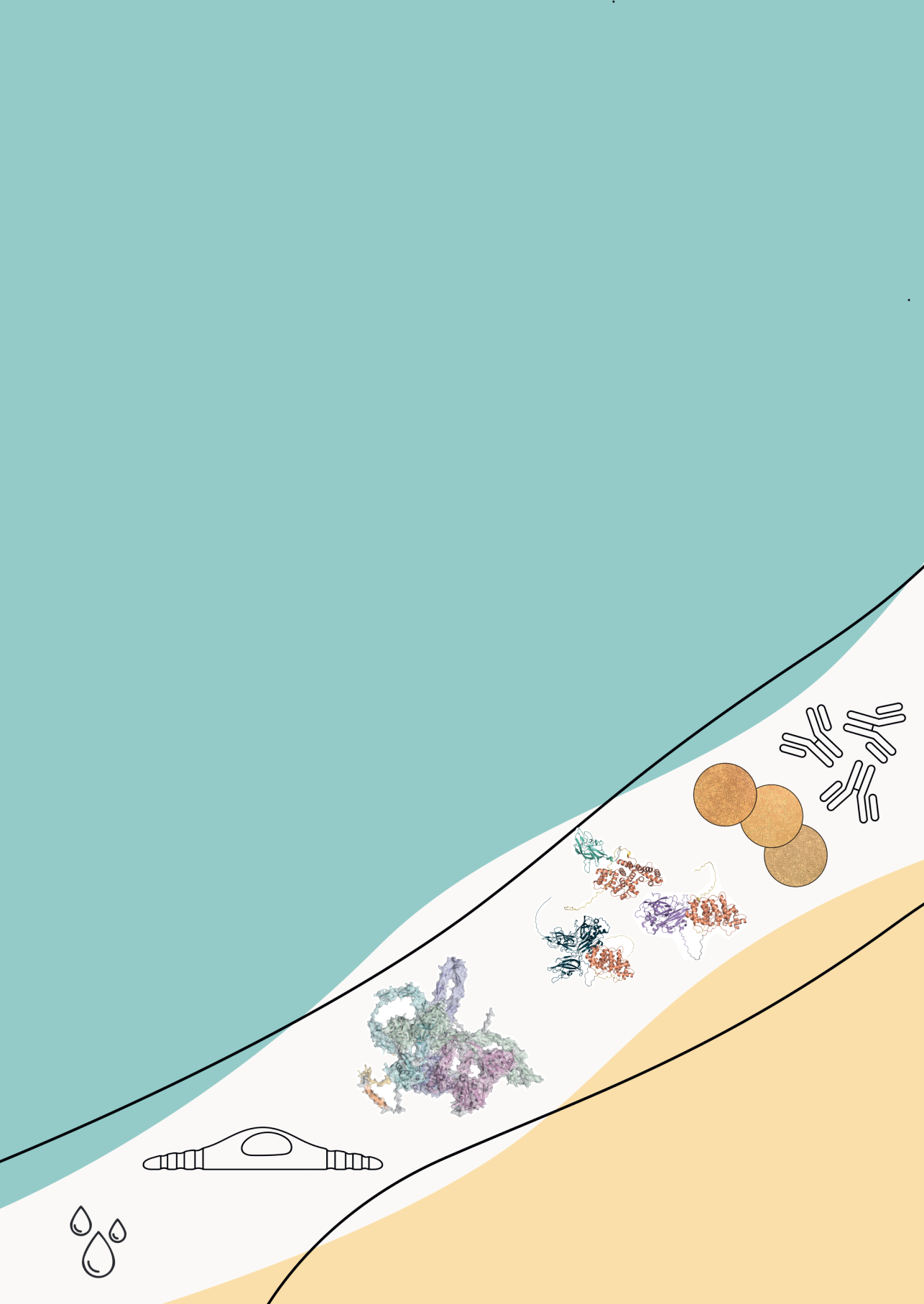
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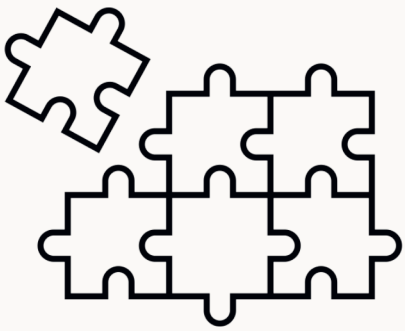
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Appendix



English summary

The treatment of hemophilia has been greatly improved over the past decades. Non-factor replacement therapies such as the bi-specific antibody emicizumab are now available for prophylactic treatment of hemophilia A patients. FVIII administration is however still needed for treatment of breakthrough bleeds and/or surgery putting patients at risk of developing FVIII inhibitors. It has been shown that around 25% of patients with severe hemophilia A develop FVIII inhibitors in response to FVIII replacement therapy. The development and persistence of FVIII inhibitors remains an unresolved challenge. Inhibitory antibodies directed towards FIX develop in around 3% of patients with hemophilia B. Our current knowledge on the mechanism driving immune responses to FVIII and FIX is still very limited.

In **Chapter 1** we provide an overview of the etiology of hemophilia A and B. We also describe currently available treatment options for patients with hemophilia A and B. In the last part of Chapter 1 we described the development of anti-FVIII and anti-FIX antibodies in patients with hemophilia A and B in response to FVIII or FIX replacement therapy.

In **Chapter 2** we describe the properties of liver sinusoidal endothelial cells (LSEC) which have been shown to synthesize the vast majority of circulating FVIII. LSEC are highly fenestrated cells and lack a basal membrane which allows for a rapid exchange of nutrients present in blood and hepatocytes and stellate cells. Many scavenger receptors are expressed on LSEC which serve to rapidly clear cellular debris and lipoproteins from the circulation. Interestingly, stabilin-2, one of the major scavenger receptors on LSEC has been implicated in clearance of the FVIII-VWF complex. Importantly, LSEC have evolved as an unique population of antigen presenting cells which can promote the differentiation of naïve CD4+ T cells into regulatory T cells thereby promoting tolerance. This unique property positions LSEC as a potential target for innovative therapies to induce tolerance to FVIII.

In **Chapter 3** we studied the interaction of stabilin-2 with FVIII. We demonstrate that stabilin-2 is able to bind to FVIII in a von Willebrand factor independent manner. We also show that the Link domain of stabilin-2 contributes to the binding of FVIII.

In **Chapter 4**, we explored a novel strategy for inducing immune tolerance to FVIII by exploiting the physiological clearance of red blood cells (RBCs). Recombinant proteins in which FVIII domains (C2, A2 or A3-C1-C2) were fused to Annexin V (AnxA5) were designed and shown to bind to phosphatidylserine (PS) exposed on

RBCs. FVIII-AnxA5 fusion protein loaded RBCs were rapidly endocytosed by macrophages. Since macrophage-mediated RBCs turnover is an inherently tolerogenic process, this approach can potentially create a favorable environment for induction of FVIII-specific tolerance.

In **Chapter 5** we focus on inhibitor development in hemophilia B. We pulsed monocyte derived dendritic cells with FIX and analyzed the repertoire of FIX derived peptides that was presented on HLA-DR. Peptides derived from different domains of FIX were presented on HLA-DR. Surprisingly, we identified a single peptide 295-NVIRIIPHHNYNA-306 that was presented by multiple donors with different HLA-DR alleles. This promiscuously presented FIX peptide may be used for the design of novel therapies focusing on prevention and/or eradication of FIX inhibitors in patients with hemophilia B.

In **Chapter 6**, we analyze the potential of modulating the interaction of anti-FVIII antibodies with specific Fc receptors. We mutated six amino acids in the Fc region of EL14-IgG1, an anti-FVIII antibody that targets the C2 domain. This modification specifically enhances the binding to the inhibitory FcγRIIB receptor that is highly expressed on LSEC as well as other antigen presenting cells. Based on our findings we argue that anti-FVIII antibody that can bind with high affinity to the inhibitory FcγRIIB receptor as well as next generation FVIII-Fc fusion proteins with increased affinity for FcγRIIB may be used to more efficiently restore tolerance when compared to currently available treatment strategies.

Finally, in **Chapter 7** we discuss the findings reported in this PhD thesis with reference to other studies. Knowledge obtained on FVIII clearance mechanisms as well as antigen presentation of FVIII and FIX may help to design novel approaches for reducing inhibitor formation and improving long-term outcomes in patients with hemophilia.

Nederlandse samenvatting

De behandeling van hemofilie is de afgelopen decennia sterk verbeterd. Bispecifieke antilichaam therapieën zoals emicizumab zijn nu beschikbaar voor profylactische behandeling van patiënten met hemofilie A. Toediening van FVIII is echter nog steeds nodig voor de behandeling van doorbraakbloedingen en bij operaties, waardoor patiënten nog steeds het risico lopen FVIII-remmers te ontwikkelen. Het is aangetoond dat ongeveer 25% van de patiënten met ernstige hemofilie A remmende antistoffen (FVIII remmers) ontwikkelen na behandeling met FVIII. De ontwikkeling van FVIII-remmers is nog steeds een groot probleem. Remmende antilichamen gericht tegen FIX ontwikkelen zich bij ongeveer 3% van de patiënten met hemofilie B. Onze huidige kennis over het mechanisme dat immuunreacties op FVIII en FIX stuurt, is nog steeds zeer beperkt.

In **Hoofdstuk 1** geven we een overzicht van de oorzaken van hemofilie A en B. We beschrijven ook de momenteel beschikbare behandelingsopties voor patiënten met hemofilie A en B. In het laatste deel van **Hoofdstuk 1** bespreken we de ontwikkeling van anti-FVIII en anti-FIX-antilichamen bij patiënten met hemofilie A en B.

In **Hoofdstuk 2** beschrijven we de eigenschappen van lever sinusoïde endotheelcellen (LSEC) waarvan is aangetoond dat ze het grootste deel van het in bloed circulerende FVIII synthetiseren. LSEC zijn cellen zonder een basaal membraan die “fenestrae” (ronde gaten) bevatten waardoor een snelle uitwisseling van voedingsstoffen die in het bloed aanwezig zijn met hepatocyten en stellaatcellen mogelijk wordt gemaakt. Op de celmembraan van LSEC wordt een groot aantal scavenger-receptoren tot expressie gebracht, die dienen om snel cel resten en lipoproteïnen uit de circulatie te verwijderen. Interessant genoeg is stabilin-2, een van de belangrijkste scavenger-receptoren op LSEC, betrokken bij de verwijdering van het FVIII-VWF complex. LSEC vormen een unieke populatie van antigeen presenterende cellen, die de differentiatie van naïeve CD4+ T-cellen in de richting van regulerende T-cellen kunnen bevorderen, waardoor tolerantie wordt gestimuleerd. Deze unieke eigenschap maakt LSEC tot een potentieel target voor innovatieve therapieën om tolerantie gericht tegen FVIII te induceren.

In **Hoofdstuk 3** hebben we de interactie bestudeerd van stabilin-2, een van de prominente scavenger receptoren op LSEC, met FVIII. We laten zien dat stabilin-2 in staat is om aan FVIII te binden op een manier die onafhankelijk is van von Willebrand-factor. We laten ook zien dat het Link-domein van stabilin-2 bijdraagt aan de binding van FVIII.

In **Hoofdstuk 4** hebben we een nieuwe strategie ontwikkeld om immuun tolerantie voor FVIII te induceren door gebruik te maken van de fysiologische afbraak van verouderde rode bloedcellen. Recombinante eiwitten waarbij FVIII-domeinen (C2, A2 of A3-C1-C2) aan Annexin V (AnxA5) werden gekoppeld, werden gesynthetiseerd. De FVIII-AnxA5 fusie-eiwitten binden aan fosfatidylserine (PS) dat op rode bloedcellen (RBC's) aanwezig is. Met FVIII-AnxA5 fusie-eiwit geladen RBC's werden snel opgenomen door macrofagen. Omdat de door macrofagen gemedieerde afbraak van RBC's van nature een tolerogeen proces is, kan deze aanpak mogelijk een gunstige omgeving creëren voor het induceren van FVIII-specifieke tolerantie.

In **Hoofdstuk 5** richten we ons op de ontwikkeling van remmers bij hemofilie B. We hebben monocyten-afgeleide dendritische cellen gepulst met FIX en het repertoire van FIX-afgeleide peptiden geanalyseerd dat op HLA-DR werd gepresenteerd. Peptiden afkomstig uit verschillende domeinen van FIX werden op HLA-DR gepresenteerd. Verrassend genoeg identificeerden we een enkel peptide 295-NVIRIIPHHNYNA-306 dat door meerdere donoren met verschillende HLA-DR-allelen werd gepresenteerd. Dit promiscue gepresenteerde FIX-peptide kan mogelijk worden gebruikt voor het ontwerpen van nieuwe therapieën die zich richten op het voorkomen en/of de eliminatie van FIX-remmers bij patiënten met hemofilie B.

In **Hoofdstuk 6** analyseren we de binding van varianten van anti-FVIII-antilstoffen met specifieke Fc-receptoren. We hebben zes aminozuren gemuteerd in het Fc-domein van EL14-IgG1, een humane anti-FVIII-antistof gericht tegen het C2 domein van FVIII. Deze wijziging versterkt specifiek de binding aan de remmende FcγRIIB-receptor, die sterk tot expressie komt op LSEC en andere antigeen presenterende cellen. Op basis van onze bevindingen stellen we voor dat anti-FVIII-antilichamen die met hoge affiniteit aan de remmende FcγRIIB-receptor kunnen binden, evenals “next-generation” FVIII-Fc fusie-eiwitten met verhoogde affiniteit voor FcγRIIB, mogelijk kunnen worden gebruikt om tolerantie efficiënter te herstellen in vergelijking met de momenteel beschikbare behandelingsstrategieën.

Tot slot bespreken we in **Hoofdstuk 7** de bevindingen die in dit proefschrift worden gerapporteerd met verwijzing naar andere studies. Kennis over de mechanismen van FVIII-clearance en de antigeenpresentatie van FVIII en FIX kan helpen bij het ontwerpen van nieuwe benaderingen om de vorming van remmers te verminderen en de lange termijn uitkomsten bij patiënten met hemofilie te verbeteren.

Riassunto in Italiano

Il trattamento dell'emofilia è notevolmente migliorato negli ultimi decenni. Oggi sono disponibili terapie profilattiche non basate sulla sostituzione del fattore della coagulazione, come l'anticorpo bispecifico emicizumab, per i pazienti affetti da emofilia A. Tuttavia, la somministrazione di fattore VIII (FVIII) rimane necessaria per il trattamento di emorragie e/o in occasione di interventi chirurgici, esponendo i pazienti al rischio di sviluppare inibitori del FVIII. È stato dimostrato che circa il 25% dei pazienti con emofilia A grave sviluppa inibitori del FVIII in risposta alla terapia sostitutiva con FVIII. Lo sviluppo e la persistenza degli inibitori del FVIII rappresenta ancora oggi una sfida irrisolta. Gli anticorpi diretti contro il fattore IX (FIX) si sviluppano invece in circa il 3% dei pazienti con emofilia B. Le conoscenze attuali sui meccanismi che guidano la risposta immunitaria contro FVIII e FIX sono ancora limitate.

Nel **Capitolo 1** viene fornita una panoramica dell'eziologia dell'emofilia A e B. Vengono inoltre descritte le opzioni terapeutiche attualmente disponibili per i pazienti affetti da queste patologie. Nell'ultima parte del capitolo viene illustrato lo sviluppo degli anticorpi anti-FVIII e anti-FIX nei pazienti con emofilia A e B in seguito alla terapia sostitutiva con FVIII o FIX.

Il **Capitolo 2** si concentra sulle caratteristiche delle cellule endoteliali sinusoidali epatiche (LSEC), che rappresentano la principale sede di sintesi del FVIII. Le LSEC sono cellule altamente fenestrate e prive di membrana basale, caratteristica che consente un rapido scambio di nutrienti tra il sangue, gli epatociti e le cellule stellate. Sulla loro superficie sono espressi numerosi recettori scavenger, deputati alla rapida rimozione dal circolo di detriti cellulari e lipoproteine. Tra questi, stabilin-2, uno dei principali recettori scavenger delle LSEC, è stato implicato nella clearance del complesso FVIII–fattore di von Willebrand (VWF). Inoltre, le LSEC costituiscono una popolazione unica di cellule presentanti l'antigene, in grado di promuovere la differenziazione dei linfociti T CD4+ naïve in linfociti T regolatori, favorendo così la tolleranza immunologica. Questa peculiare proprietà rende le LSEC un potenziale bersaglio per lo sviluppo di terapie innovative volte a indurre tolleranza nei confronti del FVIII.

Nel **Capitolo 3** è stata studiata l'interazione tra stabilin-2 e FVIII. È stato dimostrato che stabilin-2 è in grado di legarsi a FVIII in modo indipendente dal fattore di von

Willebrand. Inoltre, è stato evidenziato che il dominio Link di stabilin-2 contribuisce al legame con il FVIII.

All'interno del **Capitolo 4** è stata esplorata una nuova strategia per indurre tolleranza immunologica nei confronti del FVIII sfruttando il processo fisiologico di eliminazione dei globuli rossi. Sono state progettate proteine ricombinanti nelle quali diversi domini del FVIII (C2, A2 oppure A3-C1-C2) sono stati fusi con l'Annexina V (AnxA5), dimostrando la capacità di legarsi alla fosfatidilserina (PS) esposta sulla superficie degli eritrociti. Gli eritrociti caricati con le proteine ricombinanti FVIII-AnxA5 sono stati rapidamente internalizzati dai macrofagi. Poiché il turnover dei globuli rossi mediato dai macrofagi rappresenta un processo intrinsecamente tollerogenico, questo approccio potrebbe creare un ambiente favorevole all'induzione di una tolleranza specifica nei confronti del FVIII.

Nel **Capitolo 5** l'attenzione è rivolta allo sviluppo degli inibitori nell'emofilia B. Cellule dendritiche derivate da monociti sono state incubate con il FIX e successivamente è stato analizzato il repertorio di peptidi derivati dal FIX presentati dalle molecole HLA-DR. Sono stati identificati peptidi provenienti da diversi domini del FIX. Sorprendentemente, è stato individuato un singolo peptide, 295-NVIRIIPHHNYNA-306, presentato da diversi donatori portatori di differenti alleli HLA-DR. Questo peptide del FIX, presentato in modo promiscuo, potrebbe essere utilizzato per progettare nuove strategie terapeutiche finalizzate alla prevenzione e/o all'eliminazione degli inibitori del FIX nei pazienti con emofilia B.

Il **Capitolo 6** analizza il potenziale della modulazione dell'interazione tra anticorpi anti-FVIII e specifici recettori Fc. Sono stati mutati sei amminoacidi nella regione Fc di EL14-IgG1, un anticorpo anti-FVIII diretto contro il dominio C2. Questa modifica aumenta selettivamente l'affinità per il recettore inibitorio FcγRIIB, altamente espresso sulle LSEC e su altre cellule presentanti l'antigene. Sulla base dei risultati ottenuti, si propone che anticorpi anti-FVIII ad elevata affinità per FcγRIIB, così come proteine di fusione FVIII-Fc di nuova generazione con maggiore affinità per questo recettore, possano ristabilire la tolleranza immunologica in modo più efficace rispetto alle strategie terapeutiche attualmente disponibili.

Infine, nel **Capitolo 7** vengono discussi i risultati presentati nella tesi di dottorato alla luce della letteratura scientifica disponibile. Le conoscenze acquisite sui meccanismi di clearance del FVIII e sui processi di presentazione antigenica del FVIII e del FIX potranno contribuire allo sviluppo di nuovi approcci terapeutici volti

a ridurre la formazione di inibitori e a migliorare gli esiti clinici a lungo termine nei pazienti affetti da emofilia.

Authors, affiliations and contributions

Chapter 2

Liver sinusoidal endothelial cells provide a highly developed exchange platform in the liver promoting clearance and tolerance

Michela Leoni[1]and Jan Voorberg[1,2]

[1]Department of Molecular Hematology, Sanquin-Academic Medical Center Landsteiner Laboratory, Amsterdam, The Netherlands

[2]Department of Pediatric Hematology, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Author contributions.

ML and JV wrote the manuscript. ML prepared the figures.

Chapter 3

Binding of blood coagulation factor VIII to stabilin-2

Michela Leoni[1*], Zsolt Szeles[1*], Paul Kaijen[1], J. Arnoud Marquart[1], Karin Fijnvandraat[1,2] and Jan Voorberg[1,3], on behalf of the EDUC8 consortium.

[1]Department of Molecular Hematology, Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherlands

[2]Department of Pediatric Hematology, Amsterdam UMC, Amsterdam, The Netherlands

[3]Department of Experimental Vascular Medicine, Amsterdam UMC, Amsterdam, The Netherlands

[*]Shared first author

Author contributions:

ML, ZS and JV designed the study. ML and ZS performed the experiments. ML and JV wrote the first draft of the manuscript. ML performed the data analysis. PK and AM assisted with the SPR experiments. KF provided clinical input for study and manuscript. All authors critically reviewed and approved the manuscript.

Chapter 4

MHC class II presentation of FVIII-AnnexinA5 fusion proteins internalized by antigen presenting cells

Mariarosaria Miranda[1†], **Michela Leoni**[1†], Carmen van der Zwaan[1], Robin van Bruggen[1], Christiaan Reutelingsperger[2], Karin Fijnvandraat[3], Arie J. Hoogendijk[1], Maartje van den Biggelaar[1], Jan Voorberg[1]*

Collaborative group: EDUC8 consortium

[†]These authors contributed equally to this work and share first authorship

[1]Department of Molecular Hematology, Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherlands;

[2]Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands;

[3]Department of Pediatric Hematology, Amsterdam UMC, University of Amsterdam, Emma Children's Hospital, Amsterdam, The Netherlands

Author contributions

MM: Writing – original draft, Writing – review & editing, Conceptualization, Investigation. ML: Writing – review & editing, Conceptualization, Investigation. CZ: Writing – review & editing, Methodology. RB: Writing – review & editing. CR: Writing – review & editing. KF: Writing – review & editing. AH: Writing – review & editing. MB: Writing – review & editing. JV: Writing – review & editing, Writing – original draft, Conceptualization, Supervision.

Chapter 5

Immunopeptidomics of blood coagulation factor IX; a core peptide derived from the protease domain is promiscuously presented on HLA-DR

Michela Leoni[1], Margot Dierickx[1], Mariarosaria Miranda[1], Pieter Langerhorst[1], Carmen van der Zwaan[1], Erik Mul[2], Arie J Hoogendijk[1], Karin Fijnvandraat[1,3] and Jan Voorberg[1,3,4]

[1]Department of Molecular Hematology, Sanquin-Academic Medical Center Landsteiner Laboratory, Amsterdam, The Netherlands.

[2]Department of Research Facilities, Sanquin Research, Amsterdam, The Netherlands.

[3]Department of Pediatric Hematology, Amsterdam UMC, Amsterdam, The Netherlands

[4]Department of Experimental Vascular Medicine, Amsterdam UMC, Amsterdam, The Netherlands

Author contributions:

ML, MD, MM, KF and JV designed the study. ML performed the experiments. ML and JV wrote the first draft of the manuscript. ML and PL performed the data analysis. PL, MD, AJH and CvdZ helped to develop the immunopeptidomics workflow, performed the final processing of the samples and acquired the MS data. EM assisted with the ImageStream experiments. All authors critically reviewed and approved the manuscript.

Chapter 6

Affinity of human FcγR receptors for FVIII-Fc and an Fc domain engineered human anti-FVIII antibody

Ilja Oomen[†1,2], Mariarosaria Miranda[†2], Michela Leoni[†2], Paul Kaijen[2], Arthur Bentlage[3], Gestur Vidarsson[3,4], Sietse Q Nagelkerke[5,6], Taco W. Kuijpers[5,6], Karin Fijnvandraat[1,2], Jan Voorberg*[1,2], Samantha Gouw* [1,7]

[†] These authors contributed equally to this work and share first authorship.

*These authors contributed equally to this work and share last authorship.

Affiliations

1. Amsterdam UMC location University of Amsterdam, Department of Pediatric Hematology, Meibergdreef 9, Amsterdam, the Netherlands
2. Sanquin Research and Landsteiner Laboratory, Department of Molecular Hematology, Amsterdam, the Netherlands
3. Sanquin Research, Immunoglobulin Research Laboratory, Amsterdam, the Netherlands
4. Utrecht University, Utrecht Institute for Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research, Department of Biomolecular Mass Spectrometry and Proteomics, Utrecht, The Netherlands.
5. Amsterdam UMC location University of Amsterdam, Department of Pediatric Immunology, Rheumatology and Infectious Diseases, Meibergdreef 9, Amsterdam, Netherlands
6. Sanquin Research and Landsteiner Laboratory, Department of Blood Cell Research, Amsterdam, the Netherlands

7. Leiden University Medical Center, Department of Clinical Epidemiology, Leiden, the Netherlands

Authorship contributions

JV and SCG designed study; IO, ML, MM performed experiments; GV provided constructs for FcγRIIb-enhanced binding antibodies; AB performed the SPR binding experiments; SQN, GV, TWK, JV and SCG supervised data analysis; JV, KF and SCG secured funding for this study; IO, MM, ML, AB, JV and SCG wrote the manuscript; all authors critically reviewed manuscript; all authors approved the final version of the manuscript.

PhD Portfolio

Name PhD student: Michela Leoni PhD period: July 2021 – December 2025 Names of PhD supervisor: Jan Voorberg		
	Year	**ECTS**
General courses		
Entrepreneurship and Innovation, EDUC8	2021-2023	2.0
Scientific Ethics and Integrity	2021	0.1
AMC World of Science	2022	0.7
Computing in R, AMC	2022	0.7
Computing in R, EDUC8	2022	1.8
Scientific communication and fund raising, EDUC8	2022	0.9
Personal Development Programme	2023-2025	0.9
Practical Biostatistics	2023	1.4
Specific courses		
Introduction to Hemostasis & Introduction to Immunology/Immunogenicity & Introduction to Hemophilia A: patient and clinician points of view, EDUC8	2021	1.8
Venous thrombosis - NVTH	2022	1.0
Arterial thrombosis - NVTH	2023	1.0
Clinical training, EDUC8	2022	1.2

Seminars, workshops and master classes		
Weekly department seminars	2021-2025	4.0
Sanquin research seminars	2021-2025	4.0
EDUC8 annual meeting	2021-2023	3.0
Presentations		
EAHAD 2021 online – e-poster	2022	0.1
NVTH 2022 Koudekerke - poster	2022	0.1
ISTH 2022 London – Oral presentation	2022	0.2
NVTH 2023 Koudekerke – poster and poster pitch	2023	0.2
(Inter)national conferences		
NVTH symposium, online	2021	0.3
BIC, Venice, Italy	2021	0.9
ECTH, Ghent, Belgium	2021	0.6
EAHAD, online	2022	0.6
NVTH symposium, Koudekerke, The Netherlands	2022	0.3
Sanquin Science Day, Amsterdam, The Netherlands	2022	0.3
ISTH, London, UK	2022	1.5
NVTH symposium, Koudekerke, The Netherlands	2023	0.3
BIC, Venice, Italy	2023	0.6
Sanquin Science Day, Amsterdam, The Netherlands	2023	0.3
Other		
NVTH PhD Day	2022	0.3

Supervising		
Bachelor student supervision	2024	2.5
Master student supervision	2025	3
Awards and Prizes		
Travel grant and complimentary registration for the EHA2024 Hybrid Congress		2024

List of publications

Miranda M, **Leoni M**, van der Zwaan C, van Bruggen R, Reutelingsperger CP, Fijnvandraat K, Hoogendijk AJ, van den Biggelaar M, Voorberg J. MHC class II presentation of FVIII-Annexin A5 fusion proteins internalized by antigen presenting cells. Front Immunol. 2025;16:1668397. doi:10.3389/fimmu.2025.1668397.

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About the author



Michela Leoni was born in Lugo (Ravenna), Italy on December 13th 1996. She grew up with her parents and

brother in Massa Lombarda, a charming little town nestled in the heart of the Emilia-Romagna region, known for its rural beauty, rich local culture, and strong culinary traditions.

In 2015 she earned her high school diploma from Liceo Scientifico Scienze Applicate F. Alberghetti in Imola and later in the year, she began her university studies in Biotechnology at the university of Bologna. As part of her Bachelor's studies she completed a research internship and wrote her thesis on a preliminary study of the transcriptional and epigenetic profile of the neuronal model of AGC1-deficiency.

After completing her Bachelor's degree in 2018 she decided to deepen her knowledge and started a Master in Pharmaceutical Biotechnology in the same university. The aim of her thesis and internship focused on the changes in the transcriptome that occur with tumorigenic progression of Gastrointestinal Stromal Tumor.

After completing her degree *cum laude,* in 2021 she moved to Amsterdam to start her PhD in Molecular Hematology at Sanquin, under the supervision of Jan Voorberg and Karin Fijnvandraat. She was part of the EDUC8 consortium, the Marie Skłodowska-Curie Innovative Training Network on Factor VIII immunogenicity, funded by the Horizon 2020 Programme. As part of this program, she moved for two months to Hamburg, Germany, to visit Topas Therapeutics, a company that is advancing a highly differentiated and versatile approach to establish immune tolerance in autoimmune and immune-mediated diseases.

What captivated Michela most during her PhD journey was the complexity of biology. From the intricacies of the immune system to the broader scientific principles, she is fascinated by how simple processes emerge and intertwine, with every scientific discovery adding a new piece to an always expanding network of knowledge.

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