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ORIGINAL ARTICLE

Factor XII contact activation can be prevented by targeting 2 unique patches in its epidermal growth factor-like 1 domain with a nanobody

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Abstract

Background: Factor (F)XII triggers contact activation by binding to foreign surfaces, with the epidermal growth factor-like 1 (EGF-1) domain being the primary binding site. Blocking FXII surface-binding might hold therapeutic value to prevent medical device–induced thrombosis.

Objectives: To unravel and prevent EGF-1–mediated FXII surface-binding with a variable domain of heavy chain–only antibody (V_HH).

Methods: FXII variants with glutamine substitutions of 2 positively charged amino acid patches within the EGF-1 domain were created. Their role in FXII contact activation was assessed using kaolin pull-down experiments, amidolytic activity assays, and clotting assays. FXII EGF-1 domain–specific V_HHs were raised to inhibit EGF-1–mediated FXII contact activation while preserving quiescence.

Results: Two unique, positively charged patches in the EGF-1 domain were identified (upstream, 73K74K76K78H81K82H; downstream, 87K113K). Neutralizing the charge of both patches led to a 99% reduction in FXII kaolin binding, subsequent decrease in autoactivation of 94%, and prolongation of clot formation in activated partial thromboplastin time assays from 36 (±2) to 223 (±13) seconds. Three FXII EGF-1–specific V_HHs were developed that are capable of inhibiting kaolin binding and subsequent contact system activation in plasma. The most effective V_HH "F2" binds the positively charged patches and thereby dose-dependently extends activated partial thromboplastin time clotting times from 29 (±2) to 43 (±3) seconds without disrupting FXII quiescence.

Conclusion: The 2 unique, positively charged patches in FXII EGF-1 cooperatively mediate FXII surface-binding, making both patches crucial for contact activation. Targeting these with FXII EGF-1–specific V_HHs can exclusively decrease FXII surface-binding and subsequent contact activation, while preserving zymogen quiescence. These patches thus have potential as druggable targets in preventing medical device–induced thrombosis.

1 | INTRODUCTION

Hemostasis is a tightly regulated biological process. Excessive coagulation can lead to thrombosis, resulting in vascular occlusion and subsequent hypoxia in the downstream tissues. Coagulation can be initiated either by tissue factor (extrinsic pathway) or by blood contact with negatively charged surfaces (intrinsic pathway). Blood contact with thrombogenic surfaces like silica, polyphosphate (polyP), or nucleic acids causes factor (F)XII binding [1,2]. When bound, FXII undergoes a conformational change toward an "open" conformation in which the activation loop is exposed [1,3–7]. In this open conformation, the activation loop is exposed, thereby enhancing the activation of FXII to active FXII (FXIIa) through cleavage after R353 by FXIIa (autoactivation), plasma kallikrein (PKa), or plasmin. Reciprocal activation of plasma prekallikrein (PK) to PKa by FXIIa is a critical feedback loop in the contact system. In contact-driven coagulation, FXIIa activates FXI on the blood-contacting surface. Further activation of the intrinsic pathway can ultimately lead to thrombin formation [8–10]. Besides initiation of the intrinsic pathway, FXIIa can initiate the kallikrein–kinin system. Here, PKa cleaves high-molecular-weight kininogen (HK), releasing the proinflammatory peptide bradykinin (BK) that mediates vascular permeability and leukocyte recruitment [2,11–15]. Excessive BK release can be caused by pathogenic mutations in FXII that introduce alternative cleavage sites (T309K) or alter FXII conformation (W268R) [16–18]. Both of these mutations accelerate the activation by PKa, which could lead to a BK-mediated inflammatory response.

The role of FXII and FXI in blood hemostasis *in vivo* is very limited, as patients with deficiencies only exhibit mild bleeding risks [11,19–21]. Furthermore, mice that are deficient in FXII or FXI are protected against obstructive thrombi in arterial thrombosis models [22,23]. However, contact activation on negatively charged surfaces of medical devices increases the risk of medical device–induced thrombosis and triggers an inflammatory reaction against the medical device [24–28]. Therefore, various strategies targeting FXII are being researched to prevent contact activation [29,30]. Among others, multiple monoclonal antibodies (mAbs) were raised to various domains of FXII [28,31–37]. Targeting FXIIa with an mAb can prevent proteolytic FXIIa activity without increasing the bleeding risk in mice [20,28,29]. However, the inhibition of FXII proteolytic activity by these mAbs results in the interference with the kallikrein–kinin system [29]. Targeting the FXII heavy chain to inhibit FXII surface-binding using mAbs has shown no success as these mAbs often trigger spontaneous FXII autoactivation and enhance the susceptibility of FXII to activation by PKa in solution [35,36,38–41]. These data suggest that therapeutic interventions to prevent FXII-driven contact activation require specific targeting of the FXII binding sequence without affecting FXII zymogen conformation [17,34,38,42,43].

The epidermal growth factor-like 1 (EGF-1) domain was recently identified as the main surface-binding domain using truncated FXII variants or chimeras of FXII and its nonbinding homolog hepatocyte growth factor activator (HGFA) [7,38,39]. Furthermore, specific amino acids that are required for surface binding of FXII were also identified more recently [40]. Based on the tertiary structure of FXII predicted by AlphaFold (Google DeepMind), we here designed and produced FXII variants to elucidate EGF-1 domain–mediated FXII surface-binding [41,42]. The predicted orientation and intramolecular interactions of domains in the heavy chain of FXII explain various biological mechanisms of these domains [43]. The EGF-1 domain in the predicted FXII structure is oriented outward and does not have interactions with other domains in the protein. Therefore, llama-derived variable domain of heavy chain–only antibodies (V$_H$Hs) specifically targeting the EGF-1 domain of FXII were raised in this study. This targeted approach holds therapeutic promise, as it would prevent FXII surface-binding and contact activation without rendering FXII more susceptible to activation by PKa (as has been the case in previous attempts in which the heavy chain of FXII was targeted using antibodies), or affecting the role of FXIIa in the kallikrein–kinin system.

2 | MATERIALS AND METHODS

An extensive description of the experimental procedures can be found in the Supplementary Materials and Methods.

2.1 | Comparison of the EGF-1 domain structures of FXII and HGFA

The EGF-1 domains of the 3-dimensional structures of FXII predicted by AlphaFold (AF-P00748-F1-model_v4) and HGFA (AF-Q04756-F1_v4) were compared using PyMOL Molecular Graphics System (Schrödinger) [44]. Two unique positively charged patches in the FXII EGF-1 domain were identified, hereafter called upstream patch or downstream patch. In mature protein numbering, the upstream patch or downstream patch consists of FXII residues K73, K74, K76, H78, K81, and H82 or residues K87 and K113, respectively.

2.2 | Molecular cloning, protein expression, and purification

Glutamine substitution variants of either (FXII Δupstream and FXII Δdownstream) or both patches (FXII Δcombined) were designed (Table 1). Briefly, BsmBI restriction sites were introduced in the

TABLE 1 Amino acid sequences of factor XII epidermal growth factor-like 1 glutamine substitution variants.

FXII variant	Mature FXII amino acid sequence (P72-K113)
WT	PKKVKDHCSKHSPCQKGGTCVNMPSGPHCL CPQHLTGNHCQK
Δupstream	PQQVQDQCSQQSPCQKGGTCVNMPSGPHCL CPQHLTGNHCQK
Δdownstream	PKKVKDHCSKHSPCQQGGTCVNMPSGPHCLC PQHLTGNHCQQ
Δcombined	PQQVQDQCSQQSPCQQGGTCVNMPSGPHCLC PQHLTGNHCQQ

The sequence covers the FXII epidermal growth factor-like 1 domain and adjacent linker sequences. At physiological pH, this sequence contains 11 positively charged amino acids (in green). FXII-specific positively charged amino acid patches were substituted for glutamine residues (Q, in red). F, factor; WT, wild type.

complementary DNA of FXII wild type (WT) to substitute the positively charged amino acids in the upstream patch for glutamine residues using Golden Gate cloning. Long oligoduplexes were inserted upon restriction with XhoI at L70 to E71 and BbvCI at L121 to R123 to replace the positively charged amino acids in the downstream patch or both patches. These sequences were transiently transfected in Expi293F cells (Gibco) were transfected via ExpiFectamine 293 (Gibco) according to the manufacturer's instructions. At day 6 after transfection, supernatant was collected from which recombinant FXII variants were purified.

2.3 | FXII kaolin binding assay

The kaolin binding capacity of the FXII variants (67.5 nM final concentration) in Freestyle 293 expression medium was investigated by incubation with kaolin (400 μg/mL final concentration) for 15 minutes at 37 °C. Samples were vortexed every 5 minutes to resuspend the kaolin, as previously described [38]. FXII autoactivation was prevented by the small-molecular protease inhibitor PPACK (50 nM final concentration). Next, kaolin was pelleted by centrifugation at 5000*g* for 5 minutes. Pellets and supernatants were analyzed by Western blotting under reducing conditions. FXII quantities were determined using band densitometry and expressed as a percentage of the input sample.

2.4 | Activation of FXII glutamine substitution variants

The ability of purified FXII glutamine substitution variants to be activated by activating surfaces was assessed in chromogenic substrate assays. Polyvinyl chloride microplates were blocked with 1% bovine serum albunin (BSA) weight per volume in HEPES-buffered saline (HBS-BSA; 10 mM HEPES, 150 mM NaCl, pH 7.4) and filtered (0.20 μm syringe filter [Sartorius]) for 30 minutes at 37 °C.

For activation by kaolin or dextran sulfate (DXS), FXII glutamine substitution variants (37.5 nM final concentration) were first mixed with plasma prekallikrein (PK; 5 μg/mL final concentration) and the chromogenic substrate H-D-Pro-Phe-Arg-pNA·2 HCl (L2120; 0.5 mM) in 0.2% HBS-BSA. Next, kaolin (20 μg/mL), DXS (2 μg/mL; M_r = 500 000), or HBS as a control was added, after which measurements were immediately started. Measurements were performed every minute at 405 nm and 37 °C for 60 minutes.

For activation by kaolin, polyP, silica, and ellagic acid, FXII glutamine substitution variants (150 nM final concentration) were reconstituted in FXII-deficient plasma and subsequently mixed with substrate L2120 (0.5 mM). Substrate conversion was measured at 405 nm, starting immediately after addition of either kaolin (40 μg/mL), polyP (0.5 mg/mL), silica (5% volume per volume [v/v]), ellagic acid (10% [v/v]), or 0.2% HBS-BSA as a control.

To investigate whether the glutamine substitutions affect the conformation of zymogen FXII, FXII variants (22.5 nM) were incubated with PKa (5 μg/mL) for 60 minutes at 37 °C. PKa was inhibited by aprotinin (2600 KIU/mL) in 0.2% BSA-HBS for 60 minutes at 37 °C. After addition of L2120 (0.5 mM), kallikrein-like activity was measured at 37 °C and 405 nm every minute for 120 minutes. Residual PKa-mediated background signal was subtracted from the FXII-containing samples to quantify FXIIa activity.

The contribution of the unique positively charged patches in the FXII EGF-1 domain to plasma clotting was assessed in an activated partial thromboplastin time (APTT) assay. FXII-deficient plasma was reconstituted to physiological levels with the FXII glutamine substitution variants (375 nM). Samples were incubated with the activator Dade Actin FS Activated PTT Reagent (50 μL, Siemens Healthineers) for 3 minutes at 37 °C. Finally, $CaCl_2$ (8.33 mM final concentration) was added and clotting times were measured on an MC10plus coagulometer (Merlin Medical).

2.5 | FXII EGF-1–specific V_HH selection

For selecting FXII EGF-1 domain–specific V_HHs, a *Lama glama* was immunized with recombinant zymogen FXII, and phage display was performed. Briefly, a negative selection was performed with the FXII truncation variant Δ1-112, which lacks the EGF-1 domain. This was followed by a positive selection to find V_HHs that were able to bind the FXII truncation variant Δ1-71, which does contain the EGF-1 domain.

2.6 | Effects of EGF-1–specific V_HHs on FXII and HK binding to kaolin

Kaolin binding was examined for both FXII and HK to demonstrate that the selected V_HHs can prevent FXII surface-binding while still allowing other protein interactions. FXII EGF-1–specific V_HHs (625 nM final concentration) were added to normal pooled plasma (NPP) supplemented with PPACK (2.5 mM final concentration). The V_HH "R2" raised against the exogenous azo-dye Reactive Red 6 was used as a negative

control. Upon addition of kaolin (150 μg/mL final concentration), the samples were incubated for 15 minutes at 37 °C. To resuspend the kaolin, samples were vortexed every 5 minutes as previously described [38]. Kaolin was spun down for 5 minutes at 5000*g*, and pellets were analyzed for kaolin binding of FXII and HK by Western blotting.

2.7 | Effects of EGF-1–specific V$_H$Hs on contact activation in plasma

To demonstrate that the EGF-1–specific V$_H$Hs can prevent contact activation in plasma, NPP (final FXII concentration, 150 nM) was supplemented with V$_H$H (15 nM, 30 nM, 75 nM, 150 nM, 300 nM, 750 nM, and 3000 nM final concentrations) or 0.2% HBS-BSA as a buffer control in a preblocked microtiter plate (1% BSA in HBS) and L2120 (0.5 mM final concentration). After incubation for 5 minutes at 37 °C, kaolin (5 μg/mL final concentration) or 0.2% HBS-BSA were added. Substrate conversion was measured for 60 minutes at 37 °C at 405 nm. The half maximal inhibitory concentration (IC$_{50}$) of the selected EGF-1–specific V$_H$Hs was determined by plotting the substrate conversion rate of the first 30 minutes and subsequent analysis using a nonlinear regression with a variables slope.

2.8 | Effect of EGF-1–specific V$_H$Hs on FXII activation by PKa in solution

To investigate whether the EGF-1–specific V$_H$Hs affect FXII zymogen conformation, the sensitivity of FXII for activation by PKa in 0.2% HBS-BSA buffer was examined. For this, FXII WT or W268R (62.5 nM final concentration) was preincubated with a 20-fold molar excess of V$_H$H (1250 nM final concentration) or buffer for 30 minutes at 37 °C, respectively. Next, FXII was exposed to PKa (1 μg/mL final concentration) for 60 minutes at 37 °C after which PKa was inhibited by aprotinin (1000 KIU/mL) for 30 minutes at 37 °C. L2120 substrate was added (0.5 mM final concentration) to determine FXIIa activity at 405 nm for 60 minutes at 37 °C. To quantify the generated FXIIa activity, the slopes of the substrate conversions were determined for the initial 30 minutes. These were fitted to a standard curve of alpha FXIIa in 0.2% HBS-BSA (0-150 nM).

2.9 | Effect of EGF-1–specific V$_H$Hs on prothrombin time and APTT

Potential effects of the EGF-1–specific V$_H$Hs on the extrinsic coagulation pathway were analyzed in a prothrombin time (PT) assay. For this, NPP (final FXII concentration 125 nM) was incubated with the selected V$_H$Hs or control VHH "R2" in various concentrations (62.5 nM, 125 nM, 250 nM, and 625 nM) or HBS as a control for 5 minutes at 37 °C. Upon addition of Dade Innovin PT reagent (Siemens Healthineers), plasma clotting times were measured on an MC10plus coagulometer.

The inhibitory effect of the EGF-1–specific V$_H$Hs on contact activation was demonstrated using APTT assays. Citrated plasma from 8 healthy donors (final FXII concentration, 125 nM) was preincubated for 3 minutes with various concentrations of V$_H$Hs (62.5 nM, 125 nM, 250 nM, and 625 nM) or HBS as a control. The intrinsic coagulation was initiated by addition of activator Dade Actin FS Activated PTT Reagent, kaolin (final concentration, 150 μg/mL), or HBS as a control. After incubation for 5 minutes, CaCl$_2$ (8.33 mM) was added and coagulation times were recorded.

2.10 | Binding characteristics of the EGF-1–specific V$_H$Hs

Surface plasmon resonance (SPR) technology (Biacore T100, Cytiva Life Sciences) was used to determine binding parameters of all V$_H$Hs with FXII WT. Furthermore, binding characteristics of the most potent inhibitory V$_H$H with FXII glutamine substitution variants were assessed. Anti-myc antibody (50 μg/mL) was immobilized on flow cell (fc)1 and fc2 of a CM5 sensor chip (Cytiva). An EGF-1–specific V$_H$H (200 nM in flow buffer; 10 mM HEPES, 150 mM NaCl, and 0.005% (v/v) Tween-20; pH 7.4) was captured in fc2. FXII variants (0 nM, 12.5 nM, 25 nM, 50 nM, and 100 nM) were injected in fc1 and fc2 at a flow rate of 30 μL/min for 90 seconds, followed by dissociation for 600 seconds. The chip was regenerated after each analyte injection with 100 mM glycine, pH 2.5, for 60 seconds at 30 μL/mL. Sensorgrams were adjusted for background signal, and data were fitted to a Langmuir 1:1 binding model with Biacore T100 evaluation software. To exclude nonspecific binding to FXI, FX, FIX, PK, HGFA, tissue plasminogen activator (tPA), or urokinase plasminogen activator (uPA), analytes were injected at a concentration of 100 nM in flow buffer. ε-Aminocaproic acid (10 mM) was added to the flow buffer to prevent nonspecific binding of tPA to the CM5 chip without affecting the binding parameters to FXII. Background-corrected binding was evaluated to determine V$_H$H specificity.

2.11 | Statistical analysis

All data were reported as mean ± SD. The groups were compared using the Dunnett's multiple comparison test, and *P* values <.05 were considered significant. Statistical analysis was performed using GraphPad Prism 9.3.0 (GraphPad Software).

3 | RESULTS

3.1 | Prediction-based design and generation of FXII variants

The EGF-1 domain and its adjacent linker regions of FXII stretch from P72 to K113 (mature protein numbering) and contain 11 positively charged amino acids (Figure 1A, B and Table 1). In contrast, only 3
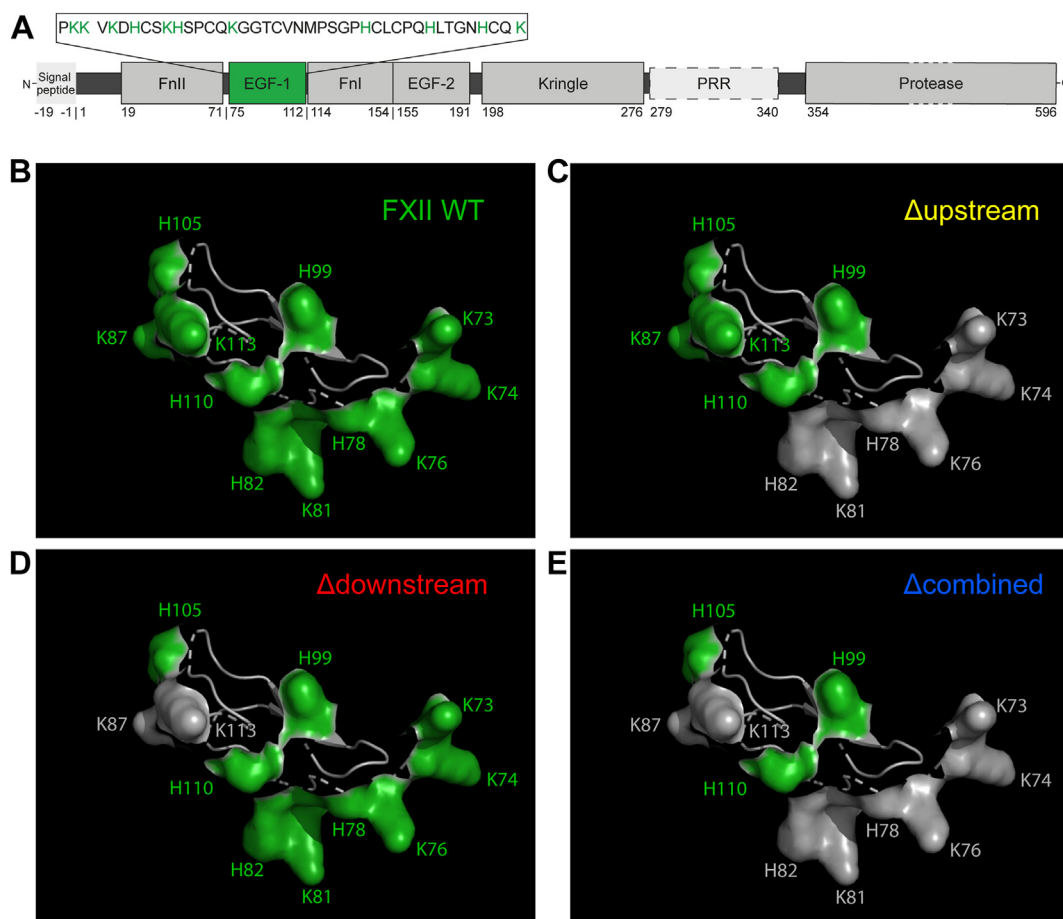
FIGURE 1 Comparison of the hepatocyte growth factor activator (HGFA) epidermal growth factor-like 1 (EGF-1) and factor (F)XII EGF-1 domains reveals 2 positively charged amino acid patches unique to the FXII EGF-1 domain. (A) A linear representation of domains and regions of wild-type (WT) FXII with mature protein numbering. The amino acids of the EGF-1 domain and its adjacent linker regions (P72, K73 and K74 N-terminal, and K113 C-terminal) are displayed with the positively charged amino acids in green. (B) The AlphaFold-predicted 3-dimensional structure (AF-P00748-F1-model_v4) of mature FXII residues P72 to K113, corresponding to the EGF-1 domain and adjacent linker regions, is displayed. This stretch in WT FXII contains 11 positively charged amino acids at physiological pH (indicated in green). Two positively charged patches are unique for FXII EGF-1 compared with HGFA EGF-1, while H99, H105, and H110 are conserved in HGFA EGF-1. The upstream patch consists of residues K73, K74, K76, H78, K81, and H82, and the downstream patch consists of residues K87 and K113. (C) Glutamine-substitution strategy of the upstream patch in which the residues of the positively charged upstream patch are substituted for glutamine residues (indicated in gray) resulting in the EGF-1 domain of the FXII Δupstream variant. (D) Glutamine-substitution strategy of the downstream patch composing the EGF-1 domain of the FXII Δdownstream variant or (E) glutamine-substitution strategy of both patches forming the EGF-1 domain of the FXII Δcombined variant. FnI, fibronectin type 1; FnII, fibronectin type 2; PRR, proline-rich region.

positively charged amino acids are situated in the EGF-1 domain of HGFA. Superimposition of the predicted EGF-1 structure of FXII and the predicted EGF-1 structure of HGFA displays 2 unique, distinct positively charged patches. The first, most N-terminal patch consists of K73, K74, K76, H78, K81, and H82 (hereafter referred to as the upstream patch). Two lysine residues, situated on 87 and 113, form the second, more C-terminal patch (hereafter referred to as the downstream patch). Three charged residues (H99, H105, and H110) in FXII EGF-1 were conserved in HGFA EGF-1. To elucidate the role of these positively charged patches, glutamine substitution of the upstream patch (Figure 1C), downstream patch (Figure 1D), or both patches combined (Figure 1E) was performed. The FXII variants were effectively expressed by Expi293F cells and remained in zymogen state after purification (Supplementary Figure S1).

3.2 | Neutralization of positively charged unique patches in FXII EGF-1 disturbs kaolin binding

FXII residues K73, K74, K76, and K81 (all present in the upstream patch) were recently identified to be necessary for FXII binding to polyP by Shamanaev et al. [40]. In kaolin binding assays, we have previously found that the FXII truncation variant lacking the EGF-1 domain (FXII Δ1-112) no longer possesses surface-binding ability [38]. Kaolin binding assays were performed to examine the kaolin binding capacities of the generated FXII variants (Figure 2A). The FXII Δupstream variant shows a 65% reduction in surface binding compared with FXII WT in kaolin binding assays (Figure 2B). The surface-binding capacity of the FXII Δdownstream variant was not affected by glutamine substitution. While glutamine substitution of
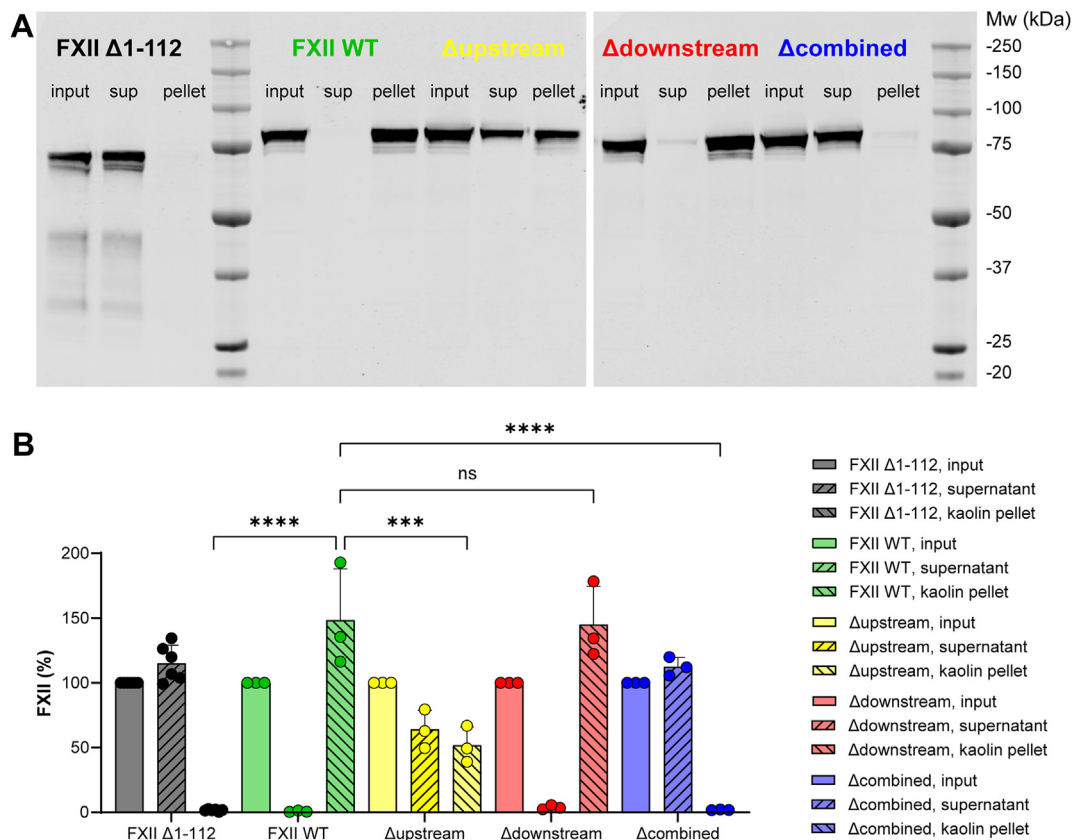
FIGURE 2 Factor (F)XII surface-binding is cooperatively affected by neutralization of the upstream patch in combination with the downstream patch. (A) FXII variants (Δ1-112, wild type (WT), Δupstream, Δdownstream, and Δcombined; 67.5 nM) in cell culture expression medium supplemented with PPACK (50 nM; to prevent FXII autoactivation) were exposed to kaolin (400 μg/mL). FXII in the kaolin fraction and supernatant was detected on Western blot (reducing conditions). Data shown here are representative for 3 independent experiments. (B) Densitometric quantification of kaolin binding of FXII variants. The bars represent the mean + SD of the relative FXII antigens in each fraction. Dots represent individual quantifications. A Dunnett's multiple comparison test was performed to compare the effect of neutralization of the unique patches in epidermal growth factor-like 1 on kaolin binding with FXII WT. ns, nonsignificant; pellet, kaolin pellet; sup, supernatant.$^{***}P <$ .001; $^{****}P <$ .0001.

the positively charged amino acids in the downstream patch on its own does not affect FXII surface-binding capacity, neutralization of the downstream patch in combination with the upstream patch heavily reduces FXII binding capacity. For FXII Δcombined, FXII binding capacity is decreased by 99% compared with that of FXII WT, indicating a cooperative binding mechanism of both patches.

3.3 | Neutralization of both unique patches reduces surface binding and subsequent contact activation causing delayed APTT clotting times, while the susceptibility to activation PKa is unchanged

Upon binding to an activating surface, zymogen FXII undergoes a conformational change to an "open" state in which R353 in the activation loop is available for cleavage [7,45]. To investigate the effects of the abrogated kaolin binding of the FXII variants on activation, FXII variants were incubated with kaolin or DXS (M_r = 500 000). PK was added to enhance measured substrate conversion, which is expressed as kallikrein-like activity. Upon exposure to an activating surface, WT

FXII displays rapid activation, whereas FXII Δupstream and FXII Δdownstream display reduced FXII activation, and FXII Δcombined, which does not bind to kaolin, displays very limited FXII activation (Figure 3A, B).

Next, it was investigated whether the reduced FXII surface-binding capacity would inhibit contact system activation in plasma when triggered through the addition of an activating surface. Hereto, FXII-deficient plasma was supplemented with WT FXII or the FXII glutamine substitution variants. No kallikrein-like activity was observed without the presence of a foreign surface (Supplementary Figure S2). However, after the addition of kaolin, polyP, silica, or ellagic acid, kallikrein-like activity was developed (Figure 3C–F). Compared with supplementation with WT FXII, all FXII variants displayed less activity upon activation by any thrombogenic surface. The Δcombined FXII variant developed the least kallikrein-like activity in a plasma environment upon exposure to any foreign surface.

In the absence of a surface, FXII WT is in zymogen conformation and thus naturally resistant against activating cleavage by PKa. Truncations and mutations in the heavy chain enhance susceptibility of FXII to PKa cleavage [7,16–18,38]. To exclude conformational changes in
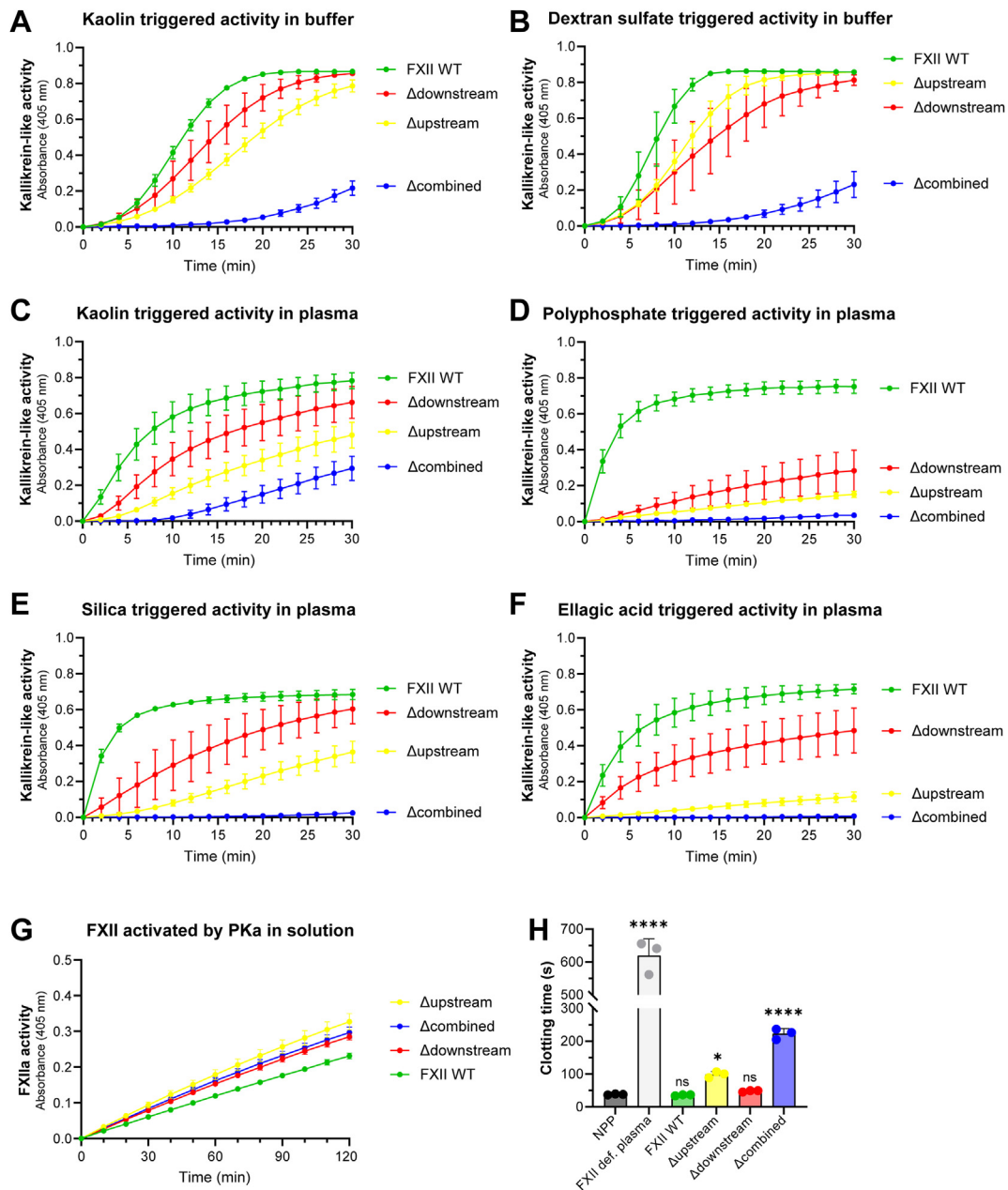
FIGURE 3 Neutralization of either upstream or downstream or both positively charged patches in epidermal growth factor-like 1 reduces surface-induced factor (F)XII activation and subsequent clot formation without affecting plasma kallikrein (PKa) cleavage in solution. (A) FXII variants (37.5 nM) in buffer with PK (5 μg/mL) were activated by (A) kaolin (20 μg/mL) or (B) dextran sulfate (2 μg/mL; M_r = 500 000). Kallikrein-like activity of FXII variants (150 nM) supplemented in FXII-deficient plasma was measured over time upon addition of (C) kaolin (40 μg/mL), (D) polyphosphate (0.5 mg/mL), (E) silica (5% volume per volume [v/v]), or (F) ellagic acid (10% [v/v]). (G) FXII variant (22.5 nM) cleavage by PKa (5 μg/mL) in solution. The data points represent the mean ± SD of 3 independent experiments, all performed in duplicate. (H) Activated partial thromboplastin time clotting times of normal pooled plasma (NPP), FXII-deficient plasma, or FXII-deficient plasma reconstituted with a FXII variant to physiological concentrations. Shown are average clotting times ± SD, and the dots represent the individual clotting times. A Dunnett's multiple comparisons test was performed to compare the clotting times of NPP with those of the FXII variants. def., deficient; ns, nonsignificant; WT, wild type. *$P < .05$; ****$P < .0001$.

FXII due to the glutamine substitutions, the generated FXII variants were mixed with PKa in buffer. The FXII Δupstream, Δdownstream, and Δcombined variants display equal susceptibility toward activation by PKa compared with WT FXII (Figure 3G). Since cleavage by PKa in solution is not enhanced by the glutamine substitutions in the EGF-1 domain, the activation loop remains properly shielded.

The potency of the FXII glutamine substitution variants to limit triggered contact activation in plasma was quantified in a modified APTT assay (Figure 3F). In this assay, FXII-deficient plasma reconstituted with WT FXII will clot after 36 seconds on average, whereas reconstitution with FXII Δupstream and FXII Δdownstream leads to a modest prolongation; reconstitution of FXII Δcombined prolongs the

clotting time to an average of 222 seconds (Figure 3F). Thus, the neutralization of the unique positively charged residues in the FXII EGF-1 domain cooperatively mediates FXII surface-binding and subsequent FXII autoactivation and contact system activation.

3.4 | FXII EGF-1–specific V$_H$H selection to interfere with FXII surface-binding

Although contact activation is not essential for hemostasis, it plays a crucial role in medical device–induced thrombosis and its prevention therefore has potential therapeutic value. The glutamine substitution FXII variants confirm the importance of FXII EGF-1 for FXII surface-binding. Therefore, 3 V$_H$Hs (F2, G2, and B1) were raised that are specific for the EGF-1 domain of FXII WT and could therefore potentially affect interaction of FXII with thrombogenic surfaces (Supplementary Figure S3). Since the V$_H$Hs were selected against the FXII EGF-1 domain, binding of FXII or HGFA in NPP to immobilized V$_H$H was examined. All selected V$_H$Hs are able to bind FXII, but not HGFA, in NPP, indicating that the selected V$_H$Hs are specific for FXII EGF-1 (Supplementary Figure S4).

3.5 | V$_H$Hs targeting the EGF-1 domain of FXII interfere with FXII surface-binding exclusively

To investigate the effects of the selected V$_H$Hs on protein binding to thrombogenic surfaces, kaolin pull-down assays were performed in NPP with 625 nM EGF-1–specific V$_H$H or "R2" as a control V$_H$H. The interaction of the EGF-1–specific V$_H$H F2 with FXII reduced kaolin binding of FXII in NPP with 73.6% compared with R2 (Figure 4A, B). Targeting FXII with EGF-1–specific V$_H$Hs G2 and B1 decreases kaolin binding of FXII with 55.9% and 40.2%, respectively. This indicates that the selected EGF-1–specific V$_H$Hs hinder FXII surface-binding through their interaction with the EGF-1 domain. To avoid side effects, the selected V$_H$Hs must exclusively hinder surface interaction with FXII EGF-1 and must not affect binding of other proteins to foreign surfaces. To investigate the effect of the FXII EGF-1–specific V$_H$Hs on HK binding to kaolin, HK was visualized in the same kaolin pull-down experiments. In all conditions, HK is able to bind kaolin equally well, indicating that the selected V$_H$Hs do not have an effect on the binding capacities of HK to bind to kaolin (Figure 4C, D).

3.6 | FXII EGF-1–targeting V$_H$Hs can prevent contact activation without affecting FXII zymogen conformation

As the FXII EGF-1–specific V$_H$Hs affect FXII surface-binding, we next examined the effect of the selected V$_H$Hs on surface-induced FXII activation in plasma. For this, NPP (in an assay final concentration of 150 nM FXII) was supplemented with multiple concentration FXII EGF-1–targeting V$_H$H (final concentration, 0-3000 nM) or "R2". Kaolin-induced

contact system activation (kallikrein-like activity; L2120 conversion by FXIIa and PKa) in NPP and contact activation were measured for 120 minutes at 37 °C. The EGF-1–targeting V$_H$Hs F2 and G2 already decreased kallikrein-like activity at a molar ratio of 1:2 (V$_H$H:FXII; final V$_H$H concentration, 75 nM; Figure 5A). When F2 and G2 were administered at equimolar concentrations to FXII (150 nM), these V$_H$Hs decreased kallikrein-like activity by 70.9% and 51.6%, respectively, in comparison with addition of R2 (Figure 5B). B1 did not affect kaolin-induced FXIIa activity in NPP at equimolar concentrations. Only at a V$_H$H to FXII molar ratio of 20 (final V$_H$H concentration, 3000 nM), B1 exhibited a 33.9% inhibition of contact activation in NPP (Figure 5C). Upon addition of F2 or G2 at this concentration to NPP, contact activation was inhibited with 85.3% or 70.6%, respectively. Based on the inhibitory effects on kaolin-induced FXII activation in NPP, the IC$_{50}$ was determined for F2 (72.91 ± 5.26 nM) and G2 (70.18 ± 3.61 nM).

Previous attempts using mAbs directed against the heavy chain of FXII were done before to selectively prevent FXII-driven contact activation. The mAbs in these attempts induced conformational changes in FXII zymogen and thereby enhanced FXII activation through cleavage at R353 by PKa [17,27,35]. It is therefore important that therapeutically targeting FXII does not alter FXII zymogen conformation. To assess the initiation of kallikrein-like activity in NPP upon addition of the selected FXII EGF-1–specific V$_H$Hs, multiple doses of a FXII EGF-1–targeting V$_H$H or R2 were administered to NPP. None of these V$_H$Hs induce generation of kallikrein-like activity in NPP (Figure 5D–F). Furthermore, the effects of the EGF-1–targeting V$_H$Hs on FXII zymogen conformation in buffer were investigated. For this, FXII WT (final concentration, 62.5 nM) in buffer was incubated with one of the V$_H$Hs (final concentration, 1250 nM, 20 times higher than FXII WT). Subsequently, PKa-initiated FXIIa activity was measured and compared with that of the pathogenic FXII mutation W268R, which disturbs zymogen quiescence and thereby activates the kallikrein–kinin system [46]. None of the selected FXII EGF-1–specific V$_H$Hs abolish proteolytic FXIIa activity (Figure 5G, H). FXIIa activity of FXII WT in the absence of a V$_H$H was similar to that of FXII WT supplemented with R2 or F2. This suggests that the FXII EGF-1–specific V$_H$H F2 does not affect FXII zymogen conformation and, as such, does not induce activation of the kallikrein–kinin system. Increased FXIIa activity was observed when FXII WT was incubated with G2 or B1. Interaction with FXII WT to one of these V$_H$Hs accelerates PKa-induced FXIIa activity and alters FXII zymogen conformation, albeit not to the extent of FXII W268R.

3.7 | Plasma clotting times can be prolonged with selective EGF-1 targeting V$_H$Hs

Since the selected EGF-1–specific V$_H$Hs decrease FXII binding but do not initiate FXII activation in plasma, the downstream effects of these V$_H$Hs on plasma clotting were assessed in PT and APTT assays. The FXII EGF-1–specific V$_H$Hs (final concentration, 0-625 nM in HBS) or "R2" was administered to NPP (final FXII concentration, 125 nM). None of the FXII EGF-1 specific V$_H$Hs had an effect on the clotting
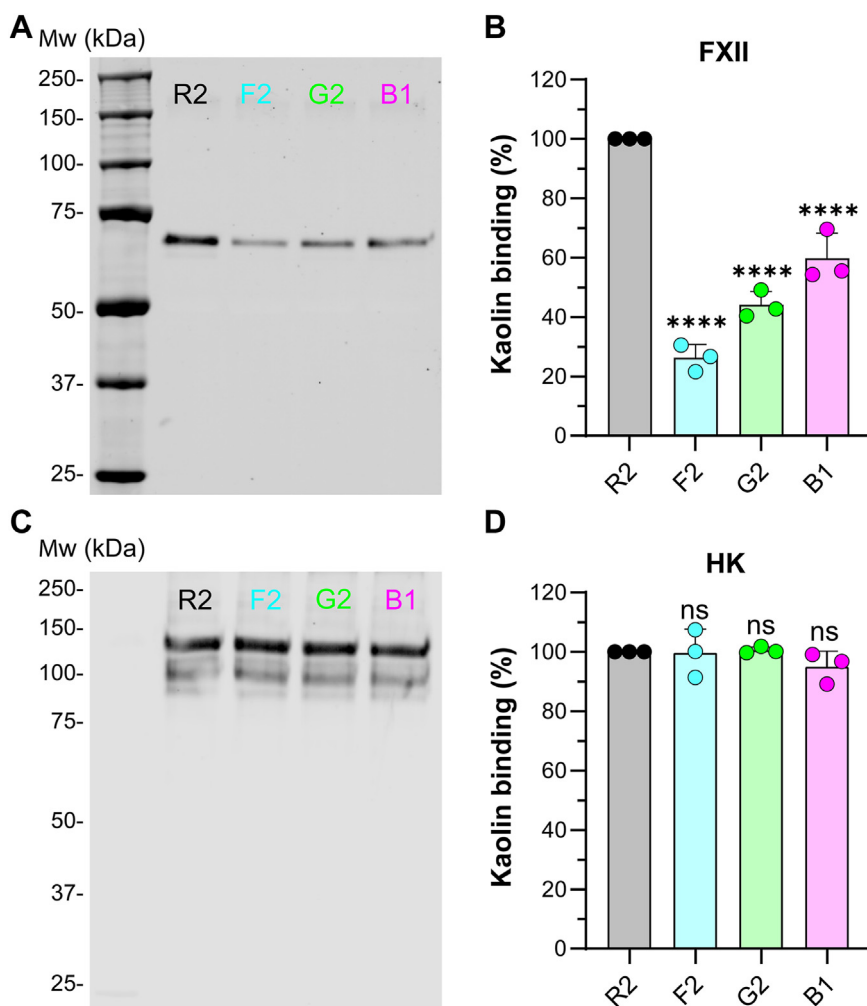
FIGURE 4 Factor (F)XII epidermal growth factor-like 1 (EGF-1)–specific variable domains of a heavy chain–only antibody (V$_H$Hs) specifically decrease FXII binding to kaolin but do not affect high-molecular-weight kininogen (HK) binding to kaolin. (A, C) Normal pooled plasma (final concentration FXII was 125 nM) supplemented with PPACK (final concentration, 2.5 mM) and 625 nM FXII EGF-1–specific V$_H$Hs or R2 (negative control) were incubated with kaolin. After 30 minutes, the kaolin was pelleted by centrifugation to analyze kaolin binding of (A) FXII at 700 nm and (C) HK at 800 nm on the same Western blot. The Western blot is representative for 3 independent experiments. (B, D) Densitometric quantification of kaolin binding of (B) FXII or (D) HK. Shown are FXII and HK antigen quantities relative to R2 (±SD) on the kaolin pellet, and the dots represent the individual quantifications. A Dunnett's multiple comparison test was performed to compare the effect of the EGF-1–specific V$_H$Hs with R2 on kaolin binding of FXII and HK. ns, nonsignificant. ****$P < .0001$.

time in a PT assay setup (Figure 6A). For APTT assays, the V$_H$Hs were administered to plasma of 8 healthy donors. Without addition of a surface and V$_H$Hs, an average plasma clotting time of 392.1 seconds was observed in 7 donors (Figure 6B). Addition of one of the V$_H$Hs does not decrease APTT clotting times. Contrarily, in the presence of high concentrations of F2 or G2, the clotting time of some donors was prolonged to the point of exceeding the detection limit of 995 seconds.

FXII activation was triggered using Dade Actin FS Activated PTT Reagent or kaolin to initiate plasma clotting. Addition of Dade Actin FS Activated PTT Reagent to plasma of 8 donors results in a mean plasma clotting time of 29.1 seconds in the absence of V$_H$Hs (Figure 6C). Significantly prolonged plasma clotting times were detected when F2 or G2 was administered at equimolar concentrations to FXII in NPP (final concentrations of 125 nM). With increasing concentrations, the plasma clotting times prolonged to circa 43.0 and 41.0 seconds for F2 and G2, respectively. Yet, the FXII EGF-1–specific V$_H$H B1 only significantly prolongs the clotting time to 33.6 seconds with a final in-assay concentration of 625 nM, which is 5 times higher than the final in-assay concentration of physiological FXII. Plasma

clotting times upon addition of kaolin display basal clotting times of 58.7 seconds and only F2 prolongs the clotting time significantly (Figure 6D). Significant prolonged clotting times of 72.9 or 73.7 seconds were observed upon addition of F2 with final concentrations of 250 nM and 625 nM. Kaolin-induced plasma clotting was not significantly prolonged by G2 or B1.

3.8 | Binding affinity of EGF-1–targeting V$_H$Hs to plasma proteins and FXII variants

Using SPR technology, nonspecific interactions with FXI, FX, FIX, PK, HGFA, tPA, or uPA were excluded for all selected V$_H$Hs (Supplementary Figure S5). To explain the unique characteristics of the most potent V$_H$Hs, F2, its affinity with FXII WT, FXII Δupstream, FXII Δdownstream, and FXII Δcombined was examined using SPR technology (Supplementary Figure S6). F2 binding to FXII WT was observed with a K_D of 1.11 nM. Binding affinity was reduced to 26.78 nM for the FXII Δcombined variant, indicating that F2 binds to the unique, positively charged patches of FXII EGF-1 (see Table 2).
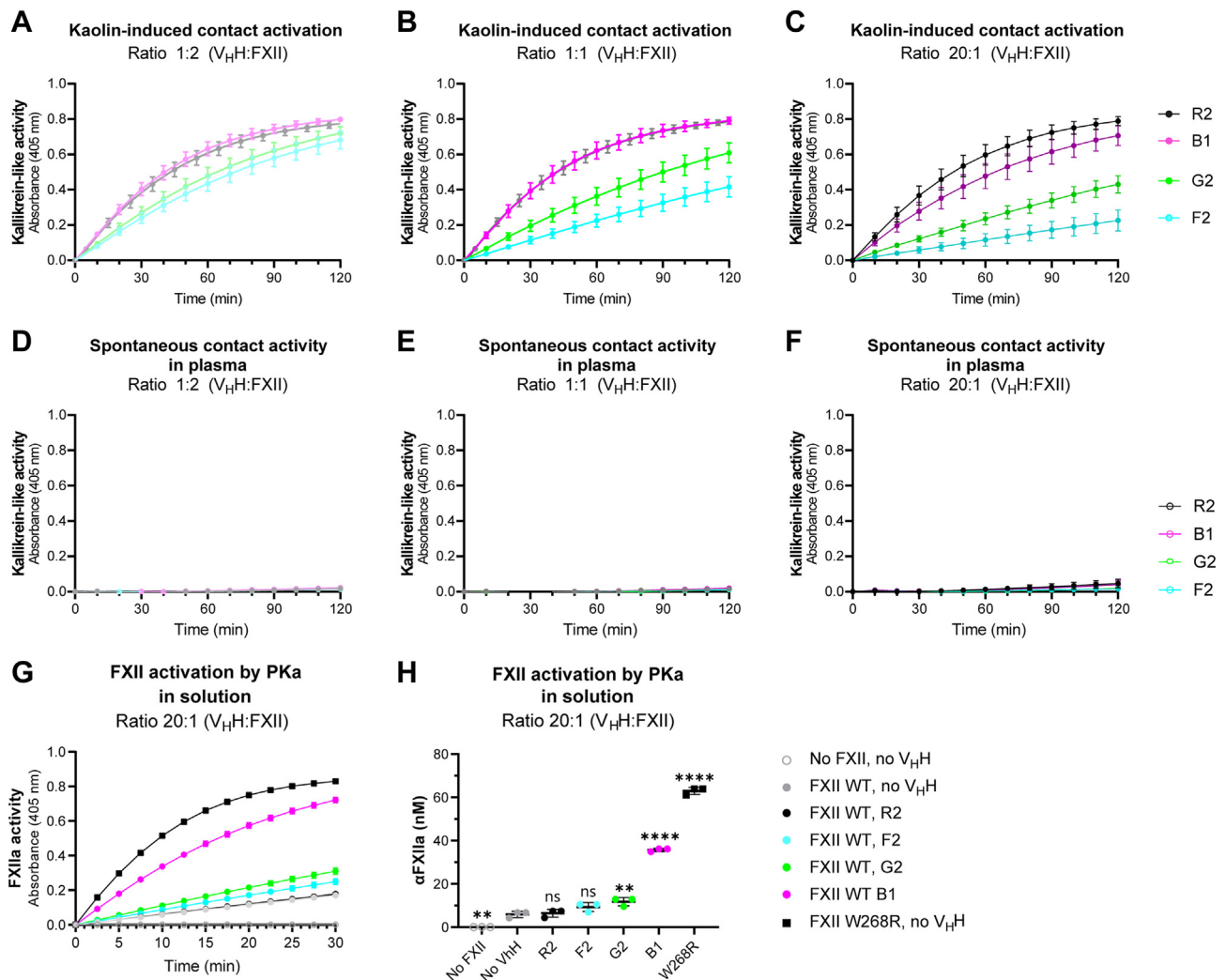
FIGURE 5 Factor (F)XII epidermal growth factor-like 1–specific variable domains of a heavy chain–only antibody (V$_H$Hs) have the potency to prevent kaolin-induced contact activation without altering the FXII zymogen conformation. (A–C) Kaolin-induced contact system activation (kallikrein-like activity; L2120 conversion by FXIIa and plasma kallikrein [PKa]) in normal pooled plasma (NPP) was measured in the presence of R2 (black; negative control), F2 (blue), G2 (green), or B1 (magenta) at (A) ratio 1:2 (V$_H$H:FXII; 75 nM final V$_H$H concentration), (B) equimolar concentration (150 nM final V$_H$H concentration), and (C) at ratio 20:1 (3000 nM final V$_H$H concentration). (D–F) Spontaneous kallikrein-like activity was measured in NPP supplemented with increasing doses of R2, F2, G2, or B1. (G) The susceptibility of FXII activation by PKa was measured in FXII wild type (WT) in buffer supplemented with buffer; a 20 times higher dose of R2, F2, G2, or B1 (final concentration, 1250 nM); or in FXII W268R supplemented with buffer. (H) FXIIa activity upon cleavage by PKa was quantified. Enzymatic activity in NPP and the effect of FXII epidermal growth factor-like 1–specific V$_H$Hs on FXIIa activity were measured for 2 hours at 37 °C. The data points represent the mean ± SD of 3 independent experiments, all performed in duplicate. The linear part of the slopes of the measured FXIIa activity was compared using simple regression. A Dunnett's multiple comparison test was performed to compare the effect of addition of any V$_H$H to FXII WT with FXII WT without addition of a V$_H$H. ns, nonsignificant. **$P < .01$; ****$P < .0001$.

4 | DISCUSSION

FXII contact activation upon binding to blood-contacting surfaces of medical devices can cause serious adverse events [24–27]. Specific inhibition of FXII contact activation might hold therapeutic value as FXII is not indispensable for blood coagulation [30]. Previous studies describe several mAbs that were raised against various domains of FXII. So far, the most successful strategy with mAbs to prevent contact activation was through targeting the protease domain of FXII with 3F7 or 5C12 [28,34]. By targeting this domain, FXIIa enzymatic activity is prevented.

Aside from the intrinsic pathway, the activation of the kallikrein–kinin system and thus BK release are also prevented [47,48]. An improved version of 3F7, garadacimab, was therefore used in a phase III trial for the prevention of hereditary angioedema attacks [49].

To specifically prevent clot formation while maintaining FXII-mediated BK generation, FXII binding to foreign surfaces must be prevented without affecting FXII enzymatic capacity. Therapeutic interventions to prevent FXII surface-binding must therefore target FXII binding site in the heavy chain of FXII. In previous work of Citarella et al. [36], residues 39 to 47 in the fibronectin type 2 (FnII) domain
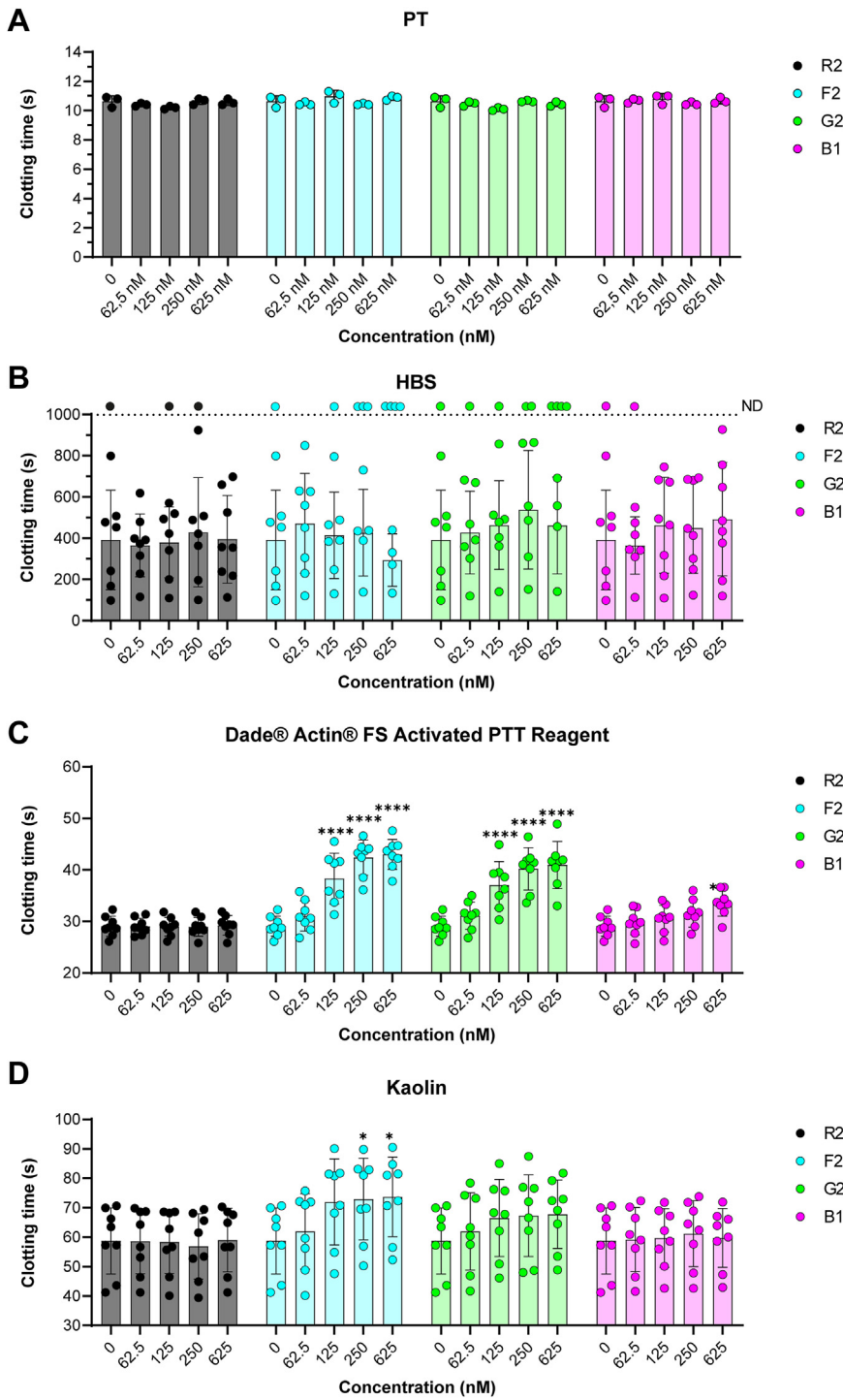
FIGURE 6 Factor XII epidermal growth factor-like 1(EGF-1)–specific variable domains of a heavy chain–only antibody (V$_H$Hs) do not initiate plasma clotting but prolong the activated partial thromboplastin time clotting times of healthy donor plasmas. Normal pooled plasma or citrated plasma of healthy donors ($n = 8$) was incubated with EGF-1–specific V$_H$Hs in final concentrations of 0, 62.5, 125, 250, or 625 nM. Plasma clotting times were assessed in (A) prothrombin time (PT) assays or in (B–D) activated partial thromboplastin time assays upon addition of (B) HEPES-buffered saline (HBS), (C) Dade Actin FS Activated PTT Reagent, or (D) kaolin (final concentration, 150 μg/mL). Shown are average clotting times (±SD) with a dot representing 1 measurable donor. Dunnett's multiple comparison tests were performed to compare the effect of the EGF-1–specific V$_H$H with that of the control V$_H$H R2 with the same dose. ND, not detected. *$P < .05$; ****$P < .0001$.

were identified as surface-binding sites in FXII. They developed the mAb KOK5 that targets these residues and thereby prevents FXII surface-binding. However, this mAb simultaneously induced FXII autoactivation, thereby mimicking surface binding. The FnII domain was later found to interact with the complement C1q receptor on endothelial cells [50,51]. Similar to KOK5, Matafonov et al. [33] raised mAb 15H8 that binds to the epidermal growth factor-like 2 and kringle domains of FXII to delay FXII activation upon addition of a surface. Yet, by exposing R353 in the activation loop upon addition of 15H8 to plasma, PKa-mediated FXII activation in solution and subsequent HK cleavage were enhanced [37,52]. It remains unclear how FXII interacts with uPA receptor on neutrophils and dendritic cells, but based on the above studies, the FnII, the epidermal growth factor-like 2, or the kringle might be involved [53,54].

Inhibiting FXII surface-binding upon blood contact with foreign materials, without affecting FXII zymogen quiescence, would be an

TABLE 2 The most potent variable domain of a heavy chain–only antibody binds to the unique, positively charged patches of epidermal growth factor-like 1 factor XII.

	K_a, 1/Ms (±SD)	K_d, 1/s (±SD)	K_D, M (±SD)
FXII WT	124 590 (±410.1)	9.87×10^{-5} (±1.25×10^{-4})	1.11×10^{-9} (±5.62×10^{-10})
FXII Δupstream	64 387 (±23 760)	2.82×10^{-4} (±4.65×10^{-5})	4.62×10^{-9} (±9.62×10^{-10})
FXII Δdownstream	79 863 (±6709)	1.11×10^{-4} (±1.50×10^{-5})	1.44×10^{-9} (±3.00×10^{-10})
FXII Δcombined	19 957 (±5055)	5.27×10^{-4} (±1.02×10^{-4})	2.68×10^{-8} (±3.10×10^{-9})

Binding parameters of F2 to FXII variants were assessed using surface plasmon resonance technology with immobilized F2 on a CM5 chip. F2 was selected to recognize epidermal growth factor-like 1 in FXII WT and therefore has the highest association constant and lowest dissociation constant for WT. Binding affinity of F2 for FXII Δupstream or FXII Δdownstream was slightly reduced. Its binding affinity for Δcombined was decreased over 24 times. F, factor; WT, wild type.

elegant strategy to prevent contact activation and thus medical device–induced thrombosis. Recent studies demonstrate a crucial role of the EGF-1 domain for FXII binding to negatively charged surfaces [38,39]. Moreover, some crucial amino acids in the EGF-1 domain were identified last year by Shamanaev et al. [40]. However, the AlphaFold predicted FXII tertiary structure highlights some novel information that has not been described before, but does have critical importance for FXII binding and therapeutic target discovery. Using the predicted 3-dimensional structure of FXII and its nonbinding homolog HGFA, we identified differences in the EGF-1 domain. When comparing the EGF-1 domains, 2 positively charged patches were found to be unique for FXII. Shamanaev et al. [40] identified some amino acids that are found in the upstream patch, which consists of K73, K74, K76, H78, K81, and H82. The second unique patch of positively charged amino acids, here called the downstream patch, was not identified previously. Here, we studied the importance of both unique positively charged patches for FXII binding to negatively charged surfaces, following activation and subsequently FXII-dependent plasma clotting. Glutamine substitution variants were made to neutralize 1 or both charged patches in EGF-1. We found that the patches have a cooperative effect on FXII surface-binding and subsequent contact activation. The cellular interactions with FXII EGF-1 remain unknown. In the FXII Δcombined variant, contact activation on negatively charged surfaces is not abolished entirely, suggesting a secondary binding role for the FnII or kringle domain. Contrary to these domains, the identified patches in EGF-1 do not have intramolecular interactions. Mutagenesis in these domains therefore did not induce conformational changes that affect FXII function, which was confirmed by similar PKa-mediated activation of the FXII variants compared with FXII WT. We cannot entirely exclude conformational changes within the EGF-1 domain caused by neutralization of the unique positively charged patches.

For therapeutically blocking FXII-driven contact activation, specific targeting of the EGF-1 domain is required as this is its main surface-binding domain and is predicted to have no intramolecular interactions with domains other than its flanking domains [43]. Previously described mAbs targeting various domains of the FXII heavy chain do not target the EGF-1 domain [32,33,35]. Therefore, 3 FXII EGF-1 specific V_HHs were raised in this study. All selected V_HHs decrease FXII binding to thrombogenic surfaces and inhibit FXII contact activation at high concentrations without inducing FXIIa activity in plasma. Furthermore, there were no nonspecific binding partners found for all selected V_HHs and none of the FXII EGF-1 specific V_HHs initiate plasma clotting or affect the PT assay. At high concentrations, all selected V_HHs decreased kaolin-induced FXIIa activity. Two of the selected EGF-1–specific V_HHs have a dose-dependent prolonging effect on plasma clotting times initiated with Dade Actin FS Activated PTT Reagent. The most potent of these, F2, does not affect FXII conformation of FXII and thus does not induce FXII autoactivation or enhance susceptibility for activation. F2 is therefore our lead candidate. This V_HH binds to the unique positively charged patches in FXII EGF-1; it has a decreased binding affinity for the FXII variants. This FXII EGF-1–specific V_HH therefore may have antithrombotic effects, without affecting the kallikrein–kinin system, proving that selective inhibition of FXII contact activity is possible.

Taken together, 2 unique positively charged patches in the EGF-1 domain of FXII cooperatively mediate FXII binding to foreign surfaces. Blocking this interaction by targeting the EGF-1 domain with EGF-1–targeting V_HHs can reduce FXII surface-binding and contact activation. In the presence of a FXII EGF-1–specific V_HH, time to clot formation *in vitro* is prolonged while FXII proteolytic activity and zymogen conformation are not affected. This suggests that targeting FXII with EGF-1–specific V_HHs could prevent blood coagulation upon contact with foreign surfaces, while its potential to initiate the kallikrein–kinin system remains unaffected.

AUTHOR CONTRIBUTIONS

R.F. and C.M. were involved in the conceptualization of this study. R.F. and S.S. performed research experiments. H.E.O. contributed to the interpretation of the data. R.F. wrote the manuscript. H.E.O. and C.C.C. revised the manuscript. All authors were involved in reviewing and editing.

DECLARATION OF COMPETING INTERESTS

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REFERENCES

[1] Maas C, Oschatz C, Renné T. The plasma contact system 2.0. *Semin Thromb Hemost.* 2011;37:375–81.

[2] Renné T, Stavrou EX. Roles of factor XII in innate immunity. *Front Immunol.* 2019;10:2011.

[3] Ratnoff OD, Saito H. Amidolytic properties of single-chain activated Hageman factor. *Proc Natl Acad Sci U S A.* 1979;76:1461–3.

[4] Griffin JH, Cochrane CG. Mechanisms for the involvement of high molecular weight kininogen in surface-dependent reactions of Hageman factor. *Proc Natl Acad Sci U S A.* 1976;73:2554–8.

[5] de Maat S, Tersteeg C, Herczenik E, Maas C. Tracking down contact activation - from coagulation in vitro to inflammation in vivo. *Int J Lab Hematol.* 2014;36:374–81.

[6] de Maat S, Maas C. Factor XII: form determines function. *J Thromb Haemost.* 2016;14:1498–506.

[7] Shamanaev A, Litvak M, Gailani D. Recent advances in factor XII structure and function. *Curr Opin Hematol.* 2022;29:233–43.

[8] Macfarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature.* 1964;202:498–9.

[9] Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. *Science.* 1964;145:1310–2.

[10] Grover SP, Mackman N. Intrinsic pathway of coagulation and thrombosis. *Arterioscler Thromb Vasc Biol.* 2019;39:331–8.

[11] Renné T, Schmaier AH, Nickel KF, Blombäck M, Maas C. In vivo roles of factor XII. *Blood.* 2012;120:4296–303.

[12] Schmaier AH, McCrae KR. The plasma kallikrein-kinin system: its evolution from contact activation. *J Thromb Haemost.* 2007;5:2323–9.

[13] Müller F, Renné T. Novel roles for factor XII-driven plasma contact activation system. *Curr Opin Hematol.* 2008;15:516–21.

[14] Maas C, Renné T. Regulatory mechanisms of the plasma contact system. *Thromb Res.* 2012;129(Suppl 2):S73–6.

[15] Björkqvist J, Jämsä A, Renné T. Plasma kallikrein: the bradykinin-producing enzyme. *Thromb Haemost.* 2013;110:399–407.

[16] de Maat S, Clark CC, Boertien M, Parr N, Sanrattana W, Hofman ZLM, Maas C. Factor XII truncation accelerates activation in solution. *J Thromb Haemost.* 2019;17:183–94.

[17] Hofman ZLM, Clark CC, Sanrattana W, Nosairi A, Parr NMJ, Zivkovic M, Krause K, Mahnke NA, Scheffel J, Hack CE, Maurer M, de Maat S, Maas C. A mutation in the kringle domain of human factor XII that causes autoinflammation, disturbs zymogen quiescence, and accelerates activation. *J Biol Chem.* 2020;295:363–74.

[18] de Maat S, Björkqvist J, Suffritti C, Wiesenekker CP, Nagtegaal W, Koekman A, van Dooremalen S, Pasterkamp G, de Groot PG, Cicardi M, Renné T, Maas C. Plasmin is a natural trigger for bradykinin production in patients with hereditary angioedema with factor XII mutations. *J Allergy Clin Immunol.* 2016;138:1414–23.e9.

[19] Tillman BF, Gruber A, McCarty OJT, Gailani D. Plasma contact factors as therapeutic targets. *Blood Rev.* 2018;32:433–48.

[20] Kokoye Y, Ivanov I, Cheng Q, Matafonov A, Dickeson SK, Mason S, Sexton DJ, Renné T, McCrae K, Feener EP, Gailani D. A comparison of the effects of factor XII deficiency and prekallikrein deficiency on thrombus formation. *Thromb Res.* 2016;140:118–24.

[21] Duga S, Salomon O. Congenital factor XI deficiency: an update. *Semin Thromb Hemost.* 2013;39:621–31.

[22] Renné T, Pozgajová M, Grüner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med.* 2005;202:271–81.

[23] Wang X, Smith PL, Hsu MY, Gailani D, Schumacher WA, Ogletree ML, Seiffert DA. Effects of factor XI deficiency on ferric chloride-induced vena cava thrombosis in mice. *J Thromb Haemost.* 2006;4:1982–8.

[24] Campbell DJ, Dixon B, Kladis A, Kemme M, Santamaria JD. Activation of the kallikrein-kinin system by cardiopulmonary bypass in humans. *Am J Physiol Regul Integr Comp Physiol.* 2001;281:R1059–70.

[25] Raffini L. Anticoagulation with VADs and ECMO: walking the tightrope. *Hematology Am Soc Hematol Educ Program.* 2017;2017:674–80.

[26] Tillman B, Gailani D. Inhibition of factors XI and XII for prevention of thrombosis induced by artificial surfaces. *Semin Thromb Hemost.* 2018;44:60–9.

[27] Buchtele N, Schwameis M, Schellongowski P, Quehenberger P, Knöbl P, Traby L, Schmid M, Schoergenhofer C, Herkner H, Jilma B, Staudinger T. Prevalence and clinical impact of reduced coagulation factor XII activity in patients receiving extracorporeal membrane oxygenation. *Crit Care Med.* 2021;49:e1206–11.

[28] Larsson M, Rayzman V, Nolte MW, Nickel KF, Björkqvist J, Jämsä A, Hardy MP, Fries M, Schmidbauer S, Hedenqvist P, Broomé M, Pragst I, Dickneite G, Wilson MJ, Nash AD, Panousis C, Renné T. A factor XIIa inhibitory antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk. *Sci Transl Med.* 2014;6:222ra17.

[29] Davoine C, Bouckaert C, Fillet M, Pochet L. Factor XII/XIIa inhibitors: their discovery, development, and potential indications. *Eur J Med Chem.* 2020;208:112753.

[30] Fredenburgh JC, Weitz JI. New anticoagulants: moving beyond the direct oral anticoagulants. *J Thromb Haemost.* 2021;19:20–9.

[31] Pixley RA, Stumpo LG, Birkmeyer K, Silver L, Colman RW. A monoclonal antibody recognizing an icosapeptide sequence in the heavy chain of human factor XII inhibits surface-catalyzed activation. *J Biol Chem.* 1987;262:10140–5.

[32] Ravon DM, Citarella F, Lubbers YTP, Pascucci B, Hack CE. Monoclonal antibody F1 binds to the kringle domain of factor XII and induces enhanced susceptibility for cleavage by kallikrein. *Blood.* 1995;86:4134–43.

[33] Matafonov A, Leung PY, Gailani AE, Grach SL, Puy C, Cheng Q, Sun MF, McCarty OJT, Tucker EI, Kataoka H, Renné T, Morrissey JH, Gruber A, Gailani D. Factor XII inhibition reduces thrombus formation in a primate thrombosis model. *Blood.* 2014;123:1739–46.

[34] Wallisch M, Lorentz CU, Lakshmanan HHS, Johnson J, Carris MR, Puy C, Gailani D, Hinds MT, McCarty OJT, Gruber A, Tucker EI. Antibody inhibition of contact factor XII reduces platelet deposition in a model of extracorporeal membrane oxygenator perfusion in nonhuman primates. *Res Pract Thromb Haemost.* 2020;4:205–16.

[35] Heestermans M, Naudin C, Mailer RK, Konrath S, Klaetschke K, Jämsä A, Frye M, Deppermann C, Pula G, Kuta P, Friese MA, Gelderblom M, Sickmann A, Preston RJS, Nofer JR, Rose-John S, Butler LM, Salomon O, Stavrou EX, Renné T. Identification of the factor XII contact activation site enables sensitive coagulation diagnostics. *Nat Commun.* 2021;12:5596.

[36] Citarella F, te Velthuis H, Helmer-Citterich M, Hack CE. Identification of a putative binding site for negatively charged surfaces in the fibronectin type II domain of human factor XII—an immunochemical and homology modeling approach. *Thromb Haemost.* 2000;84:1057–65.

[37] Kohs TCL, Lorentz CU, Johnson J, Puy C, Olson SR, Shatzel JJ, Gailani D, Hinds MT, Tucker EI, Gruber A, McCarty OJT, Wallisch M. Development of coagulation factor XII antibodies for inhibiting vascular device-related thrombosis. *Cell Mol Bioeng*. 2021;14:161–75.

[38] Clark CC, Hofman ZLM, Sanrattana W, Den Braven L, De Maat S, Maas C. The fibronectin type II domain of factor XII ensures zymogen quiescence. *Thromb Haemost*. 2020;120:400–11.

[39] Shamanaev A, Ivanov I, Sun MF, Litvak M, Srivastava P, Mohammed BM, Shaban R, Maddur A, Verhamme IM, McCarty OJT, Law RHP, Gailani D. Model for surface-dependent factor XII activation: the roles of factor XII heavy chain domains. *Blood Adv*. 2022;6:3142–54.

[40] Shamanaev A, Litvak M, Cheng Q, Ponczek M, Dickeson SK, Smith SA, Morrissey JH, Gailani D. A site on factor XII required for productive interactions with polyphosphate. *J Thromb Haemost*. 2023;21:1567–79.

[41] Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Zídek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, et al. Highly accurate protein structure prediction with AlphaFold. *Nature*. 2021;596:583–9.

[42] Varadi M, Anyango S, Deshpande M, Nair S, Natassia C, Yordanova G, Yuan D, Stroe O, Wood G, Laydon A, Zídek A, Green T, Tunyasuvunakool K, Petersen S, Jumper J, Clancy E, Green R, Vora A, Lutfi M, Figurnov M, et al. AlphaFold protein structure database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Res*. 2022;50:D439–44.

[43] Frunt R, El Otmani H, Gibril Kaira B, De Maat S, Maas C. Factor XII explored with AlphaFold - opportunities for selective drug development. *Thromb Haemost*. 2023;123:177–85.

[44] Schrödinger LLC. The PyMOL Molecular Graphics System, 8 2015. https://pymol.org/. [accessed November 12, 2021].

[45] Maas C, Renné T. Coagulation factor XII in thrombosis and inflammation. *Blood*. 2018;131:1903–9.

[46] Scheffel J, Mahnke NA, Hofman ZLM, de Maat S, Wu J, Bonnekoh H, Pengelly RJ, Ennis S, Holloway JW, Kirchner M, Mertins P, Church MK, Maurer M, Maas C, Krause K. Cold-induced urticarial autoinflammatory syndrome related to factor XII activation. *Nat Commun*. 2020;11:179.

[47] Cao Z, Biondo M, Rayzman V, Hardy M, McDonald A, Busfield S, Nolte MW, Wilson M, Nash A, Panousis C. Development and characterization of an anti-FXIIa monoclonal antibody for the treatment of hereditary angioedema. *J Allergy Clin Immunol*. 2015;135:AB194.

[48] Cao H, Biondo M, Lioe H, Busfield S, Rayzman V, Nieswandt B, Bork K, Harrison LC, Auyeung P, Farkas H, Csuka D, Pelzing M, Dower S, Wilson MJ, Nash A, Nolte MW, Panousis C. Antibody-mediated inhibition of FXIIa blocks downstream bradykinin generation. *J Allergy Clin Immunol*. 2018;142:1355–8.

[49] Craig TJ, Reshef A, Li HH, Jacobs JS, Bernstein JA, Farkas H, Yang WH, Stroes ESG, Ohsawa I, Tachdjian R, Manning ME, Lumry WR, Saguer IM, Aygören-Pürsün E, Ritchie B, Sussman GL, Anderson J, Kawahata K, Suzuki Y, Staubach P, et al. Efficacy and safety of garadacimab, a factor XIIa inhibitor for hereditary angioedema prevention (VANGUARD): a global, multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet*. 2023;401:1079–90.

[50] Mahdi F, Madar ZS, Figueroa CD, Schmaier AH. Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes. *Blood*. 2002;99:3585–96.

[51] Kaira BG, Slater A, McCrae KR, Dreveny I, Sumya U, Mutch NJ, Searle M, Emsley J. Factor XII and kininogen asymmetric assembly with gC1qR/C1QBP/P32 is governed by allostery. *Blood*. 2020;136:1685–97.

[52] Ivanov I, Matafonov A, Sun MF, Mohammed BM, Cheng Q, Dickeson SK, Kundu S, Verhamme IM, Gruber A, McCrae K, Gailani D. A mechanism for hereditary angioedema with normal C1 inhibitor: an inhibitory regulatory role for the factor XII heavy chain. *Blood*. 2019;133:1152–63.

[53] Göbel K, Pankratz S, Asaridou CM, Herrmann AM, Bittner S, Merker M, Ruck T, Glumm S, Langhauser F, Kraft P, Krug TF, Breuer J, Herold M, Gross CC, Beckmann D, Korb-Pap A, Schuhmann MK, Kuerten S, Mitroulis I, Ruppert C, et al. Blood coagulation factor XII drives adaptive immunity during neuro-inflammation via CD87-mediated modulation of dendritic cells. *Nat Commun*. 2016;7:11626.

[54] Stavrou EX, Fang C, Bane KL, Long AT, Naudin C, Kucukal E, Gandhi A, Brett-Morris A, Mumaw MM, Izadmehr S, Merkulova A, Reynolds CC, Alhalabi O, Nayak L, Yu WM, Qu CK, Meyerson HJ, Dubyak GR, Gurkan UA, Nieman MT, et al. Factor XII and uPAR upregulate neutrophil functions to influence wound healing. *J Clin Invest*. 2018;128:944–59.

SUPPLEMENTARY MATERIAL

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