



Factor XII-Driven Inflammatory Reactions with Implications for Anaphylaxis

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Anaphylaxis is a life-threatening allergic reaction. It is triggered by the release of pro-inflammatory cytokines and mediators from mast cells and basophils in response to immunologic or non-immunologic mechanisms. Mediators that are released upon mast cell activation include the highly sulfated polysaccharide and inorganic polymer heparin and polyphosphate (polyP), respectively. Heparin and polyP supply a negative surface for factor XII (FXII) activation, a serine protease that drives contact system-mediated coagulation and inflammation. Activation of the FXII substrate plasma kallikrein leads to further activation of zymogen FXII and triggers the pro-inflammatory kallikrein-kinin system that results in the release of the mediator bradykinin (BK). The severity of anaphylaxis is correlated with the intensity of contact system activation, the magnitude of mast cell activation, and BK formation. The main inhibitor of the complement system, C1 esterase inhibitor, potently interferes with FXII activity, indicating a meaningful cross-link between complement and kallikrein-kinin systems. Deficiency in a functional C1 esterase inhibitor leads to a severe swelling disorder called hereditary angioedema (HAE). The significance of FXII in these disorders highlights the importance of studying how these processes are integrated and can be therapeutically targeted. In this review, we focus on how FXII integrates with inflammation and the complement system to cause anaphylaxis and HAE as well as highlight current diagnosis and treatments of BK-related diseases.

Keywords: contact system, factor XII, kallikrein-kinin system, bradykinin, mast cells, heparin, polyP, anaphylaxis

BACKGROUND OF THE PLASMA CONTACT SYSTEM

The factor XII (FXII)-driven contact system is a network of proteases and inhibitors that integrates four major pathways: (1) the complement system, (2) the coagulation cascade, (3) the fibrinolytic system, and (4) the kallikrein-kinin system (1). The name “plasma contact system” comes from FXII being activated when it comes into “contact” with anionic surfaces, which leads to a conformational rearrangement resulting in the active protease factor XIIa (FXIIa). FXIIa initiates a series of downstream events that mediate the interface between of inflammation and coagulation (2, 3). FXIIa activates two serine proteinases, factor XI (FXI) and plasma prekallikrein (PK) that drive the coagulation and kallikrein-kinin systems, respectively. The

non-enzymatic cofactor, high-molecular-weight kininogen (HK) is cleaved by activated plasma kallikrein (PKa) to release the pro-inflammatory oligopeptide bradykinin (BK) (4). Recent data have linked FXIIa-driven formation of BK and the downstream activation of the G-protein-coupled receptor B2 (B2R) potentially signaling to anaphylaxis and other immunologic disorders (**Figure 1**) (5, 6).

Proteins and Molecules of the Contact System

Factor XII

Factor XII circulates in plasma as a zymogen with a concentration of 40 µg/ml (375 nM) (1). Coming into contact with anionic surfaces causes the zymogen form of FXII to undergo a conformational change in the presence of zinc ions. Conformational rearrangements induce auto-activation, which leads to small amounts of FXIIa (7). Due to activation of FXII zymogen, the single-chain polypeptide is converted into a two-chain molecule,

composed of a heavy chain [353 amino acid (aa)] and a light chain (243 aa). The two chains stay connected with each other by a disulfide bond between Cys340 and Cys367 residues. The heavy chain is responsible for binding to anionic surfaces and, similar to HK heavy chain (8), links the zymogen to proteoglycans of cell surfaces (9). The catalytic domain is located within the C-terminal light chain of the protease. In humans, single-chain (sc)FXII has measurable, although much lower, proteolytic activity than complete FXIIa and its potential importance *in vivo* remains to be shown (10). FXIIa initiates the intrinsic coagulation cascade, which leads to the generation of thrombin and fibrin to produce clots in the blood (11). Furthermore, FXIIa converts PK to the active protease PKa, which reciprocally activates more FXII (7). In addition, PKa can initiate a further proteolysis of FXIIa into a ~30 kDa light chain fragment, termed β-FXIIa. The cleavage takes place at the peptide bond Arg353–Val354 and consequently, the active site released from the heavy chain and thus from surfaces. This small, soluble β-FXIIa variant retains its proteolytic activity toward PK, but not to FXI (**Figure 1**), offering an explanation

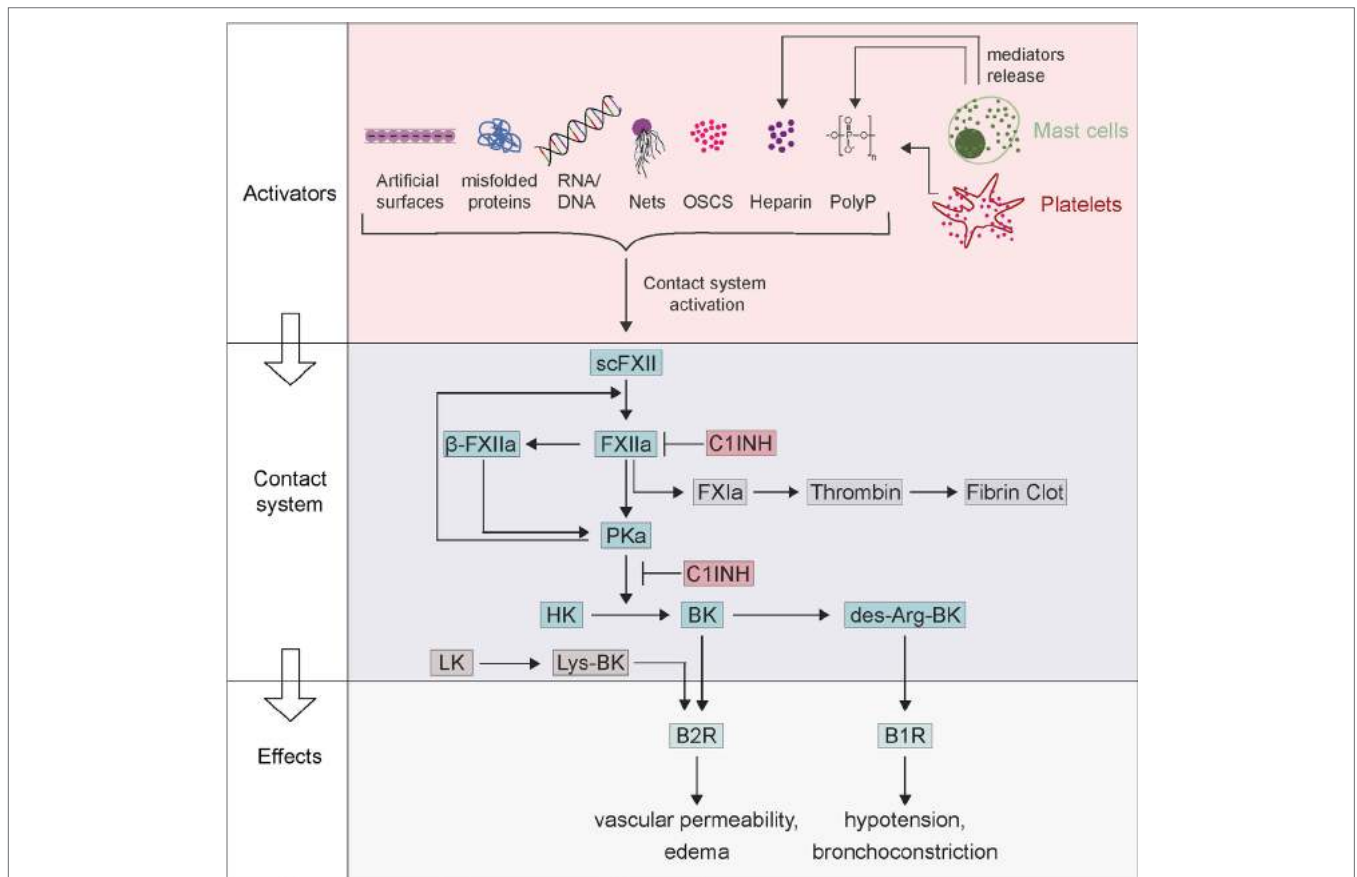


FIGURE 1 | Factor XII (FXII)-driven contact system in activation of anaphylaxis. Zymogen scFXII becomes activated to FXIIa either by endogenous activators [misfolded proteins, RNA/DNA, neutrophil extracellular traps (NETs), polyP, oversulfated chondroitin sulfate-contaminated heparin (OSCS-heparin) and heparin] or by artificial surfaces. Anaphylaxis can activate mast cells with the release of their mediators (polyP and heparin), which also leads to FXIIa. FXIIa proceeds to activate prekallikrein, which reciprocally cleaves both FXIIa into β-FXIIa and high-molecular-weight kininogen (HK) to bradykinin (BK). BK binds receptor B2 (B2R) and triggers inflammation, edema, and symptoms of anaphylaxis. BK can be further proceeding to des-Arg-BK and mediates B1 receptor (B1R) activation resulting in hypotension and bronchoconstriction. The contact system can be inhibited by the C1INH that inhibits both FXIIa and plasma kallikrein.

for selective activation of the kallikrein–kinin pathway in the absence of coagulation (12).

Plasma Kallikrein

Prekallikrein has a plasma concentration of 35–50 µg/ml (580 nM) and exists as two different glycosylated forms with molecular weights of 85 and 88 kDa, respectively. Similar to FXII, a limited proteolysis activates zymogen PK and the active form is composed of a heavy chain (residues 1–371, 55 kDa) linked by a disulfide bond and a light chain (residues 378–619, 30 kDa). The heavy chain contains four apple domains and PK/PKa binding to HK is mediated by apple domains 1, 2, and 4 (13, 14). The PK light chain contains the peptidase domain with the substrates being HK, FXII, plasminogen, and urokinase-type plasminogen activator. Interestingly, the kallikrein–kinin system is linked to thrombosis, fibrinolysis, and the rennin–angiotensin system through the conversion of plasminogen to plasmin by PKa (7).

High-Molecular-Weight Kininogen

In humans, the non-enzymatic cofactor HK is generated from a single gene but undergoes alternative splicing to form high- (HK) and low-molecular (LK) weight kininogen. Murine HK contains two kininogen genes and both transcripts undergo alternative splicing which results in four kininogens. HK, but not LK, binds to cell surface glycosaminoglycans and the interaction is improved by zinc ions (15, 16). There is no detectable spontaneous HK activation due to HK protection from proteolytic cleavage by glycosaminoglycans binding. Therefore, cell surface presents a reservoir for BK production (7, 17).

Activation of BK *via* the FXII-Driven Contact System

Bradykinin is a nonapeptide composed of the sequence Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg and functions as an inflammatory mediator. BK is the product of the kallikrein–kinin system following activation of FXII. FXIIa leads to proteolysis of PK, and the resulting PKa cleaves HK to generate BK (**Figure 1**). In contrast to PK, tissue kallikrein liberates kallidin (Lys–BK) from LK (18). Released BK binds with high-affinity (8–12 nM) to B2R. Upon binding of BK or kallidin, the activated B2R induces an increase of intracellular calcium ($[Ca^{2+}]_i$) that stimulates the endothelial nitric oxide synthase resulting in increased protein kinase G activity (19, 20). B2R signaling triggers vasodilatation, increase of vascular permeability, mobilization of arachidonic acid, and chemotaxis in granulocytes (21). BK increases vascular permeability *via* opening tight junctions of endothelial cells (22). B2R is constitutively expressed in multiple tissues such as endothelial cells, sensory fibers, smooth muscle cells, and epithelial cells, among others. Furthermore, expression of the B2R is enhanced by cytokines, cyclic adenosine monophosphate, estrogen, and glucocorticoids. Pathologic B2R activation contributes to various allergic, inflammatory, and infectious diseases such as sepsis, anaphylaxis, traumatic brain edema, rhinitis, capillary leak syndrome, or ischemia/reperfusion injuries (6, 23, 24). BK has a short half-life (<30 s) in plasma because it is quickly degraded by both plasma and endothelial peptidases. To overcome limitations

in analyzing BK in patient samples elegant assays that measure BK-free HK (cleaved HK) have recently been developed (25). The angiotensin-converting enzyme (kinase 2), carboxypeptidases M and N (kininase 1), and the neutral endopeptidase (Neprilysin) process BK at two distinct sites (Pro7–Phe8 and Phe5–Ser6) leading to the inactive peptides BK1–7 and BK1–5 (26). Carboxypeptidase N removes the C-terminal BK arginine residue resulting in the metabolite des-Arg9–BK. This peptide stimulates the G-protein-coupled kinin B1 receptor (B1R) (27). Under normal physiological conditions, B1R is minimally expressed, but expression is rapidly upregulated in response to stimuli such as tissue injury or an increase in inflammation (20). Pharmacological inhibition of some mitogen-activated protein kinases and NF-κB interfere with B1R expression. Interestingly, all kallikrein–kinin system components are found within the central nervous system (CNS), and BK is formed and contributes to brain trauma and ischemia (28). Recently, a role for B1R in brain immune inflammation in a mouse model of Alzheimer's disease was identified, possibly with microglial/macrophage involvement (29). Blocking B1R reduces brain infarction and edema formation in mice, while B2R deficiency had no effect on stroke outcome in mice (30). Furthermore, murine models indicate a role of FXIIa and BK in CNS autoimmunity, including multiple sclerosis (31) and pharmacologic interference with BK formation and/or signaling might ameliorate secondary brain injury (32).

THE CONTACT SYSTEM INTEGRATES WITH ACTIVATED MAST CELLS, THE COMPLEMENT SYSTEM AND MEDIATES ANAPHYLAXIS

Anaphylaxis is a multisystem syndrome of a rapid onset of symptoms and an immunologic response to allergens (33) that is predominantly driven by activated mast cells. Mast cells are found near blood vessels and areas susceptible to foreign antigens, such as tissue mucosa, and serve as multifunctional effector cells in the immune system (34). In most cases, the initiation of anaphylaxis is due to an antigen (allergen) that interacts with high-affinity receptors for immunoglobulin E (FcεRI), which are located on mast cells and basophils. Allergen-binding leads to intracellular signaling that results in the release of granules (35). These components, which are synthesized by mast cells and other immune cells such as macrophages or neutrophils, interact with circulating plasma proteins or tissue factors. Among the liberated compounds is histamine, which increases vascular permeability and vasodilation, leads to plasma leakage and reduced intravascular volume (36). This induces a drop in blood pressure that can lead to a lethal outcome.

Mediators of the Mast Cells and Activators of FXII-Driven Contact System

Mast cells critically contribute to anaphylaxis. The link between mast cells and anaphylaxis was established once it was discovered that mast cells were abundant in protein and mediators such as tryptase, chymase, and other cytokines, as well as newly synthesized lipid-derived molecules such as prostaglandins,

platelet-activating factor (PAF), cytokine tumor necrosis factor α , and leukotrienes (37). These mediators play an important role in the development of anaphylaxis; however, the mechanisms of inducing anaphylaxis vary widely (6). For instance, PAF activates inflammatory and thrombotic pathways by causing platelet activation and liberates vasoactive substances, resulting in increased endothelial permeability. Uncontrolled PAF activities can result in sepsis, shock and are important in disseminated intravascular coagulation (38, 39). Prostaglandins lead to smooth muscle relaxation and act as vasodilators. Interestingly, they can also inhibit platelet adherence. Levels of urinary prostaglandin D2 correlate with severity of anaphylaxis (40) and leukotriene production accompanies histamine and prostaglandin production. Their release triggers smooth muscle contractions and vasodilation, leading to bronchoconstriction and hypotension. Cysteinyl leukotrienes are termed slow-reacting substance of anaphylaxis and are up to 1,000-fold more potent than histamine but have a slower onset and long-lasting activities (41–43). The overlap of these pathways leads to synergistic pathologic effects that also result in activation of complement and contact system pathways, highlighting the importance of developing effective therapeutics for this potentially lethal condition. In this section, some of the main mediators that induce inflammation and/or coagulation through contact system-mediated pathways will be discussed in greater detail.

Histamine

Released histamine causes increased angioedema, anaphylaxis, or chronic spontaneous urticaria and is also involved in allergic responses. Histidine decarboxylase is the only enzyme capable of producing histamine (44). Upon mast cell release, histamine promotes recruitment of T_H2 helper cells and dendritic cells along with antigen presentation (35). Mast cell secretory granules also contain heparin and proteoglycans, which are heavily negatively charged, in contrast to histamine, which is positively charged. Both components can interact within granules and upon mast cell activation, heparin proteoglycans and histamine are released with similar kinetics (45). Furthermore, histamine and heparin have been shown to interact in purified systems (34, 46), but there is no evidence for a physiologically relevant interaction *in vivo*. In urticaria patients, the occurrence of angioedema was reduced with antihistamine therapy (47). In addition, there were no increased plasma BK levels in four patients with an acute histamine-sensitive angioedema (48) arguing that BK and histamine have the capacity for inducing edema by independent pathways. For angioedema with unknown derivation (idiopathic angioedema) and for hereditary angioedema (HAE), histamine receptor antagonists are clinically applied, but approximately one in six patients exhibiting idiopathic angioedema do not respond to antihistamine treatments (49). This suggests that other mediators are involved in the trigger and the outcome of hereditary forms of angioedema.

Serotonin

Serotonin, a biogenic amine, is a mast cell granule constituent. However, confocal microscopy revealed that distinct mast cell granules contain both histamine and serotonin (50, 51). In absence

of endogenous histamine, serotonin is increased in immune cells including mast cells (44). One explanation could be that mast cells can selectively release serotonin without releasing histamine (52) *via* high-affinity serotonin-binding proteins used to sequester serotonin from secretory vesicles (53). Serotonin functions as a regulator of immune and inflammatory responses and is partially mediated through direct interactions with macrophages (54).

Heparin

Another major component of mast cell granules is heparin, which is released following IgE/antigen activation (5). Heparin-driven FXII contact activation triggers the kallikrein–kinin system, releases BK to stimulate B2R in human plasma and leads to edema *in vivo* (55). Heparin levels are elevated in patients with anaphylaxis while PK and HK plasma levels are low in anaphylaxis, indicating that the contact system is indeed activated. In contrast to other contact system activators, mast cell heparin does not activate the coagulation pathway, possibly because heparin binds to antithrombin III, thereby increasing its inhibitory activity toward thrombin (6).

In 2007, heparin contaminated with synthetic oversulfated chondroitin sulfate-contaminated heparin (OSCS-heparin) was accidentally given to patients in the United States and Germany. This commercially available contaminated heparin resulted in adverse clinical events in the heparin therapy for hundreds of individuals (56). Within several minutes of intravenous infusion of contaminated heparin, there was a drastic reaction in patients causing edema, hypotension, swelling of the larynx and other related symptoms including death (56). The OSCS-contaminated heparin potently activates FXII *via* the kallikrein–kinin system through BK formation in human plasma (57), demonstrating the importance of understanding the mechanisms that induce BK in patients.

Polyphosphate

Polyphosphate (polyP) is a polymer of linear linked phosphate units *via* energy-rich phosphoanhydrous bonds. PolyP is pro-inflammatory and procoagulant and is found in secretory granules of platelets, basophils, and mast cells that resemble acidocalcisomes in prokaryotes (51, 58). Mast cell activation leads to a release of polyP that activates the FXII-driven contact system (51) while FXII- or B2R-deficient mice do not exhibit activated mast cell-induced edema and hypotension (5).

Polyphosphate was first found in prokaryotes and is involved in metabolism, structural behavior and stress responses. The polymer can be from a few up to thousands of residues long (11). In artificial systems, dissolved long-chain polyP (>500 residues) activates FXII more potently than short-chain polyP (<100 residues); however, these long-chain polymers have low solubility under physiological conditions (59). The hypothesis that size determines the activity of polyP for activating FXII has been challenged by the fact that polyP form calcium-rich nanoparticles *in vivo*. Independent of the size of the individual, polyP monomer polyP, packed into particles potently activates FXII (60). PolyP is unstable in plasma (61) and technology to specifically analyze the polymer has been developed (62). Recently, intravital microscopy visualized release of polyP nanoparticles

from platelet dense granules. PolyP nanoparticles accumulate on the procoagulant platelet surface *in vivo*. The polyP particles are retained on the platelet surface where they potentially initiate FXII contact activation (63, 64). FXII activation by exposed procoagulant polyP offers a rationale for the critical role of FXIIa in mediating platelet driven coagulation/clot formation that is well established since decades the field (65–70). In addition to polyP particles, small amounts of short-chain soluble polymers are released into the supernatant from activated platelets (71) and activate an array of procoagulant mechanisms (72). The role of these FXII-independent mechanisms, however, remains enigmatic *in vivo*. PolyP colocalizes with serotonin and calcium in the acidic secretory granules of mast cells (51). Taken together, polyP in mast cells is released in a mechanism similar to that of platelets. These data suggest that the release of heparin coupled with polyP inhibits the procoagulant properties of polyP while retaining the pro-inflammatory capability.

Contact System Cross Talk with the Complement System

An important component of the immune response is the complement system, which is composed of soluble proteins circulating as precursors in the plasma. There are three distinct pathways that can activate the complement system: (1) the classical pathway, (2) the lectin pathway, and (3) the alternative pathway. The classical pathway is activated *via* binding of C1q to antibodies complexed with antigens. In some cases, the interaction of C1q with certain pathogens can lead to a direct surface binding without the presence of antibodies. The C1 complex contains C1q, which is further bound to two molecules each of the zymogens C1r and C1s (73). The mannose-binding lectin (MBL) pathway is initiated when mannan-binding lectin-associated serine proteases (MASP-1 and MASP-2) bind and are activated *via* MBL, ficolins or collectins to carbohydrates on the bacterial cell wall (12). The alternative pathway is initiated *via* spontaneous activation of C3b that leads to binding on the pathogenic surface. The activation of all three pathways is driven by a series of limited-proteolysis reactions that convert the proenzymes to an active enzymes (74) culminating in generation of C3 convertase (73). The convertase cleaves C3 to C3a and C3b and can generate more C3 molecules to amplify production of C3b. C3b is involved in the production of C5 convertase, which functions as an opsonization marker for bacteria to be phagocytosed by macrophages and neutrophils (75). The cleavage of C5 by C5 convertase yields C5a and C5b in a similar fashion as C3. C3a and C5a, known as anaphylatoxins, are pleiotropic inflammatory mediators and proteolytically released from C3 and C5 (74). In host defense responses, the membrane attack complex (MAC) is produced by C5b-mediated formation of C5b-9 complex. This MAC induces lysis of pathogens or cells *via* incorporation into the cell membranes (12).

The complement system has the capacity to trigger anaphylactic shock, mainly *via* C3a and C5a activity. These anaphylatoxins induce degranulation of mast cells, which leads to the release of histamine. In addition, they also increase vascular permeability and induce contraction of smooth muscle cells (76, 77). Complement activation was found to trigger anaphylactic shock

in mice exposed to peanut extract through C3 activation. In accordance with this finding, the authors showed that mice deficient in C3 or its receptor C3aR had almost no response to the peanut extract (78).

There is extensive cross talk between the complement and contact systems at several levels (Figure 2). They share the major endogenous inhibitor, C1INH that inhibits the initial step of both cascades. While C1INH inhibits FXIIa activation of the contact system, all three-activation pathways of the complement are also inhibited by distinct mechanisms. The classical pathway is inhibited by C1INH-mediated inactivation of C1r and C1s (79), two subunits of the C1 complex that is also known to be activated by FXIIa (Figure 2) (80, 81). By covalent binding to MASP-1 and MASP-2, C1INH also inhibits the lectin pathway (82). Finally, the alternative pathway is inhibited by reversible binding of C1INH to C3b (83). Interestingly, *in vitro* activation of FXII by OSCS activates C3 and C5 in human plasma in addition to the kallikrein–kinin system. In FXII-deficient plasma, activation was abrogated with no effect on normal complement activation, an effect that was rescued by addition of purified FXII to FXII-deficient plasma (57). There are multiple inhibitors of the complement system that are expressed on cell surfaces. The inhibitory effect of antithrombin, however, is much enhanced by glycosaminoglycans, such as heparin and heparan sulfate (84, 85). Further interactions between the complement and the kallikrein–kinin system have been discovered. For example, PKa has been found to trigger the generation of C3a fragments in humans (86) and C5a due to limited proteolysis of C5 in rabbits (87). While this last reaction was confirmed using anti-PK IgG

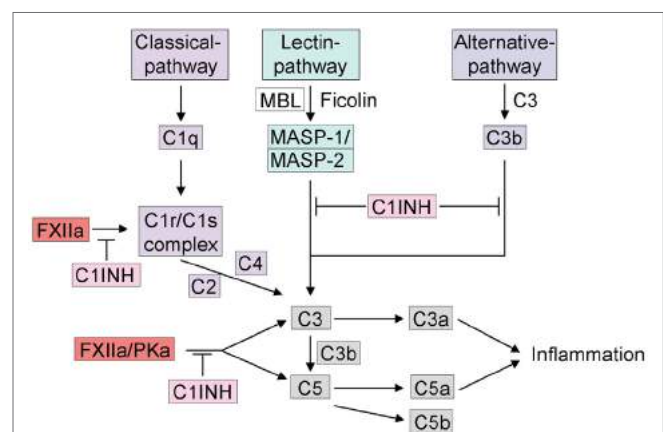


FIGURE 2 | Cross talk between the kallikrein–kinin system and the complement system. The complement system can be activated by three different pathways: (1) C1q initiates the activation of the classical pathway, (2) the mannose-binding lectin (MBL) or ficolins trigger the lectin pathway for glycosylation on the surface of pathogens. Activation of either the classical pathway or MBL generates C3 convertase. (3) If C3 is spontaneously hydrolyzed, the alternative pathway is activated and generates activated C3b. C3 and C5 release C3a and C5a, which can trigger inflammation. The activation of the complement system *via* the kallikrein–kinin system is indicated, mainly FXIIa and plasma kallikrein are involved in activation of C3 and C5. FXIIa can trigger the C1r/C1s complex. C1INH inhibits the complement system in all three different pathways and furthermore *via* the inhibition of the kallikrein–kinin system.

or soybean trypsin inhibitor, this result has yet to be confirmed in humans. The cross talk between complement and the contact system has become increasingly more relevant because many types of molecules are produced that play an important role in pathologies such as angioedema (12) and anaphylaxis.

BK IN ANAPHYLAXIS AND INFLAMMATORY DISEASES

Anaphylaxis

Anaphylaxis can result from serious allergic reactions and immunologic response to allergens and may lead to life-threatening swelling episodes (33). Its onset is in the range of a few minutes if the allergen entered *via* the circulatory system to a couple of hours if the allergen was ingested. Depending on the type of the response, symptoms of anaphylaxis include hypotension, vascular leakage, or even cardiac arrhythmia and bronchial constriction in severe cases (6). There are common triggers for anaphylactic reactions such as food, medications or insect venom with 1–15% of the population being susceptible to anaphylaxis (88).

Recent work from our group has shown that this increased vascular permeability was mediated by heparin-initiated BK formation in mice (5). In this study, it was shown that targeting FXII or B2R abrogated heparin-mediated leukocyte adhesion to the endothelium and inhibited mast cell-triggered hypotension. Ablation of FXII or B2R protected against mast cell-mediated leakage in response to allergens and heparin-induced edema. Furthermore, our group has also demonstrated that deficiency or targeted inhibition of FXII, PK, HK, B2R, but not B1R, resulted in a protective effect against anaphylaxis in an allergen/IgE mice model. In *F12^{-/-}* mice, this protective effect could be abolished by restoration of plasma FXII levels, confirming the involvement of the contact system in this model of anaphylaxis. Analysis of human plasma from anaphylactic patients revealed activation of the contact system. The degree of anaphylaxis associated with levels of mast cell degranulation, heparin levels in the plasma, the amount of contact activation, and subsequent BK formation (6).

Abnormal blood coagulation as a result of IgE-triggered hypersensitivity has been known for years. Activated partial thromboplastin time, a measure of FXIIa-driven coagulation is delayed in patients with anaphylaxis and anaphylactic shock (89, 90). In contrast, the prothrombin time, which utilizes the FXII-independent extrinsic pathway of coagulation, remains unchanged in patients with allergen-mediated anaphylaxis, suggesting that they mediate their effect only *via* the intrinsic coagulation pathway (11). The plasma of IgE/Ag-challenged mice does not clot due to a heparin concentration of >4 µg/ml, which is sufficient for initiation of BK formation (5). Therefore, minute amounts of heparin may produce BK on the mast cells surface.

Hereditary Angioedema

Dysregulation of the contact system leads to HAE, an autosomal dominant disorder that results in recurrent episodes of angioedema of the skin or tissue mucosa. Before the use of prophylactic drugs, laryngeal edema and upper airway obstruction were lethal in up to one-third of patients (91). It is unknown how prevalent

HAE is across the world but current estimates propose as many as 1/10,000–1/150,000 individuals in Europe (92). HAE is caused by either reduced C1INH levels (HAE type I) (93), a defective C1INH protein (HAE type II) (94), or hyperactive FXII (HAE type III). In HAE type III patients, C1INH functions normally and circulates at a normal concentration in plasma. However, a single point mutation in FXII (position 309) leads to enhanced FXIIa activity by a mechanism that recently has been unraveled (95). A defective FXII glycosylation at that single site (Thr309 that is mutated to Arg or Lys) is the underlying cause of excessive FXII activation in HAE type III, suggesting that HAE type III is a disease model for gain of function FXII contact activation (96). Edema in HAE type III is not associated with thrombosis (96), supporting a role of mast cell heparin in activating mutant FXII similar to anaphylactic reactions (6). C1INH deficiency increases the ability of FXIIa to convert PPK to PKa (97), since C1INH inhibits over 90% of plasma FXIIa (94). In murine models, cross-breeding C1INH-null with B2R-null mice completely rescues the leakage phenotype, confirming that BK triggers edema formation (98). During acute swelling attacks, C1INH infusions, B2R antagonists, and PK inhibitors have all been shown to effectively block generation of BK (20).

Inflammatory Diseases

Vasodilation and vascular permeability are two processes are involved in many inflammatory diseases (48), leading to local swelling attacks of the dermis and submucosa (99). As an important regulator of those processes, the contact system has been studied in several inflammatory diseases. In rheumatoid arthritis and irritable bowel diseases, for example, high levels of PKa and BK have been observed. Furthermore, it was shown in rodent rheumatoid arthritis models that inhibition of the contact system interferes with arthritis. Moreover, HK deficiency in rats resulted in less acute and chronic arthritis (100). The precise role of the kinin receptors in rheumatoid arthritis has remained a matter of discussions. While B2R receptor deficiency did not affect arthritis in a mouse model of anti-collagen antibody-induced arthritis, combined deficiency of B2R and B1R attenuated arthritis (101). In support of these observations, there are similar findings in irritable bowel disease. Patients with ulcerative colitis (UC) showed decreased plasma levels of PKa and HK, which indicated proteolysis of these precursors and therefore contact system activation (102). Both kinin receptors are expressed in UC patients in intestinal epithelial cells. During active UC, however, B1R is significantly upregulated and seems to be the main receptor by which BK exerts its deleterious effect in UC (18). Interestingly, in a murine dextran sulfate induced colitis model C3, deficiency conferred protection from disease development indicating a role for the complement system in the disease (103). In the same study, the authors showed that treatment with C1INH would also reduce the severity of the disease in WT mice. A rat enterocolitis model confirmed the clinical observation of decreased plasma PKa and HK and intestinal inflammation could be reduced by treatment with BK antagonists or HK deficiency in a PG-PS model. There are many different animal models that display intestinal inflammation and contact system inflammation but use different triggering agents (56, 104, 105), suggesting that the

contact system is an integral part of the process. Taken together, these data indicate that contact activation can be detected in most inflammatory diseases and is mostly mediated through BK production and its receptors.

Diagnostics Related to the Kallikrein–Kinin System

Diagnostics for anaphylaxis are well described in Montanez et al. (106). In the case of BK-related anaphylaxis, there are some more *in vitro* assays available. But measuring the concentration of BK is very challenging, due to rapid degradation of BK and des-Arg⁹-BK (27 ± 10 and 643 ± 436 s, respectively) (107). Therefore, a number of enzymatic assays have been designed to circumnavigate this issue by measuring more stable BK-related products such as cleaved HK levels (108, 109). Other assays focus on C1INH inhibitory capacities by measuring free C1s activity (110) and C1INH–protease complexes levels (111).

Amidase Activity Assay

This assay measures the activity of free, active C1s amidase (e.g., not bound to C1INH) by the kinetic or endpoint colorimetric assay, using the substrate H-D-Pro-Phe-Arg-pNA (110). It was demonstrated that spontaneous amidase activity was increased in plasma from patients with BK-dependent disorders compared to plasma from normal patients. They confirmed increased BK production by detecting HK cleavage *via* Western blot, which also correlated with increased kininogenase activity (112).

Cleaved Kininogen Assay

A direct indicator of BK release is cleaved HK (25, 113). The reconstitution of liver-synthesized, novel protein is slow. Due to the slow recovery of plasma HK levels, the observed distribution of HK and HK degradation products gives a robust readout of the *in vivo* BK production and allows for the detection of active angioedema (114). The cleaved kininogen assay could be used in injury cases where the role of contact system is developing, such as in transfusion-related acute lung injury and other detrimental blood reactions (115).

Treatments with Drugs against BK Formation

The standard treatment for anaphylaxis is adrenaline, but since mast cell and contact system activation correlate with the severity of the response (116), other drugs inhibiting BK formation could be also considered. For example, specific inhibitors of the kallikrein–kinin system have been shown to be effective at preventing BK-mediated HAE attacks. Some severe side effects can exist, such as a hypersensitivity to the drug that can induce anaphylaxis. The variations in physiological responses demonstrate the need for detailed mechanistic studies of therapeutics that target the contact system. Some of the current therapeutics in clinical trials will be discussed in more detail.

Icatibant

Icatibant (Firazyr[®]; Shire) is a synthetic decapeptide containing five non-proteinogenic amino acids (H-D-Arg-Arg-Pro-Hyp-

Gly-Thi-Ser-D-Tic-Oic-Arg-OH) which resembles the BK-peptide and selectively blocks B2R. In contrast to BK, icatibant has a relatively long half-life (1–2 h) (117). Several *in vitro* and *in vivo* pharmacological assays showed that icatibant binds with a high-affinity to B2R in guinea pig models (118). Furthermore, the anaphylaxis associated BK-induced bronchoconstriction in guinea pig models was inhibited with icatibant (119). Consistent with animal model data, clinical trials showed the efficacy of icatibant and a strong decrease of HAE attacks in treated patients (114, 120). Some side effects were observed in 90% of the patients treated with icatibant, such as temporally local pain, swelling, and erythema at the injection site.

Ecallantide

Ecallantide (Kalbitor[®]; Dyax, USA) is a potent recombinant protein modeled after the human tissue factor pathway inhibitor Kunitz 1 domain that inhibits PK (121). To test the efficacy and safety in acute attacks, there were two double-blind, placebo-controlled studies performed in 160 patients with HAE. The results of these studies were comparable but the measurement of the patients' reported outcomes was different (122, 123). A known risk of ecallantide treatment for acute HAE attacks is hypersensitivity and subsequent anaphylaxis. The clinical relevance and post-marketing surveillance are required to determine the therapeutic and clinical value (121).

C1INH

Some drugs are available to cover the inappropriate function of C1INH or C1INH deficiency. These are plasma-derived (pd)C1INHs [Berinert[®] (CSL Behring), Cetor[®] (Sanquin), and Cinryze[®] (ViroPharma)] or recombinant human (rh)C1INH (Rucostat[®] in Europe, Rhucin[®] in the USA, Pharming Group NV). The pdC1INHs prepared and pasteurized from fractionated plasma. rhC1INH is expressed in the mammary gland of transgenic rabbits. Interestingly, both synthetic proteins produced C1INHs differ in their glycosylation pattern. rhC1INH contains less glycosylation than pdC1INH due to its production in a heterologous system. Because of the differences in glycosylation patterns, the rhC1INH can be cleared within 3 h from the circulation, in comparison to pdC1INH, which takes more than 24 h. It is important to know if patients have a rabbit allergy since this could induce anaphylaxis upon treatment with rhC1INH (121). To confirm the safety of these products, more long-term data are necessary.

Avoralstat

Avoralstat (BCX4161) is developed by BioCryst Pharmaceuticals Ltd. (Durham, NC, USA) and is a small molecule kallikrein inhibitor of oral administration. It is an effective and specific inhibitor of PK, as indicated in preclinical studies. One promising study in phase IIa was performed with statistically significant mean attack reduction for HAE type I and type II (124).

DX-2930

DX-2930 is a recombinant human monoclonal antibody against PK produced by Dyax Corp (Burlington, MA, USA) that was

developed using phage display. DX-2930 acts as a long-acting inhibitor and could be used to prevent HAE attacks (124).

Anti-FXIIa Antibody (3F7)

3F7 is a recombinant, fully humanized antibody (3F7) which neutralizes FXIIa by blocking the protease activity of the catalytic domain (125). 3F7 blocks the intrinsic clotting cascade in human plasma and thrombosis formation in mouse models. Consistent with the selective role of FXII in thrombosis but not in hemostatic mechanisms, 3F7 thromboprotection is similar to that of heparin but there is no change in bleeding. 3F7 interferes with FXII activation in response to an array of contact activators including polyP and heparin (126). In humanized mouse models of HAE type III, 3F7 inhibits FXIIa and as a consequence prevents edema in animal models. Supporting a potential use of 3F7 to treat anaphylaxis and HAE, the addition of the antibody abolished BK formation in patients' plasma of HAE type III (96).

CONCLUSION

The FXII-driven contact system plays a role in anaphylaxis and angioedema *via* its ability to increase inflammation and vessel permeability. During the onset of these pathologies, the mast cell activation releases pro-inflammatory mediators including polyP and heparin that can activate the contact system. This contact system activation triggers the kallikrein-kinin system and the complement pathways that intertwine at many levels, for

example frequently used control mechanisms, cross-activation, and commonly used binding proteins. The abnormal production of BK leads to HAE and also plays a role in anaphylaxis that both can lead to acute, life-threatening attacks of edema. Therefore, it is of interest to study the common pathways between these pathologies. There are several novel drugs emerging to interfere with contact system activation and possibly other pathologies involving HK, BK, and C1INH. Further clinical studies of the contact system are required to better understand the connection between the contact system and inflammatory-related pathologies like HAE and anaphylaxis.

AUTHOR CONTRIBUTIONS

All authors have made significant intellectual contributions of the review. LB, HW and ATL drafted the original manuscript. SRJ and TR critically analyzed and gave suggestions for the concept and revision that improved content of the text and figures. All authors approved the final version of the manuscript.

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***IN VIVO* ROLES OF FACTOR XII**

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Abstract

Coagulation factor XII (FXII, Hageman Factor, EC=3.4.21.38) is the zymogen of the serine protease, factor XIIa (FXIIa). FXII is converted to FXIIa through autoactivation induced by "contact" to charged surfaces. FXIIa is of crucial importance for fibrin formation *in vitro*, but deficiency in the protease is not associated with excessive bleeding. For decades, FXII was considered to have no function for coagulation *in vivo*. Our laboratory developed the first murine knockout model of FXII. Consistent with their human counterparts, FXII^{-/-} mice have a normal hemostatic capacity. However, thrombus formation in FXII^{-/-} mice is largely defective and the animals are protected from experimental cerebral ischemia and pulmonary embolism. This murine model has created new interest in FXII because it raises the possibility for safe anticoagulation, which targets thrombosis without influence on hemostasis. We recently have identified platelet polyphosphate (an inorganic polymer) and mast cell heparin as *in vivo* FXII activators with implications on the initiation of thrombosis and edema during hypersensitivity reactions. Independent of its protease activity, FXII exerts mitogenic activity with implications for angiogenesis. The goal of this review is to summarize the *in vivo* functions of FXII, with special focus to its functions in thrombosis and vascular biology.

The factor XII-driven plasma contact system

Fibrin formation may be initiated by two distinct pathways, either triggered by exposure of blood to a damaged vessel wall (extrinsic) or to blood-borne (intrinsic) factors. The intrinsic pathway of coagulation is initiated by factor XII (FXII, Hageman factor), in a reaction involving high molecular weight kininogen (HK) and plasma kallikrein (PK). These factors are collectively referred to as the plasma contact system¹⁻⁶. Contact with negatively charged surfaces induces a conformational change in zymogen FXII resulting in a small amount of active FXII (FXIIa)⁷. FXIIa cleaves PK to generate active plasma kallikrein, which in turn reciprocally activates FXII⁸. FXIIa triggers fibrin formation through activation of factor XI (FXI) and also liberates the inflammatory mediator bradykinin (BK) from HK through cleavage by PK³. Binding of BK to the kinin B2 receptor (B2R) activates pro-inflammatory signaling pathways that dilate vessels, induce chemotaxis of neutrophils and increase vascular permeability⁹. Thus, the FXIIa-driven contact system has proinflammatory and procoagulant activities via the kallikrein kinin-system (KKS) and the intrinsic coagulation pathway, respectively (**Figure 1**). The serpin C1 esterase inhibitor (C1INH) is the major plasma inhibitor of FXIIa and plasma kallikrein and controls proteolytic activity of the contact system¹⁰. Besides C1INH, antithrombin III (ATIII) and PAI-1 also have FXIIa-blocking activity¹¹. *In vitro*, FXIIa triggers activation of the classical complement pathway and initiates the fibrinolytic system via PK-mediated urokinase activation (**5**). Whether FXIIa has the capacity to trigger activation of the complement and fibrinolytic systems *in vivo* remains uncertain.

Factor XII is dispensable for hemostasis

The enzymology of the FXII-driven contact system *in vitro* is well understood. However, its *in vivo* contributions are just beginning to emerge. Factor XII-contact activation *in vitro* provides the mechanistic basis for one of the most commonly used diagnostic coagulation tests, the activated partial thromboplastin time (aPTT) which is extensively used in clinical practice (>500 million assays/per year worldwide) for preoperative screening, the diagnostics of thrombosis-related autoimmune diseases and monitoring of anticoagulation therapy. Despite its contribution to fibrin formation *in vitro*, FXII-initiated coagulation *in vivo* was not considered to be of significance. This premise is based on the observation that FXII deficient individuals and animals do not exhibit a clinically relevant bleeding phenotype: individuals with partial or severe FXII deficiency do not bleed excessively from sites of injury despite a marked prolongation of the aPTT^{12,13}. This apparent discrepancy between the essential role of FXII for contact-driven fibrin formation in test tubes that eventually lacks a correlation *in vivo* puzzled investigators for decades. Similar to FXII deficiency, individuals lacking the contact proteins PK or HK, do not have impaired hemostasis and are commonly diagnosed during routine coagulation screening when a prolonged aPTT is discovered. In sharp contrast, patients deficient in FXI have a mild trauma-induced bleeding disorder (sometimes called "Hemophilia C") that is mostly restricted to tissues with high fibrinolytic activity. Severe FXI deficiency is a rare inherited abnormality in the general population (seen with a one in a million people prevalence), but is more common in specific populations such as Ashkenazi Jews (one in 450)¹⁴.

This lack of a bleeding tendency observed with FXII deficiency is in sharp contrast to deficiencies of other components of the coagulation cascade such as factor VII (FVII), tissue factor (TF) and factors VIII or IX (causing the bleeding disorders Hemophilia A and B, respectively) has led to the reasonable hypothesis that fibrin formation *in vivo* is initiated largely, if not exclusively, through the extrinsic pathway of coagulation. Notably, complete ablation of TF expression causes embryolethal intrauterine bleeding in mice. Human TF deficiency has not been described, indicating that TF is essential for development and/or survival¹⁵. The dominant role of VIIa/TF-driven coagulation for hemostasis is supported by the "revised model of coagulation" which shows that FXI, the substrate of FXIIa during contact-initiated clotting, can also be activated by thrombin, independently of FXII¹⁶. Although FXII is dispensable for fibrin formation in hemostatic reactions severe deficiencies (<10% of

normal plasma levels) in the coagulation protein are invariably rare¹⁷, suggesting that FXII has critical roles that warrant future studies. Interestingly, FXII is a rather modern protein in terms of evolution. Copies of the FXII gene are absent in infra-mammalian vertebrates such as birds or fish¹⁸, which have a closed circulatory system, supporting the notion that FXII is not required to seal vessel injuries.

Factor XII has an essential contribution to thrombosis

Injury to a blood vessel triggers activation of blood platelets and the plasma coagulation system, leading to formation of a blood clot consisting of platelets and fibrin. To investigate the role of FXII *in vivo*, FXII^{-/-} mice were generated and characterized in experimental thrombosis models^{19,20}. Identical to FXII deficient humans, FXII^{-/-} mice have a normal hemostatic capacity as assessed by a tail-bleeding assay and could undergo surgical procedures without excessive bleeding²¹. Completely unexpected, intravital fluorescence microscopy and blood flow measurements in three distinct arterial beds revealed a severe defect in thrombus formation in FXII-deficient mice when challenged by chemical (FeCl₃), mechanical and Rose Bengal/laser of vascular injury models^{21,22}. The thrombo-protective phenotype of FXII^{-/-} mice in combination with their normal hemostatic capacity, challenges the previously accepted notion of a “coagulation balance”. Although FXII has a crucial role in fibrin formation during “pathological” thrombosis, it does not contribute to “physiological” hemostatic fibrin formation at sites of vessel injury. Reconstitution of FXII^{-/-} mice with human FXII normalized both their prolonged aPTT and the defective thrombotic response. The reconstitution experiments indicate that FXII operates similarly in mice and humans. Indeed, the contact system is highly conserved among mammalian species²³. Thrombus formation in FXII heterozygous mice (having 50% of normal FXII plasma levels) was similar to wild-type animals (having 100%), indicating that half of normal plasma concentration is sufficient for vessel-occlusive clot formation. Modulating FXII expression levels by antisense nucleotides (ASO) showed that reduction of >75% FXII antigen plasma levels is required to produce thrombo-protection in a model of stasis-induced venous thrombosis²⁴.

The decisive role of FXII for experimental models of thrombus formation extends to thromboembolic disease: FXII-deficient mice are protected from cerebral ischemia in an experimental stroke model²⁵. These thrombo-protective effects are conferred by an impaired FXII-dependent fibrin formation in the microvasculature of the ischemic tissue. Indeed, mice lacking FXI are similarly protected from vessel-occlusive fibrin formation, suggesting that FXII impacts on pathologic clotting predominantly via the intrinsic pathway^{26,27}. This notion is supported by observations that in models of lethal pulmonary embolism, survival of mice with combined deficiency in FXII and FXI (FXII^{-/-}/FXI^{-/-}) is similar to animals with deficiency in FXII or FXI alone²⁸. Given the results from mouse models the concept that pathological thrombus formation represents a disequilibrium between the enzymatic reactions that produce a clot at a site of vascular injury, likely needs revision. Indeed, pharmacological inhibition of FXII activation may provide an attractive approach for the management of thrombotic disease. For instance, the peptide-based inhibitor PCK (Phe-Pro-Arg-chloromethylketone) provides protection from cerebral ischemia in wild type mice without causing excessive bleeding at a site of surgical injury²⁵. Consistently, a recombinant infestin-4-based inhibitor that specifically targets FXIIa, provides protection from cerebral ischemia in experimental stroke models, albeit it did not reduce the hemostatic capacity of inhibitor-treated mice²⁹. Cumulatively, the findings in animal models raise the exciting possibility that targeting FXII may offer a safe and powerful strategy for prevention or treatment of pathological thrombosis without the associated risk of hemorrhage that accompanies currently used anticoagulants^{30,31}. However, the detailed association of FXII deficiency and risk for thrombosis is probably more complex. The instability of arterial thrombi observed in FXII-deficient mice²¹, defective polyphosphate-driven fibrin formation in FXII-deficient human and mouse plasma^{32,28}, as well as case reports of pulmonary emboli seen in FXII-deficient individuals³³ raises an intriguing hypothesis: FXII

deficiency may protect from arterial thrombosis as high shear stress interferes with propagation of unstable thrombi into the vessel lumen. In contrast, larger thrombi may develop in veins under low shear conditions. However, reduced stability of these thrombi increases their risk to embolize.

Analysis of a large registry involving \approx 9,000 patients from Austria supports this dual function of FXII in thrombosis. There is an inverted U-shaped association of FXII plasma levels and overall mortality and mortality from cardiovascular disease³⁴. Clearly more epidemiological studies are necessary to define the precise consequences of FXII deficiency in arterial and veno-occlusive disease.

Mechanisms of contact activation *in vivo*

FXII activation by non-physiologic negatively charged surfaces such as glass is well-established. Various materials have been identified that have the potency of triggering FXII activation (reviewed in³⁵). Amongst the best-characterized agents that initiate FXII-dependent clotting are the white clay minerals kaolin and celite (silica-rich compounds), which are commonly employed in aPTT coagulation assays. Moreover, kaolin is also used as a hemostatic agent *in vivo* to terminate blood loss after injury in combat victims. Although the idea of employing FXII activators as hemostatic agents for sealing injuries is attractive, exposure of flowing blood to kaolin triggers thromboembolic events and produces excessive heat at the wound site, resulting in additional burn injury and subsequent tissue necrosis³⁶.

Initiated by the discovery that FXII is crucial for occlusive thrombus formation²¹, there was a renewed search for candidate endogenous activators of this protease. RNA that is released from injured cells was shown to activate FXII in plasma. Administration of RNase (an enzyme that degrades RNA) to mice prior to experimental challenge has thrombo-protective effects in a FeCl₃-induced carotid artery injury model in mice³⁷. RNA is released from various types of disintegrating cells. The regulatory mechanisms that allow RNA-driven FXII in thrombosis but not at injury sites at the vessel wall remain to be identified. However, oligonucleotides such as RNA and nucleotide-associated proteins such as histones³⁸ contribute to procoagulant states associated with infections and sepsis. FXII binding to collagen is known to enhance coagulation *in vitro*³⁹. This interaction is dependent on repetitive negative charge exposed by collagen fibrils⁴⁰. When added to platelet-rich plasma, equine type I collagen (Horn) promotes thrombus formation under flow in an FXII-dependent manner⁴¹. In contrast, FXII activity does not contribute to thrombus formation under flow over atherosclerotic plaque material⁴². The presence of platelets dramatically enhanced the FXII-dependent procoagulant capacity of collagen⁴¹, suggesting that it also promotes FXII activation indirectly through release of platelet-derived activators²⁸. In line with this observation, it has been found that activated platelets support FXII activation in a mechanism that is dependent on integrin α IIb- β 3 signaling⁴³. Moreover, activated platelets support activation of the kallikrein-kinin system⁴⁴, as well as fibrin formation in an FXII-dependent manner⁴⁵, even in the presence of thrombin- and TF-inhibition^{44,46}. Taken together, these studies link platelet activation and FXII-driven fibrin formation and raise the question: how do procoagulant platelets activate FXII?

Polyphosphate drives FXII activation during thrombosis

Platelet polyphosphate (polyP) is an inorganic non-branched polymer that is released upon platelet activation. Polyphosphate was originally identified in non-mammalian cells and is highly enriched in platelet dense granules⁴⁷. Synthetic polyP is used as water softener in technical processes and has been shown to modulate plasma coagulation by multiple mechanisms involving FXII²⁸, FXI⁴⁸, the fibrinolytic system⁴⁹, factor V⁵⁰, and through alterations of the fibrin structure⁴⁹ in *ex vivo* experiments (recently reviewed in⁵¹). Platelet polyP is a non-branched polymer of 60-100 orthophosphate residues that directly binds to FXII (and HK) with high affinity²⁸. Polyphosphate potently initiates coagulation in human plasma and in mice in an FXII-dependent manner. Targeting polyP with phosphatase (an

enzyme that cleaves phosphoester bonds and degrades polyP) largely abolishes the formation of occlusive thrombus formation *in vivo*. Conversely, synthetic polyP restored defective clot formation in platelet-rich plasma from Hermansky-Pudlak patients, who (amongst others) lack the storage pool for platelet polyP²⁸ suggesting that polyP may be utilized as a hemostatic agent. Together, these findings identify polyP as the endogenous platelet-derived FXII activator *in vivo*⁵². While triggering FXII-dependent clotting, polyP/FXII also activates the kallikrein-kinin system leading to plasma kallikrein-mediated BK formation²⁸. Cumulatively, the identification of polyP as a platelet-derived procoagulant agent provides a long-sought link between primary and secondary hemostasis⁴¹ and may represent a new paradigm for the treatment of thromboembolic and inflammatory diseases⁵³.

Tissue factor exposed at subendothelial sites of vessel injury initiates fibrin production. However, tissue factor pathway inhibitor (TFPI) that is released from platelets and endothelial cells rapidly terminates TF-activity (**Figure 2A**). Additionally, adherent platelets shield the transmembrane TF protein at the injury site. There is a requirement to stimulate ongoing fibrin production within the three-dimensionally developing thrombus. PolyP released by procoagulant platelets triggers FXII activation and drives fibrin formation via the intrinsic coagulation pathway (**Figure 2B**). Deficiency in platelet polyP-stimulated FXII-driven fibrin formation, interferes with fibrin fiber thickness^{32,49} resulting in mechanical clot instability²⁸ and likely increases the risk of thrombus embolization. Blood-borne TF on circulating monocytes, neutrophils⁵⁴ and microparticles⁵⁵ that are incorporated into the developing thrombus in a P-selectin-dependent manner⁵⁶ provides an alternative FXII-independent mechanism for triggering fibrin production. The relative importance of TF/FVIIa-versus polyP/FXII-initiated fibrin formation and their contribution to thrombosis has not been directly compared and may differ depending on vascular beds and types of vessel injury.

Factor XII zymogen as a growth factor

Although most investigations focus on FXII as a serine protease, zymogen FXII has mitogenic activities on cultured cells independent of its enzymatic activity. FXII's heavy chain consists of a fibronectin type II and I domain, two EGF-like domains, a kringle domain and a proline-rich region adjacent to its catalytic domain⁵⁷. The EGF-like domains in FXII, like those in single chain urokinase and tissue-type plasminogen activator share structural similarities with homologous domains in hepatocyte growth factor (HGF)⁵⁸. Both FXII and FXIIa are mitogenic on cultured HepG2 cells and phosphorylate mitogen-activated protein kinase (MAPK) in HepG2 and vascular smooth muscle cells⁵⁹. FXII zymogen stimulates 5-bromo-2'-deoxy-uridine incorporation through the ERK1/2 and Akt S473 phosphorylation pathway in endothelial cells in a uPAR-dependent manner^{60,61}. The domain 2 region of uPAR where FXII binds is a regulatory site of the uPAR interactome (55a). In addition to FXII, ScuPA, HK/HKa, vitronectin, and PAI-1/uPA, bind to this region to overlapping, but not identical sequences, on uPAR's domain 2 as well. Consistently, FXII stimulates aortic sprouts from wild type mice but not from uPAR^{-/-} aorta and initiates new vessel formation into matrigel plugs in wild type but not in uPAR deficient animals. Vice versa, there is less number of vessels in skin punch biopsies in a FXII^{-/-} mouse model both constitutively and in a wound healing model⁶². In contrast, in another FXII^{-/-} mouse strain there are no obvious vascular abnormalities in histological analyses¹⁹.

These combined data indicate that zymogen FXII, like single chain urokinase, functions as a growth factor that mediates cell signaling leading to proliferation and stimulating angiogenesis, indicating a new *in vivo* activity for zymogen XII in post-natal angiogenesis after ischemia, inflammation and injury (**Figure 3**).

Role of Factor XII in human thrombotic disease

A severe human FXII deficiency is rare and, as a consequence, there is a lack of epidemiological studies that systematically compare the incidence or severity of thromboembolic events (i.e. stroke, myocardial infarction, pulmonary embolism) in humans

with low FXII plasma levels. Based on the animal studies, one would expect that FXII deficiency would protect from thrombosis in patients. In contrast, there is a long history of anecdotal reports suggesting that FXII deficiency may actually predispose to thrombosis^{63,64}. FXII deficiency associated with increased risk of thrombosis dates back to the death of the index patient with FXII deficiency John Hageman, who deceased from a pulmonary embolism⁶⁵. In brief, the railroad worker John Hageman fell from a boxcar and fractured his left hemipelvis. He was kept at bed rest for a week and was subsequently allowed to walk on crutches. A few days later he was found gasping for breath, pulseless and passed away within minutes. Autopsy showed massive thrombi occluding his left and right pulmonary arteries and several large thrombi were recovered, which presumably had originated from his lower extremity veins⁶⁵. It is difficult to link FXII-deficiency to this lethal pulmonary embolism event, since the trauma and subsequent immobilization represent established FXII-independent risk factors for venous thrombosis. Indeed, careful re-analysis has identified other risk factors in FXII-deficient patients with thrombosis arguing against FXII deficiency as an independent prothrombotic risk factor⁶⁶. Consistently, larger epidemiological studies in the Netherlands and Switzerland did not find a correlation between FXII deficiency and increased thrombotic risk. However, none of these studies had analyzed whether FXII deficiency conferred thrombo-protection^{67,68}. In contrast, recent clinical studies from Israel have analyzed the incidence of ischemic stroke and deep vein thrombosis in humans with severe deficiency of FXI (the direct substrate of FXIIa in the intrinsic coagulation pathway). Similarly to FXI-null mice, FXI-deficient humans are largely protected from cerebral ischemia⁶⁹ and venous thrombosis⁷⁰, supporting the decisive role of the intrinsic coagulation pathway for thrombosis in humans.

Hereditary angioedema

FXII has the capacity of activating the classical complement pathway in plasma⁷¹. A simultaneous activation of the contact and complement system often occurs in pathological conditions. Hereditary angioedema (HAE [MIM #106100]) is a life-threatening tissue swelling disorder that develops in individuals, who are quantitatively or qualitatively deficient in C1-esterase inhibitor (C1INH; HAE type I and II, respectively). C1INH deficiencies facilitate excessive activation of the FXII-driven complement and contact system cascades and the development of edema in HAE type I and II patients⁷². In addition to these two well-known HAE types, a third variant exists that almost exclusively affects women. HAE type III patients have normal levels of fully functional C1INH but suffer from angioedema nonetheless⁷³. Clinically, all types of HAE are characterized by recurrent episodes of acute swelling involving the skin, oropharyngeal, laryngeal or gastrointestinal mucosa. The pathophysiology of the observed increased vascular permeability in HAE has remained controversial. Elegant studies with genetically modified mice demonstrated that edema formation in C1INH-dependent HAE forms is due to pathological contact system activation⁷⁴. Genetic ablation of C1INH expression results in excessive BK production and excess BK signaling which increases vascular permeability in humans⁷⁵ and mice⁷⁴. In contrast, in combined C1INH and bradykinin B2 receptor gene-deficient mice vascular leak is normal. Comprehensive studies have identified BK as the principal mediator of vascular leakage in HAE-related swelling attacks in patients⁷⁶. Hence, HAE types I and II are treated by infusion of C1INH⁷⁷ or BK B2-receptor antagonists (Icatibant)⁷⁸. Alternatively, recombinant plasma kallikrein inhibitors (Ecallantide) may be used to interfere with acute swelling episodes in HAE patients⁷⁹. In contrast to C1INH-dependent forms of HAE, the disease mechanism of HAE type III was enigmatic. Using genome-wide linkage analyses in affected families, HAE type III was shown to be an autosomal dominant disease associated with a single missense mutation (c.1032CrA) in the gene of FXII⁸⁰. Consecutive independent studies involving other families found HAE type III to be associated with a different mutation affecting the same nucleotide of the FXII gene, c.1032CrG⁸¹. Both point mutations translate into amino acid exchanges Thr328Lys and Thr328Arg (numbering includes the signal peptide), respectively, on the protein level. The aPTT assay yields normal values in all types of HAE patients and fails to

detect affected individuals. Factor XII plasma levels in HAE type III patients are in the normal range⁸⁰, suggesting that a yet unknown mechanism triggers edema predominantly in women. HAE patients experience recurrent attacks of swelling but the stimuli that trigger these periodic episodes of excessive vascular leakage are poorly defined⁷².

Mast cell-mediated activation of Factor XII

Until recently it was believed that mast cell-mediated vascular leakage is predominantly, if not exclusively, mediated by the release of histamine and targeting histamine signaling is widely used therapeutically to treat edema formation associated with aberrant mast cell activity⁸². In allergic disease, BK is generated and contributes to increased vascular permeability^{78,83,84}. Mast cells are highly effective sentinel cells that are found close to blood vessels and are especially common at sites of potential infection⁸⁵. A hallmark of mast cell activity in host defense and allergic reactions is increased vascular permeability. In addition to histamine, mast cell secretory granules also contain highly sulfated polysaccharides with heparin as a major constituent. This glycosaminoglycan is synthesized exclusively by mast cells⁸⁶ and has been identified as a FXII contact activator *in vitro*⁸⁷. Heparin released from allergen-activated mast cells initiates formation of BK in a FXII-dependent manner⁸⁸. Minute amounts of heparin ($\geq 4\mu\text{g/mL}$) are sufficient to activate FXII. FXI is not activated under these circumstances suggesting the presence of a regulatory mechanism for prekallikrein-directed activity of FXIIa. Heparin also protects FXIIa from inhibition by C1INH⁸⁹.

Intravital confocal laser scanning microscopy and tracer extravasation experiments identified BK as the active mediator for increasing leakage in heparin-driven edema in the skin⁸⁸. From mast cell-mediated hypersensitivity reactions in mouse models, it was estimated that heparin-driven bradykinin formation accounts for a significant portion (about 50%) of total mast-cell evoked increase in vascular permeability. Consistently, small molecule inhibitors of FXIIa or kinin B2 receptors both interfere with experimental mast cell-triggered leakage. Based on these experimental findings, targeting heparin-initiated BK formation may represent a promising strategy to counteract aberrant mast cell activation in a broad variety of diseases.

Contaminated heparin

The identification of mast cell heparin as an endogenous FXII contact activator in hypersensitivity reactions is reminiscent of reports that had associated therapeutic heparin infusion and contact system activation in a series of life threatening complications. For decades, heparin has been widely used as an anticoagulant drug. This polysaccharide prevents the formation and extension of blood clots in the circulatory system by increasing AT III activity. Starting November 2007, there was a dramatic increase in heparin-induced adverse reactions in the United States and Germany, such as lethal acute hypersensitivity reactions in patients intravenously receiving commercial heparin of specific lots from a single manufacturer (http://www.fda.gov/cder/drug/infopage/heparin/adverse_events.htm). Conservatively, more than 150 patients died from anaphylactic hypotension associated with intravenous heparin treatment. Comprehensive analyses identified a non-natural contaminant occurring in suspect preparations of heparin that was characterized as over-sulfated chondroitin sulfate (OSCS)⁹⁰. OSCS-contaminated heparin has a greatly increased potency for activating FXII and triggering PK-mediated BK formation in human plasma and in a model of experimental hypotonic shock *in vivo*⁹¹. These catastrophic reactions in patients are reminiscent of experimental shock models induced by dextran sulfate (DXS)-stimulated BK-formation in pigs. Infusion of high-molecular weight DXS (500 kDa) induced transient systemic hypotension⁷ and Icatibant (an antagonist of the kinin B2 receptor) blocked this effect on blood pressure⁹². The FXII-activating property seems to be dependent on negative charge density of the polysaccharide rather than on a defined structure. Indeed, potency of FXII-driven contact activation in a reconstituted system decreased from dextran sulfate and OSCS (with an average 4 sulfate residues per disaccharide)^{7,93}, to mast cell heparin (with an average of ≈ 2.7 sulfate residues per disaccharide)⁸⁷, whereas heparan sulfate (with an

average of ≈ 1 sulfate residue per disaccharide) was inactive. The potency to activate the plasma contact system also greatly varies among diverse heparin preparations⁹⁴ reflecting differences in purification procedures, sources of the polysaccharides and experimental settings. Of note, although intravenous heparin infusion may trigger BK generation, infusion of the polysaccharide even at high concentrations in a bolus normally does not induce hypotension nor cause edema. BK that is generated in venous vessels is rapidly and almost completely degraded by ACE and other kininases that are abundantly expressed in lung microvessels before reaching precapillary vascular beds, which regulate blood pressure.

Differential activities of Factor XII

Dextran sulfate (DXS) is a polysulfated polysaccharide of linked glucose moiety. DXS-mediated FXII activation is critically dependent on the chain-length and degree of sulfation of the polyanion⁷. High molecular weight DXS (500 kDa) is a potent stimulator of FXII activation, whereas shorter DXS polymers fail to independently activate FXII but do support cleavage of FXII by plasma kallikrein⁹⁵. Although long-chain DXS induces BK-mediated hypotension *in vivo*⁹², it does not trigger intravascular coagulation. This indicates that some FXII-activators do not have the capacity of triggering coagulation and reveal selectivity in the responses to FXII contact-activating surfaces. Indeed, several FXII contact activators initiate unilateral activation of the kallikrein-kinin system. For instance, other polysaccharides besides DXS such as OCSC or heparin specifically initiate BK formation without triggering a procoagulant activity. Misfolded protein aggregates, the toxic protein species amongst others found in the cerebrospinal fluid of Alzheimer's disease patients and the plasma of patients suffering from amyloidosis trigger FXII activation⁹⁶. These hazardous protein aggregates specifically initiate BK formation via activation of the kallikrein kinin-system but do *not* trigger activation of the intrinsic pathway of coagulation. The mechanism for selective activation of PK without activation of homologous FXI⁹⁷ is not entirely clear but might reflect higher plasma concentrations of PK as of FXI. Furthermore, increased affinity of heparin-dependent plasma inhibitors such as antithrombin III (AT III) for activated FXI *versus* PK might direct FXII-activation driven by charged polysaccharides to BK formation⁹¹. Notably, both PK and FXI are surface-bound via HK and share a conserved HK-binding site⁹⁷⁻⁹⁹. Additionally, surface characteristics of the contact activator might be decisive for PK and/or FXI activation. FXII binds differently to negatively charged surfaces such as polyP as compared to its binding to misfolded protein aggregates. Anionic surface binding is thought to be mediated through the type II fibronectin domain, the 2nd EGF domain and kringle domain^{100,101}, whereas interaction with misfolded protein aggregates is mediated by the fibronectin type I domain¹⁰². Differences in FXII binding may also modulate the conversion of α -FXIIa to β -FXIIa by kallikrein activity and shift FXII activity towards kinin formation^{103,104}.

Future perspectives

The discovery that pathological thrombus formation in FXII^{-/-} mice is largely defective in models for experimental arterial thrombosis and ischemic stroke has created a new interest in this protein, especially since it raises the possibility of treating thrombosis without compromising hemostasis. Recently, several *in vivo* contact activators of FXII have been identified including platelet polyphosphate, over-sulfated chondroitin sulfate, nucleotides, misfolded protein aggregates and mast cell heparin. These various activators may be involved in the development of thrombotic and/or inflammatory diseases. Additionally, investigations on the growth factor function of FXII have only begun. Cumulatively, these discoveries may help elucidate the physiologic function(s) of FXII *in vivo*, which has remained persistently mysterious since the protein was discovered over 50 years ago. Further investigations will expectedly reveal novel roles of FXII through which certain disease states such as thrombosis, inflammation and infections can be therapeutically modified.

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Figure Legend

Figure 1: The factor XII (FXII)-driven contact system. Contact with negatively charged surfaces activates coagulation factor XII (FXII) on endothelial cells, leukocytes, bacteria, and thrombocytes and initiates pro-coagulant and pro-inflammatory proteolytic reactions. Activated FXII triggers fibrin formation through the factor XI (FXI)-mediated intrinsic pathway of coagulation. Simultaneously, activation of prekallikrein by FXIIa leads to generation of the vasoactive peptide bradykinin (BK) by plasma kallikrein (PK)-mediated cleavage of high molecular weight kininogen (HK).

Figure 2. The role of polyphosphate/FXII in thrombosis. A) Initially, the tissue factor (TF)/FVIIa driven “extrinsic” coagulation pathway triggers fibrin formation at sites of injury. FXII has no function during this stage. Tissue factor pathway inhibitor (TFPI) is released from endothelial cells and adherent platelets and blocks TF activity. B) In the developing thrombus, activated platelet-released polyP triggers fibrin production via activation of FXII that drives the “intrinsic” coagulation cascade. PolyP/FXII-driven fibrin formation operates distant from the injured vessel wall and hence, does not contribute to hemostasis.

Figure 3. Model of zymogen FXII signaling pathway. FXII binds to domain 2 of uPAR and induces uPAR to communicate intracellularly through β_1 integrins. Monoclonal antibody 6S6 to β_1 integrin blocks this pathway. Cell stimulation through uPAR and integrin requires an interaction with one or more of the ErbB receptor kinases since the tyrosine inhibitors AG1478 or PP3 block FXII signaling. The MEK inhibitor PD98059 blocks FXII-induced ERK1/2 phosphorylation. LY294002, a PI3 kinase inhibitor, blocks FXII-induced Akt phosphorylation. Cross-talk between pERK1/2 and pAkt systems also occurs. Cleaved forms of HK (HKa) block binding of FXII to endothelial cells. Inhibition of any step of the FXII signaling pathways blocks cell proliferation and angiogenesis in HUVEC and aortic segments, respectively (modified from⁵⁶).

Figure 1

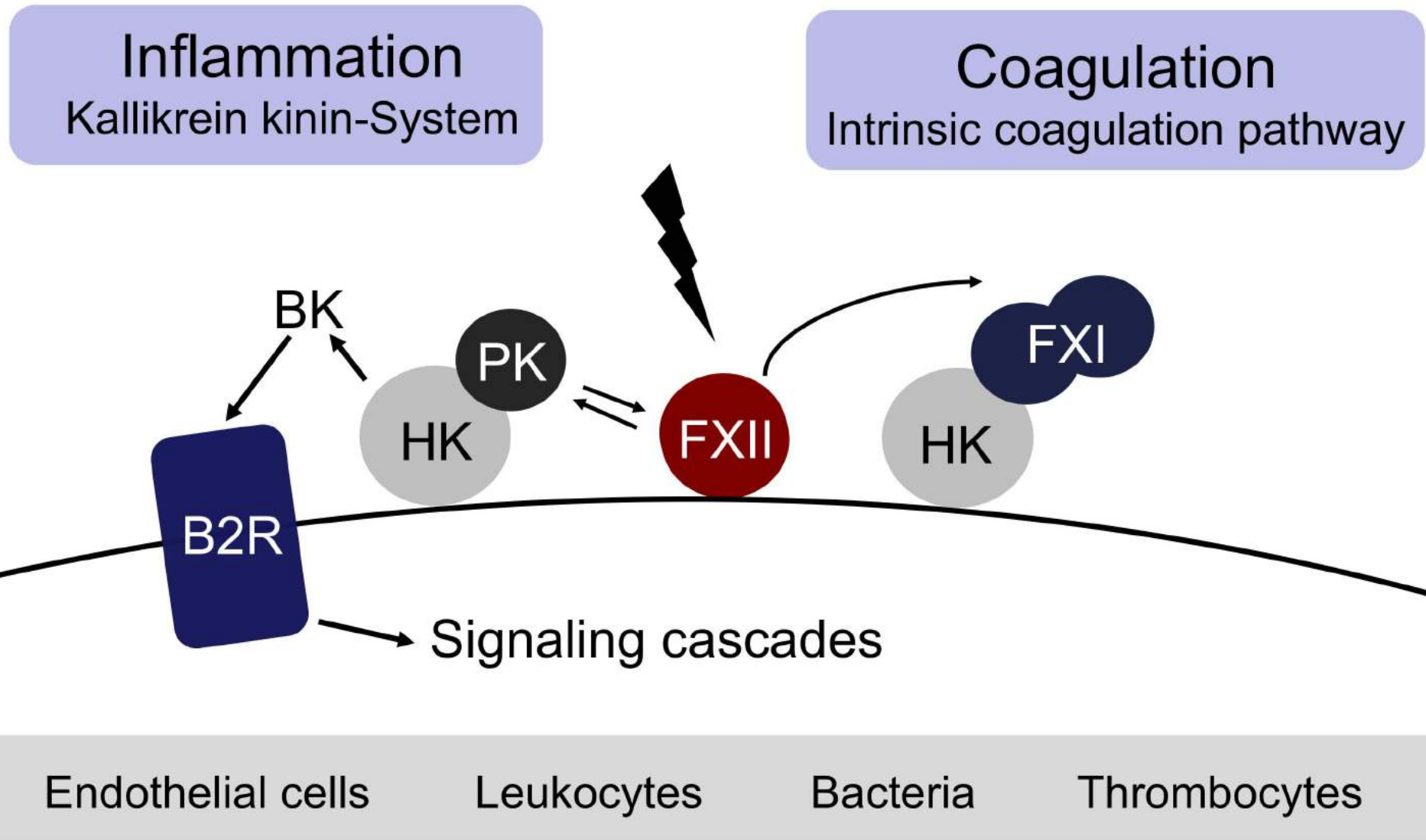
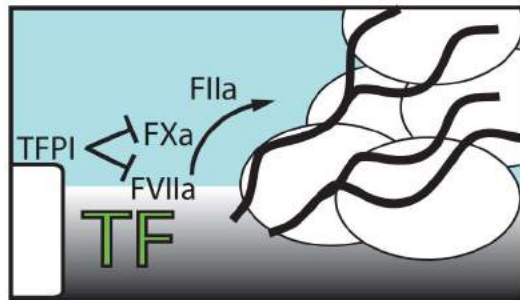
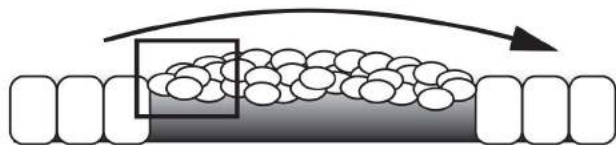


Figure 2

A



B

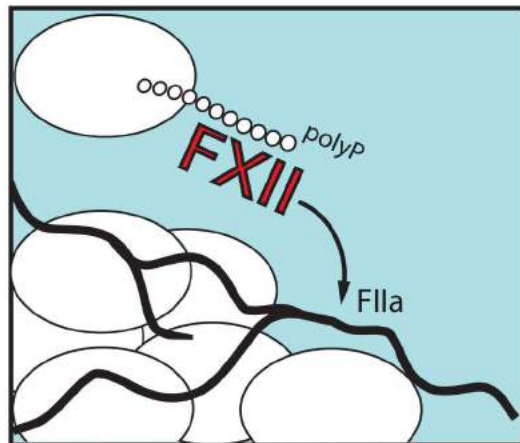
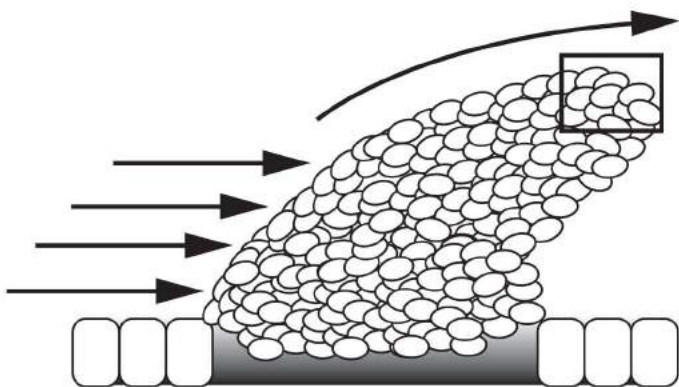


Figure 3

FXII

