Defective thrombus formation in mice lacking coagulation factor XII

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Blood coagulation is thought to be initiated by plasma protease factor VIIa in complex with the membrane protein tissue factor. In contrast, coagulation factor XII (FXII)–mediated fibrin formation is not believed to play an important role for coagulation in vivo. We used FXII-deficient mice to study the contributions of FXII to thrombus formation in vivo. Intravital fluorescence microscopy and blood flow measurements in three distinct arterial beds revealed a severe defect in the formation and stabilization of platelet-rich occlusive thrombi. Although FXII-deficient mice do not experience spontaneous or excessive injury-related bleeding, they are protected against collagen- and epinephrine-induced thromboembolism. Infusion of human FXII into FXII-null mice restored injury-induced thrombus formation. These unexpected findings change the long-standing concept that the FXII-induced intrinsic coagulation pathway is not important for clotting in vivo. The results establish FXII as essential for thrombus formation, and identify FXII as a novel target for antithrombotic therapy.

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Abbreviations used: aPTT, activated partial thromboplastin time; FXII, coagulation factor XII; PFP, platelet-free plasma; PRP, platelet-rich plasma; PS, phosphatidylserine; TF, tissue factor.

Injury to a blood vessel triggers activation of blood platelets and the plasma coagulation system, leading to formation of a blood clot containing platelets and fibrin. Although clot formation is critical for limiting posttraumatic blood loss, this process may also occlude diseased vessels leading to diseases such as myocardial infarction and stroke that are still leading causes of death in industrialized nations. In the waterfall or cascade models of fibrin clot formation, proposed in 1964 by Macfarlane and by Davie and Ratnoff, respectively (1, 2), plasma coagulation proceeds through a series of sequential activations of plasma serine proteases culminating in the generation of thrombin, which converts plasma fibrinogen to fibrin. Thrombin also activates platelets, and activated platelets, in turn, facilitate thrombin generation by exposing procoagulant phosphatidylserine (PS) on the outer surface of their membranes (for review see reference 3).

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The original cascade/waterfall models described two distinct pathways for initiating coagulation, triggered by either vessel wall (extrinsic) or bloodborne (intrinsic) factors, that converge on a common pathway leading to thrombin generation and fibrin formation. Initiation of coagulation through the extrinsic pathway occurs when the plasma protease factor VIIa comes into contact with the integral membrane protein tissue factor (TF), which is present in subendothelial layers of the vessel but not on the vessel's luminal surface. TF on circulating microvesicles may also contribute to coagulation by sustaining thrombin generation on the surface of activated platelets (for review see reference 4). The intrinsic pathway of coagulation is initiated when coagulation factor XII (FXII), also referred to as Hageman factor, comes into contact with negatively charged surfaces in a reaction (contact activation) involving the plasma proteins high molecular mass kininogen and plasma kallikrein. Although FXII is activated by a variety of polyanions, including constituents of subendothelial matrix (glycosaminoglycans and collagens), sulfatides, nucleosomes, and nonphysiological

materials (glass, ellagic acid, kaolin, and cilica; references 5 and 6) the mechanisms responsible for FXII activation in vivo are unknown. Induction of fibrin clot formation through contact activation–mediated activation of FXII is the basis of the activated partial thromboplastin time (aPTT) assay, a commonly used method for the global assessment of plasma coagulation in clinical settings.

Despite its obvious importance to blood coagulation in vitro, the pathophysiologic significance of the FXII-triggered intrinsic pathway of coagulation has been questioned for >50 yr, based on the important clinical observation that hereditary deficiency of FXII is not associated with abnormal bleeding. The absence of a bleeding phenotype in FXII deficiency, in contrast to deficiencies of components of the extrinsic cascade such as factor VII and TF (7, 8), has led to the reasonable hypothesis that fibrin formation in vivo is initiated largely, if not exclusively, through factor VIIa–TF (4, 9). The factor VIIa–TF–initiated model for hemostasis is supported by the observations that factor VIIa–TF can activate factor IX (10), and that factor XI, a major substrate for activated FXII (FXIIa) during contact-initiated clotting, can be activated by thrombin independently of FXII (11).

We used FXII-deficient mice to assess the in vivo significance of the intrinsic pathway of coagulation in thrombus formation. In agreement with observations in FXII-deficient humans, these mice have normal bleeding times and show no spontaneous bleeding. However, in vivo fluorescence microscopy revealed that even though the initial adhesion of platelets at sites of injury is not affected by FXII deficiency, the subsequent formation and stabilization of three-dimensional thrombi is severely impaired. This defect is observed in several locations in the vascular system in response to different types of injury and is completely reversed by the infusion of human FXII. These findings demonstrate that FXII-enhanced thrombin generation, or another unidentified FXII-dependent process, is required for arterial thrombus formation in vivo.

RESULTS
Factor XII is essential for contact activation, but not for controlling bleeding after injury

We have previously reported the development of an FXII-deficient mouse line (12). Mice homozygous for the FXII null allele ($FXII^{-/-}$) are healthy, fertile, and phenotypically indistinguishable from their wild-type littermates. Similar to humans, $FXII^{-/-}$ mice do not suffer from spontaneous hemorrhage or pathological vascular thrombosis. Consistent with our initial characterization of these animals, we found that $FXII^{-/-}$ mice do not have prolonged bleeding compared with wild-type mice in a tail bleeding time assay (369.5 ± 201.7 s and 355.9 ± 176.1 s, respectively; Fig. 1 A). Additionally, a detailed histological analysis did not reveal any correlation of abnormal bleeding or thrombosis in various tissues such as the lung, kidney, liver, small bowel, heart, brain, and spleen (unpublished data). Similarly, as described

for humans lacking FXII (13), $FXII^{-/-}$ mice did not suffer from inadequate bleeding during the various surgical challenges performed in the study despite having prolonged plasma aPTT (71 ± 18 s) and recalcification times (412 ± 78 s; wild-type values are 25 ± 4 s and 210 ± 31 s, respectively; Fig. 1 B). Furthermore, as is the case with FXII-deficient humans, the prothrombin time of $FXII^{-/-}$ mouse plasma, which evaluates TF-initiated coagulation, is similar to wild-type plasma collected by retroorbital puncture (8.9 ± 1.3 s and 9.1 ± 1.3 s, respectively; Fig. 1 B). Peripheral blood cell counts in $FXII^{-/-}$ and wild-type mice did not differ. The addition of normal human plasma to FXII-deficient murine plasma, and vice versa, corrected the defects in aPTT assays, indicating that human and murine FXII function similarly (in 50% mixtures the aPTT was 130% of wild type, and >90% FXII-containing plasma completely restored the aPTT prolongation found in FXII-deficient species). Using an aPTT assay based on human FXII-deficient plasma, it was determined that $FXII^{-/-}$ mice have <1% of the FXII level of wild-type mice. Western blots of plasma confirmed the absence of FXII in the plasma of $FXII^{-/-}$ mice, whereas levels of the contact factors high molecular mass kininogen and prekallikrein were normal (Fig. 1 C). To test the contribution of FXII to clotting in vitro, we determined the recalcification time in the presence of resting platelets or platelets stimulated with the calcium ionophore A23187, a very potent stimulus of PS exposure and microparticle formation, before induction of clot formation. In wild-type blood, the addition of A23187-stimulated platelets decreased the clotting time ∼2.5-fold compared with resting platelets. This effect was not detectable in FXII-deficient blood, suggesting that activated platelets may promote clot formation in a FXII-dependent manner (Fig. 1 D). Similar results were observed in human FXII-deficient plasma (not depicted).

Platelets interact with and contribute to the activation of several coagulation factors and the central coagulation product thrombin is a potent platelet activator (for review see references 14, 15). To discriminate between platelet and FXII-induced effects on thrombus formation, we induced clotting using either kaolin, which classically activates FXII but has no direct effect on platelets, or collagen, which activates both FXII and platelets. In the presence, but not in the absence, of platelets, collagen was superior to kaolin for clot formation in wild-type plasma (Fig. 1 E). In contrast, in platelet-rich plasma (PRP) derived from mice deficient in the FcR γ chain, which also lack the activating collagen receptor GPVI (16) in their platelets and, thus, cannot be activated by collagen, the relative potency of kaolin and collagen was similar to platelet-free plasma (PFP). On the other hand, in plasma lacking FXII, in vitro clot formation in response to either agonist was severely defective in the presence or absence of platelets. Further experiments confirmed that the clotting defect in FXII-deficient plasma in response to collagen was based on impaired thrombin formation (Fig. 1 F). In summary, these results demonstrated that activated/
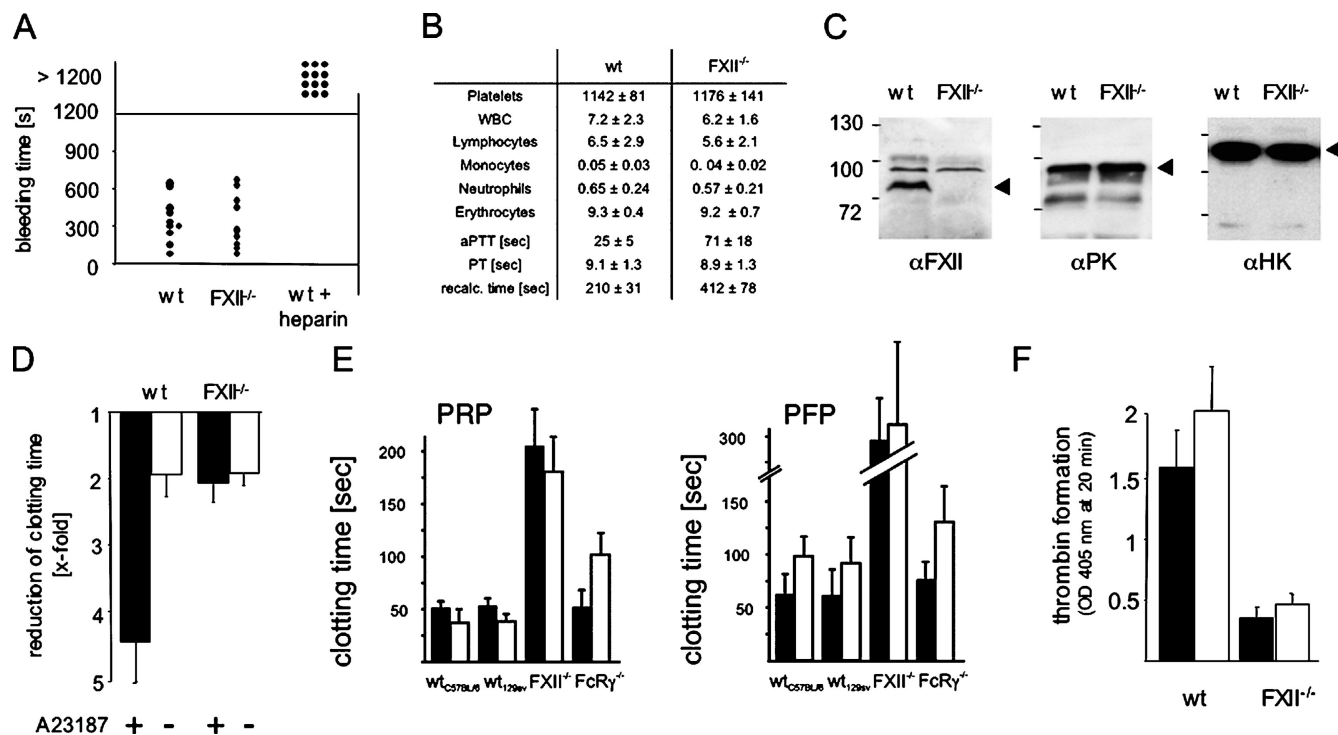
Figure 1. Hemostasis and coagulation in FXII deficient mice. (A) Tail bleeding times for wild-type ($n = 12$) and FXII$^{-/-}$ ($n = 11$) mice. Each symbol represents one individual. (B) Peripheral blood counts in thousands/microliter and coagulation parameters for FXII$^{-/-}$ and wild-type mice. Values are mean ± SD for 10 mice of each genotype. WBC, white blood cells; aPTT, activated partial thromboplastin time; PT, prothrombin time. (C) Western blots of wild-type or FXII deficient plasma (0.3 μl per lane) using primary antibodies recognizing the contact proteins FXII (αFXII), plasma kallikrein (αPK), and high molecular mass kininogen (αHK). The arrowheads point to FXII (left), plasma kallikrein (middle), and high molecular mass kininogen (right). Numbers indicate molecular mass standards. (D) Wild-type PFP or FXII-deficient plasma was reconstituted with platelets stimulated with calcium ionophore A23187 (+) or buffer (−), and recalcification clotting times were determined. Reduction of the clotting time is given relative to PFP substituted with buffer ($n = 6$). (E) Recalcification clotting times were determined in PRP (left) and PFP (right) from wild-type (129Sv and C57BL/6), FXII$^{-/-}$, and FcRγ$^{-/-}$ mice after activation with kaolin (black bars) or collagen (white bars). Mean ± SD from six experiments is given. (F) Thrombin generation in PRP (white bars) and PFP (black bars) from wild-type and FXII$^{-/-}$ mice initiated by the addition of 30 μg/ml of collagen. Thrombin formation was analyzed using the chomogenic substrate S-2238, and absorbance was detected at a 405-nm wavelength after incubation for 20 min. Data shown are mean ± SD ($n = 6$).

procoagulant platelets promote FXII-induced thrombin and clot formation. It will be the subject of further studies to distinguish whether FXII may be directly activated by procoagulant platelets and/or whether activated platelets are merely recruited downstream of FXII into the growing thrombus. Furthermore, the results demonstrate that FXII$^{-/-}$ mice and FXII-deficient humans have similar plasma coagulation profiles and therefore established the FXII$^{-/-}$ animals as an appropriate model to study the impact of FXII on clot formation in vivo.

FXII contributes to collagen–induced thromboembolism in vivo

To determine the consequences of FXII deficiency in vivo, we first tested wild-type and FXII$^{-/-}$ mice in a model of lethal pulmonary thromboembolism induced by infusion of a mixture of collagen and epinephrine. All wild-type mice (19 out of 19) died within 5 min, with >95% reduction in circulating platelet counts within 2 min of challenge (Fig. 2, A

and B). Consistent with a previous report (17), FcR γ chain–deficient mice are protected from death in this model and experience only moderate reductions in platelet count. 5 out of 14 FXII$^{-/-}$ mice (37.5%) survived this challenge, although their peripheral platelet counts were reduced to a similar degree as in the wild-type controls. This suggests that the protection conferred by FXII deficiency is not caused by a platelet activation defect, but rather a defect in thrombin generation or some other FXII-related activity. Consistent with this premise, in vitro studies demonstrated that platelet aggregation in response to collagen and ADP is normal in PRP from FXII$^{-/-}$ mice (Fig. 2 C). Histologic sections of lung tissue and analysis of thrombi in the lungs are shown in Fig. 2 D. Although the majority of vessels are obstructed in wild-type mice, there is a clear reduction in the number of occluded vessels in FXII$^{-/-}$ mice (survivors and nonsurvivors). In agreement with previous observations, virtually no thrombi were found in the lungs from FcRγ$^{-/-}$ mice. These results suggest that collagen triggers both platelet and
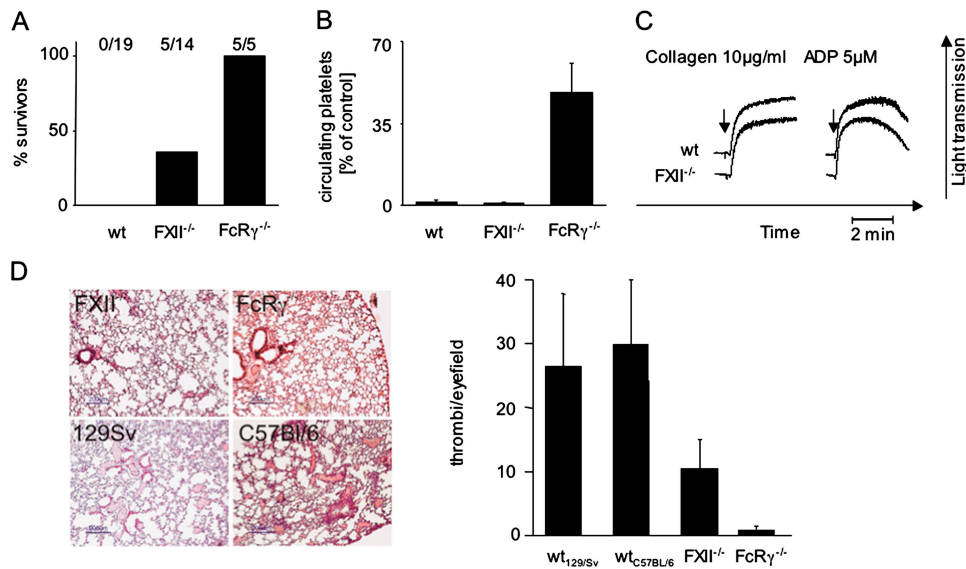
Figure 2. Collagen–induced pulmonary embolism model. (A) Mortality associated with i.v. injection of 0.8 mg/kg of collagen and 60 μg/kg of epinephrine. All wild-type mice died within 5 min. Animals alive 30 min after challenge were considered survivors. (B) Platelet counts in control ($n = 19$), FXII$^{-/-}$ ($n = 14$), and FcRγ$^{-/-}$ ($n = 5$) mice 2 min after infusion of collagen/epinephrine. (C) Heparinized PRP from wild-type and FXII$^{-/-}$ mice was stimulated with 10 μg/ml of collagen or 5 μM ADP, and light transmission was recorded in a standard aggregometer. Representative results for each genotype are shown ($n = 6$). (D) Hematoxylin and eosin–stained sections from lungs of mice 2 min after collagen/epinephrine infusion. Thrombi per visual field were counted at 20×. Mean ± SD for 100 fields is shown. Bar, 200 μm.

FXII activation, which synergize in this model to form occlusive pulmonary thrombi.

Defective arterial thrombus formation in FXII$^{-/-}$ mice

Pathological thrombus formation in arteries is thought to be initiated by fissuring or other disruption of an atherosclerotic plaque, leading to local activation of platelets and plasma coagulation on exposed subendothelial layers of the vessel. We studied the effect of FXII deficiency on thrombus formation in wild-type and FXII$^{-/-}$ mice using three models of arterial injury. In the first model, oxidative injury was induced in mesenteric arterioles, and thrombus formation was examined by in vivo fluorescence microscopy. Wild-type and FXII$^{-/-}$ mice received 10^8 fluorescently labeled platelets from donors of the same genotype, and injury was induced by topical application of filter paper saturated with 20% ferric chloride (FeCl$_3$). FeCl$_3$ induces formation of free radicals, leading to disruption of the vascular endothelium. Platelets rapidly interacted with the injured vessel wall, and 5 min after injury the number of firmly adherent platelets was similar in FXII and wild-type mice (Fig. 3 A). In wild-type mice, additional platelets were recruited into the growing thrombus. Thrombi >20 μm developed in 100% of wild-type vessels tested (17 out of 17) within 10 min of injury (Fig. 3 B), ultimately leading to complete occlusion in 94.1% (16 out of 17) of vessels within the 40-min observation period (mean occlusion time: 25.6 ± 8.9 min; Fig. 3 C). In contrast, formation of microaggregates or thrombi did not occur in 50% (7 out of 14) of vessels in FXII$^{-/-}$ mice (Fig. 3 B). In the remaining vessels (7 out of 14), thrombi formed that were un-

stable and detached from the vessel wall (Fig. 3 D). In no FXII$^{-/-}$ vessel did a thrombus >20 μm in diameter remain attached to the injury site for >1 min. Consequently, no vessel occluded within the 40-min observation period in FXII$^{-/-}$ mice (Fig. 3 C). To assess the contribution of FXII to thrombus formation more quantitatively, we tested mice heterozygous for the FXII null allele (FXII$^{+/-}$) that have half of the FXII plasma antigen and activity levels of wild-type mice and a normal aPTT (26 ± 5 s vs. 25 ± 5 s for FXII$^{+/-}$ and wild-type mice, respectively; reference 12). Platelet adhesion occurred normally in FXII$^{+/-}$ animals, and thrombi >20 μm developed in 100% of the vessels (14 out of 14) within 10 min of injury, leading to complete occlusion in 11 out of 14 vessels (mean occlusion time: 29.5 ± 7.7 min). In the other three arterioles, stable thrombi occluded >90% of the vessel lumen but did not reach complete occlusion. Collectively, these results demonstrate that FXII is required for the propagation and stabilization of platelet-rich thrombi in FeCl$_3$-injured arterioles and suggest that FXII-mediated activation of coagulation is involved in this model. To examine this possibility further, we tested factor XI–deficient mice in the same model. Factor XI is the principal substrate of FXIIa in the intrinsic pathway of the coagulation cascade in vitro, and a similar defect in thrombus formation would be expected in FXII- and factor XI–deficient mice if this pathway is operating in vivo. Similar to FXII$^{-/-}$ mice, normal platelet adhesion at the site of injury was detectable during the first 5 min after injury in factor XI–deficient mice (Fig. 3 A), with formation of thrombi in only four out of nine (44.4%) vessels studied. In the remain-
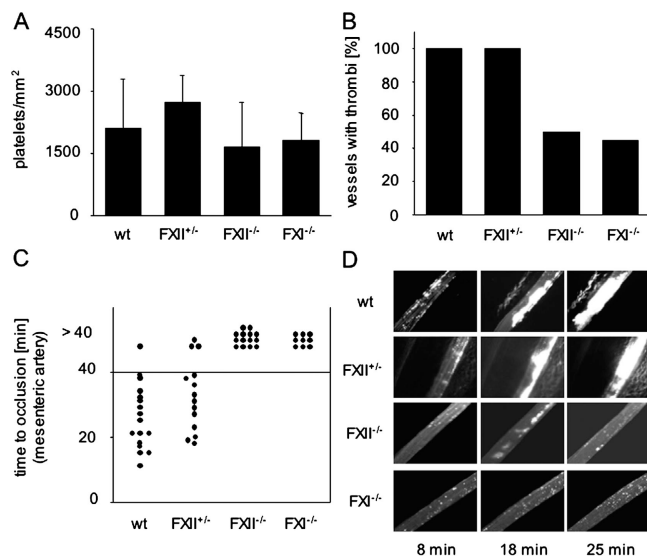
Figure 3. Defective thrombus formation in FeCl3–injured mesenteric vessels in FXII⁻/⁻ and factor XI⁻/⁻ mice. Thrombus formation in vivo was monitored on mesenteric arterioles after topical application of 20% FeCl₃. (A) Mean ± SD of platelets adherent at 5 min (129Sv wild type, $n = 14$; FXII⁺/⁻, $n = 14$; FXII⁻/⁻, $n = 11$; FXI⁻/⁻, $n = 9$). (B) The number of vessels in which one or more thrombi >20 μm in diameter formed during the 40-min observation period. (C) Time to complete occlusion after injury. Each symbol represents one monitored arteriole. (D) Representative images of one experiment.

ing vessels, microaggregates and thrombi were unstable and broke loose from the site of injury (Fig. 3 D). No vessel occluded in factor XI–deficient animals during the observation period. These data confirm and extend the previous observation that FXI-deficient mice are protected from vascular occlusion in a FeCl₃ carotid artery injury model (18).

FeCl₃-induced arterial thrombus formation is dependent on platelet activation and thrombin generation, but it is unclear how well this type of injury mimics naturally occurring arterial thrombus formation at the site of rupture of an ath-

erosclerotic plaque. As it is possible that FeCl₃-induced oxidative damage produces conditions that artifactually favor FXII-dependent processes, the effect of FXII deficiency was assessed in a second well-established arterial thrombosis model in which injury is induced mechanically in the aorta, and blood flow is monitored with an ultrasonic flow probe. After a transient increase directly after injury, blood flow progressively decreased for several minutes in all animals. In all wild-type mice (10 out of 10), this decrease resulted in complete and irreversible occlusion of the vessel within 1.6–11.1 min after injury (mean occlusion time: 5.3 ± 3.0 min; Fig. 4 A). Although a progressive reduction in blood flow was observed during the first several minutes after injury in FXII⁻/⁻ mice, occlusion occurred in only 4 out of 10 mice. Occlusive thrombi were all unstable and rapidly embolized so that blood flow was reestablished within 10–115 s after occlusion. No vessel in which flow was reestablished occluded a second time, and all FXII⁻/⁻ mice displayed essentially normal flow rates through the injured vessel at the end of the 40-min observation period. Similar results were obtained with factor XI–deficient mice, where 9 out of 11 vessels were not occluded at the end of the observation period (Fig. 4 A).

The defect in arterial thrombus formation in FXII⁻/⁻ mice was confirmed in a third model in which platelet recruitment into thrombi in the injured carotid artery is studied by in vivo fluorescence microscopy. Platelets purified from donor mice of the appropriate genotype were fluorescently labeled and injected into recipient mice. Vascular injury was induced by ligation of the carotid artery, a process that consistently causes disruption of the endothelial layer and frequent breaching of the internal elastic lamina, followed by rapid collagen-dependent platelet adhesion and thrombus formation at the site of injury (19). Large stable thrombi that did not embolize formed rapidly in wild-type animals within 5 min (thrombus area: 102.821 ± 39.344 μm²). In contrast, only small- and medium-sized aggregates formed in FXII⁻/⁻ mice, which frequently detached from the site of injury (Fig. 4, B and C). Consequently, the thrombus area was dramati-
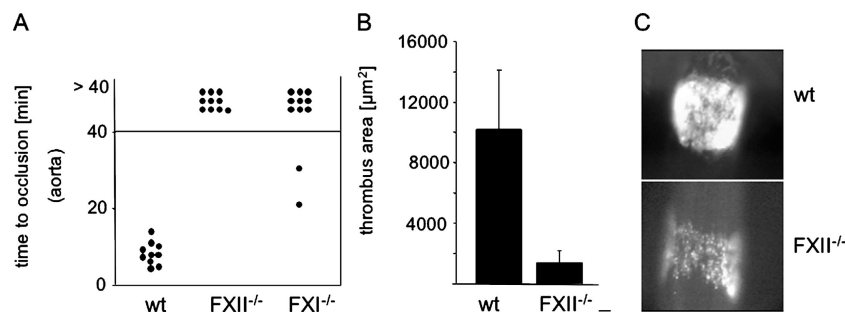

Figure 4. Defective thrombus formation in the injured aorta and carotid artery in FXII⁻/⁻ and factor XI⁻/⁻ mice. (A) Thrombosis was induced in the aorta of wild-type ($n = 10$), FXII⁻/⁻ ($n = 10$), and FXI⁻/⁻ ($n = 11$) mice by one firm compression with a forceps. Blood flow was monitored with a perivascular ultrasonic flow probe until complete occlusion. The experiment was stopped after 40 min. Each symbol represents one

individual. (B) Mechanical injury to the carotid artery was induced by ligation with a surgical filament. 5 min after removal of the filament, thrombus areas (μm²) in wild-type and FXII⁻/⁻ ($n = 10$) mice were measured and are expressed as mean ± SD. (C) The photomicrographs show representative images 5 min after injury.

cally reduced in FXII$^{-/-}$ mice 5 min after injury (8.120 ± 13.900 µm^2), whereas primary platelet adhesion on the vessel wall appeared not to be defective.

Exogenous human FXII restores arterial thrombus formation in FXII$^{-/-}$ mice

To determine whether the severe defect in thrombus formation in FXII$^{-/-}$ mice results from the absence of plasma FXII or a secondary effect of chronic FXII deficiency that indirectly alters sensitivity to prothrombotic stimuli, we studied arterial thrombus formation in FXII$^{-/-}$ mice after intravenous administration of human FXII (2 µg/g body weight). This treatment corrected the prolonged aPTT clotting time of FXII-deficient murine plasma to normal (27 ± 6 s) and completely restored arterial thrombus formation. In all FeCl$_3$-injured mesenteric arterioles in wild-type or FXII$^{-/-}$ mice treated with human FXII, thrombi >20 µm formed within 10 min after injury, and all vessels were occluded within the observation period (Fig. 5, A and B) with the exception of a single vessel in a wild-type animal. In fact, there was a slight tendency toward faster occlusion in the reconstituted FXII$^{-/-}$ mice compared with untreated wild-type control mice (mean occlusion time: 22.7 ± 8.2 min vs. 25.6 ± 8.9 min). A similar result was obtained with mechanical injury of the aorta (Fig. 5 C), with all vessels completely occluded within 10 min of injury. These results confirmed that the absence of plasma FXII protects FXII$^{-/-}$ mice from arterial thrombus formation in these models.

DISCUSSION

Although the importance of FXII for contact activation–initiated coagulation has been recognized for >50 yr (20), the protein is not considered to be an important component of the hemostatic mechanism, as humans lacking FXII do not have a bleeding diathesis. Indeed, for many years FXII has been suspected of having antithrombotic (profibrinolytic), rather than prothrombotic properties, based on reports indicating an association between FXII deficiency and venous thrombosis (21, 22) and myocardial infarction (23). How-

ever, this premise has been challenged recently by Girolami et al., who demonstrated that in most cases of thrombosis associated with FXII deficiency, other congenital or acquired prothrombotic risk factors are present (24). Indeed, large clinical studies have not identified a correlation between FXII deficiency and bleeding or thrombosis (25, 26). Current models of hemostasis stress the importance of the factor VIIa–TF complex in the initiation of thrombin formation. The importance of this pathway is highlighted by the fact that mice lacking factor VII or TF die perinatally in utero from apparent severe hemorrhage (7, 8). Although it is clear that the TF exposed at a site of vessel injury is well positioned to initiate thrombin generation, it has puzzled investigators as to how thrombin generation proceeds on the surface of a growing thrombus once the factor VIIa–TF at the wound site is covered by the clot and/or neutralized by inhibitors such as TF pathway inhibitor (27, 28). There is evidence that TF is expressed on procoagulant microparticles that are released at sites of injury or that circulate in blood. These particles can be incorporated into a growing thrombus in a P-selectin–dependent manner (29). Indeed, mice expressing low levels of TF display defective arterial thrombus formation, although it is unclear whether this is related to a lack of vessel wall or blood cell–derived TF (30, 31). However, the existence of active TF in blood is still a matter of discussion (32), and other mechanisms contributing to fibrin formation are possible.

We used three in vivo models to study platelet recruitment and thrombus formation at sites of arterial injury in FXII-deficient mice and observed a profound defect in the formation and stabilization of platelet-rich thrombi. Similar results were obtained in a pulmonary thromboembolism model. Additionally, in the FeCl$_3$ model, we consistently observed severely reduced thrombus stability not only in mesenteric arterioles but also in adjacent venules of FXII$^{-/-}$ mice, indicating that FXII may also be relevant to thrombus formation in the venous circulation, although further studies will be required to confirm this. Together, these results suggest that a TF-independent pathway may be operating in the
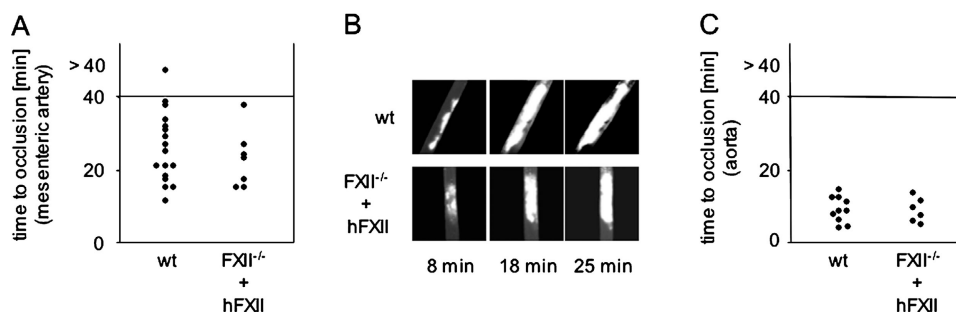

Figure 5. Thrombus formation in FXII$^{-/-}$ mice reconstituted with exogenous FXII. FXII-deficient animals received human FXII (hFXII, 2 mg/kg of body weight), and thrombus formation after FeCl$_3$-induced injury was analyzed. (A) Time to complete occlusion after injury. Each symbol represents one monitored arteriole. (B) Representative images from one experiment. (C) FXII$^{-/-}$ mice received 2 mg/kg hFXII, and aortic thrombosis was induced by compression with a forceps. Blood flow was monitored with a perivascular ultrasonic flow probe. Each symbol represents one individual.

propagation of pathologic thrombus formation in mice. The protective effect of FXII deficiency in these models is reversed by infusions of human FXII, demonstrating that the absence of plasma FXII is responsible for the observed phenotype (Fig. 5). These results appear to conflict with the reasonable proposition that the coagulation proteins involved in pathologic thrombus formation are the same as those that are also important for normal hemostasis (cessation of bleeding at a wound site). Our results support the interesting possibility that hemostasis and thrombosis may be facilitated by different, though probably largely overlapping, mechanisms. Several recent reports support this premise. Mice lacking plasma fibronectin or the secreted growth arrest–specific gene 6 product (Gas6) do not have prolonged bleeding times and do not spontaneously bleed. However, fibronectin-deficient mice do not form occlusive arterial thrombi, and Gas6-null mice are protected from thromboembolism (33, 34). Similarly, mice lacking the platelet collagen binding GPVI–FcR γ chain complex have a profound defect in injury–induced arterial thrombus formation, but only minor hemostatic abnormalities (19).

We cannot exclude the possibility that there are species-specific differences in the functions of FXII. This will be an important issue to resolve, as the models used in our studies are widely used for evaluating the importance of blood and blood vessel constituents to thrombus formation, as well as testing prospective antithrombotic agents. FXII-deficient mice, like their human counterparts, have prolonged aPTTs in the absence of a bleeding diathesis. Furthermore, plasma mixing studies demonstrate that murine FXII functions normally in human plasma in vitro, whereas infusion of human FXII into FXII$^{-/-}$ mice results in a phenotype similar to that of wild-type mice in thrombosis models. Clinical studies have associated elevated plasma FXIIa levels with an increased prevalence of coronary heart disease and other known plasma cardiovascular risk factors (35, 36), supporting the notion that an FXII-dependent pathway might contribute to thrombosis in humans. It remains to be determined if FXII activation is the cause or the consequence of the underlying vascular disease. A particular FXII single nucleotide polymorphism (46C>T) has been linked with lower FXIIa and FXII plasma levels and protection from coronary artery diseases in British patients (35). Because the same single nucleotide polymorphism has been reported to be a risk factor for ischemic stroke in the Spanish population (37), environmental and/or other genetic factors may influence the effects of FXII plasma levels on thrombotic risk. Indeed, clinical studies have shown that elevated FXIIa levels are associated with an increased risk for coronary heart disease. However, in these patients, FXIIa activation is linked with other risk factors such as elevated cholesterol, triglycerides, or fibrinogen (38). Moreover, elevated FXIIa levels have been reported as a prognostic risk factor for recurrent coronary events (39), supporting the premise that FXII contributes to thrombus formation in humans. Simi-

larly, FXIIa has been shown to efficiently activate the coagulation cascade in primates (40).

Data from heterozygous FXII$^{+/-}$ mice show that FXII at half the normal plasma level is sufficient for the formation of large and stable arterial thrombi, though with a slightly reduced rate of complete vessel occlusion (Fig. 3). This suggests that partial FXII deficiency may provide, at most, limited protection from stroke or myocardial infarction in humans. In contrast, Girolami et al. followed 21 patients with severe (homozygous) FXII deficiency with a mean observation period of 16 yr and did not observe a thrombotic event (41). Clearly, larger clinical studies will be required to define the significance of severe (homozygous) FXII deficiency for a complex disease such as arterial thrombosis.

It seems very unlikely that resistance to thrombus formation in FXII-deficient mice is an artifact of the type of injury inflicted on the vessel, or the vascular bed tested, as observations were consistent across several models and different vascular beds. Furthermore, we have no evidence that FXII functions differently in mice and humans. The mechanism through which FXII is recruited into the thrombotic process is not clear. Plaque rupture or fissuring results in the exposure of collagen fibrils and other basement membrane components to flowing blood, and it is likely that there is similar exposure in our arterial injury models, which disrupt the vascular endothelium. Early work demonstrated that collagen activates FXII (for review see reference 5), although not all investigators came to this conclusion (42). An explanation for the discrepancy may be that procoagulant activities of collagens are highly dependent on the type, available surface area, charge, and method of preparation of the collagen (43). We have observed that fibrillar collagen type I, the major collagen in blood vessels, activates FXII in plasma clotting assays (Fig. 1). Collagen may not be the only pathophysiological activator of FXII. Other candidates include substances liberated from disintegrating cells or exposed in the extracellular matrix (ECM) such as HSP90 (44) or soluble and insoluble polyanions, such as nucleosomes or glycosaminoglycans (45). Based on our results, we speculate that FXII-driven thrombin generation and fibrin deposition might proceed directly on platelet surfaces. This hypothesis is supported by two observations. First, thrombin generation initiated by collagen is enhanced in the presence of platelets (Fig. 1 E). Second, collagen, which activates platelets and FXII, is superior to kaolin in aPTT assays in PRP but not PFP, and that this difference is not observed in FXII-deficient plasma (Fig. 1 F).

Based on our results, we propose the model for pathologic arterial thrombus formation depicted in Fig. 6. At sites of vascular injury, platelets come in contact with the exposed subendothelial ECM. Platelets are initially tethered to the ECM by von Willebrand factor through platelet glycoprotein Ib (46). Activation and adhesion then proceeds through interactions between platelet collagen receptors such as GPVI and integrin α2β1 and the ECM (47). Factor VIIa–TF initiates thrombin formation, which recruits additional plate-

lets into the growing thrombus. Although FXII and factor XI may be activated during this early phase, these proteins appear to have little effect on platelet adhesion or recruitment. As the thrombus grows, the exposed ECM and TF are covered, and TF is inactivated by TF pathway inhibitor released from activated platelets (28). Under these conditions, additional mechanisms are required to maintain spatio-temporal thrombin generation to activate newly recruited platelets and consolidate fibrin formation in the growing thrombus. It is in this propagation phase that FXII$^{-/-}$ and factor XI–deficient mice appear to be defective. Therefore, we propose that FXII activation and FXII-driven thrombin formation might proceed on the surface of activated platelets (Fig. 6 B). Indeed, it has been suggested that "platelets can provide a surface, perhaps similar to that provided by such negatively charged substances as kaolin or glass" (48), that facilitate FXII activation. Because procoagulant platelets expose PS on their surface (for review see reference 49) that facilitates several coagulation protease reactions, it is tempting to speculate that this negatively charged surface might induce a conformational change in FXII, resulting in activation. Such a reaction has been described for sulfonated glycolipid micells that efficiently activate FXII in plasma (50). Therefore, the surface of activated platelets may at least partly provide the long sought after "contact activation surface." This notion is supported by the observation that A23187-activated PS-exposing platelets promote clot formation only in the presence of FXII (Fig. 1 D). It is quite possible that other structures exposed on or released from activated platelets mediate FXII activation. It will be important to identify these structures and the mechanisms underlying platelet-dependent FXII activation in detail. One approach could be to use annexin V and/or inhibitory antibodies against platelet surface molecules in clotting assays to interfere with possible sites of FXII association. It will also be interesting to directly visualize FXII recruitment into the growing thrombus in vivo and to compare it with recruitment of TF in order to better understand how these two proteins coordinate thrombus formation.

In conjunction with the observation that mice expressing low levels of TF display impaired arterial thrombus formation, our results suggest that factor VIIa–TF and FXII cooperate and synergize in an integrated manner in vivo and, thus, are both required for formation of an occlusive thrombus. The absence of a bleeding diathesis in FXII$^{-/-}$ mice indicates that FXII is not required to seal a hole in a vessel wall (hemostasis). However, FXII-mediated thrombin formation occurs in vivo and is believed to contribute to thrombus generation in sepsis-associated disseminated intravascular coagulation (51). Therefore, FXII-driven thrombin and thrombus generation may contribute to the control of bleeding when blood comes in contact with glycosaminoglycans (45) or cerebroside sulfates (50) that readily activate FXII. Furthermore, bacteria assemble contact system proteins on their surfaces and activate FXII (52). Therefore, FXII might participate in the control of blood loss on sites of infections and inflammatory responses. We are aware that this model does not satisfactorily answer the long standing question regarding the physiologic role of FXII, but our findings raise the interesting possibility that formation of a large thrombus may serve nonhemostatic functions, such as inhibition of the spread of invading pathogens or toxins within the blood stream. FXII-deficient mice will be an important tool for studying other contact system-linked diseases, such as inflammation and sepsis (52), complement activation (53, 54), and kinin-mediated vascular leakage (55).

In summary, although FXII appears to be dispensable for normal hemostasis, we have demonstrated a central role for FXII in pathologic thrombus formation in vivo. These findings establish FXII as a promising new target for antithrombotic therapies that might be associated with low or no risk of excessive bleeding.
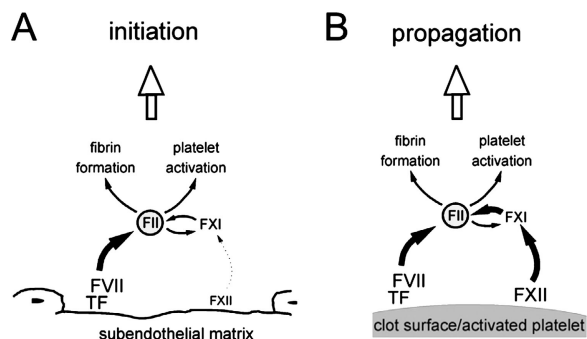
MATERIALS AND METHODS

Animals. All experiments and animal care were approved by the local Animal Care and Use Committee. Mice homozygous for null mutations in the factor XI and FXII genes were generated as previously described (12, 56). Mice deficient in the FcR γ chain (57) were purchased from Taconics. FXII$^{-/-}$ mice were inbred for nine generations to the 129/SvJ background. Factor XI$^{-/-}$ and FcRγ$^{-/-}$ mice were inbred to C57BL/6J for more than nine generations. Corresponding wild-type C57B/6J or 129/SvJ mice were used as controls.

Platelet preparation and aggregometry. Murine platelets were prepared according to established protocols (58). Platelet aggregation in 200 μl PRP (0.5×10^6 platelets/μL) stimulated with 10 μg/ml collagen or 5 μM ADP was determined by changes in light transmission using a standard aggregometer (Fibrintimer 4; APACT Laborgeräte und Analysensysteme). Changes through 10 min were expressed as arbitrary units with 100% transmission represented by platelet-poor plasma.

Tail bleeding time. Mice were anesthetized by i.p. injection of tribromoethanol (0.15 ml/10 g of body weight; Sigma-Aldrich), and the distal

Figure 6. A model of arterial thrombus formation. (A) Initiation of thrombus formation at sites of vascular lesions is predominantly caused by TF and collagens exposed in the subendothelial matrix. TF in complex with FVII initiates thrombin formation, which promotes fibrin formation and platelet activation. The contribution of FXII in this early phase of thrombus formation is minor. (B) FXII activity on the exposed surface of the thrombus contributes to thrombin generation and additional platelet activation, propagating thrombus growth. Accordingly, FXII, as well as factor XI, deficiency severely impairs thrombus formation.

3-mm segment of the tail was removed with a scalpel. Bleeding was monitored by gently absorbing the bead of blood with a filter paper at 15-s intervals without touching the wound. Bleeding was stopped manually if it continued for >20 min.

Preparation of platelets for intravital microscopy. Heparinized blood was centrifuged at 250 g for 10 min, and PRP was gently transferred to a fresh tube. Platelets were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and adjusted to a final concentration of 200×10^6 platelets/250 μl as described previously (59).

FeCl₃–induced arterial thrombosis model. 4–5-wk-old mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol and 2-methyl-2-butanol (0.15 ml/10 g of body weight from a 2.5% solution; Sigma-Aldrich). 10^8 CFSE-labeled platelets per mouse were injected through the tail vein. The mesentery was externalized through a midline abdominal incision. 35–60-μm-diameter arterioles were visualized at $10\times$ with an inverted microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.) equipped with a 100-W fluorescent lamp source (HBO) and a CCD camera (CV-M300; Visitron Systems GmbH) connected to an S-VHS video recorder (AG-7355; Panasonic). After topical application of a filter paper (2×1 mm) saturated with 20% FeCl₃ for 1 min, arterioles were monitored for 40 min or until complete occlusion (blood flow stopped for >1 min) occurred as described previously (60). Platelet adhesion was defined as the number of fluorescently labeled platelets bound to the vessel wall 5 min after injury. A thrombus was defined as a platelet aggregate >20 μm in diameter. In some experiments, human FXII (American Diagnostica, Inc.) was injected i.v. directly before the experiment.

Intravital microscopy of thrombus formation in the carotid artery. Intravital microscopy of injured carotid arteries was performed as previously described (19). In brief, mice were anesthetized by i.p. injection of ketamine/xylazine (100:5 mg/kg; Parke-Davis and Bayer AG, respectively). Polyethylene catheters (Portex) were implanted into the right jugular vein, and 2×10^8 fluorescent platelets/250 μl were infused i.v. Carotid injury was induced by ligation with a surgical filament. Before and after vascular injury, fluorescent platelets were visualized in vivo by video microscopy of the right common carotid artery using a microscope (Axiotech; Carl Zeiss MicroImaging, Inc.) with a $20\times$ water immersion objective and a 100-W mercury lamp (HBO) for epiillumination. Platelet adhesion and thrombus formation were observed for 5 min after injury, and videotaped images were evaluated using a computer-assisted image analysis program (Visitron) as previously described (61).

Collagen/epinephrine–induced pulmonary thromboembolism. Mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol and 2-methyl-2-butanol (0.15 ml/10 g of body weight from a 2.5% solution), and a mixture of 0.8 mg/kg of collagen and 60 μg/kg of epinephrine was injected into the jugular vein (62). Platelet counts were determined by flow cytometry on a FACSCalibur (Becton Dickinson). Results are expressed as mean ± SD or as percentage of control.

Histopathologic analyses. Mice were killed, and lungs were rapidly removed and fixed at 4°C for 24 h in buffered 4% formalin, pH 7.4. Tissues were dehydrated and embedded in paraffin (Histolab Products AB), cut into 4–μm sections, and stained with Mayer's hematoxylin and eosin (Sigma-Aldrich).

Aorta occlusion model. The abdominal cavities of anesthetized mice were opened with longitudinal incisions, and an ultrasonic flow probe (Transonice Systems, Inc.) was placed around the abdominal aortas. Thrombus formation was induced by a single firm compression of the vessel with forceps immediately downstream from the flow probe. Blood flow was monitored until complete occlusion occurred or for 40 min.

Preparation of antimurine FXII antibody. Murine FXII heavy chain was expressed as a GST fusion protein in *Escherichia coli* BL21, and poly-clonal anti-FXII antibodies were raised in rabbits according to standard immunization protocols.

SDS-PAGE and Western blotting. 0.3 μl plasma/lane was separated by 12.5% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with the anti–high molecular mass kininogen antibody MBK3 (donated by W. Müller-Esterl, University of Frankfurt, Frankfurt, Germany), the anti–plasma kallikrein antibody AS176 (63), and anti–murine FXII antibodies in 1:1,000 dilutions, respectively. Bound antibodies were detected using peroxidase-conjugated secondary antibodies followed by a chemiluminescence detection method.

Measurement of thrombin generation. Thrombin generation was measured according to the method of Aronson with minor modifications as previously described (64) using the chomogenic substrate S-2238 (H–D–Phe–Arg–NH–NO₂–HCl; Chromogenix). The absorbance of the released product was measured spectrophotometrically at 405 nm. Measurements were obtained in triplicate at each time point.

Coagulation assays. Washed platelets from WT or FXII$^{-/-}$ mice were resuspended in Tyrode buffer supplemented with 4 mM Ca^{2+} and 5 μM Ca^{2+} ionophore A23187 (Sigma-Aldrich) for 10 min before suspension in PFP. Clot formation was initiated by recalcification with 100 μl/25 mM CaCl₂ solution in a "Kugelkoagulometer" (KC10; Amelung) at 37°C, and the time to clot formation was recorded using a coagulation timer (KC4; Amelung). For the determination of the recalcification clotting time, 100 μl of citrate anticoagulated mouse plasma (0.38% sodium citrate), was incubated with 100 μg each of Horm type collagen (Nycomed), kaolin (final concentrations, 30 μg/ml), or buffer for 120 s at 37°C before addition of CaCl₂.

Coagulation analysis. Global coagulation parameters were determined with an automated blood coagulation system (BCS; Dade Behring) with reagents according to the protocols for human samples detailed by the manufacturer (http://www.dadebehring.com). Peripheral blood counts were determined on the Sysmex XE 2100 (Diamond Diagnostics) according to standard protocols.

Statistical evaluation. Statistical analysis was performed using the unpaired Student's t test.

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