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A hemophilia A mouse model for the *in vivo* assessment of Emicizumab function

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Key points

- Emicizumab (≥ 1.5 mg/kg) partially corrects blood loss in a hemophilia A bleeding model, with a FVIII-equivalence of 9 U/dL
- Emicizumab provides additive hemostatic activity when given in combination with low dose Factor VIII

ABSTRACT

The bispecific antibody Emicizumab is increasingly used for hemophilia A-treatment. However, its specificity for human factors IX and X (FIX, FX) has limited its *in vivo* functional analysis to primate models of acquired hemophilia. Here we describe a novel mouse model allowing to examine Emicizumab function. Briefly, FVIII-deficient mice receive Emicizumab intravenously 24h before performing a tail clip-bleeding model. A second infusion with human FIX and FX is administered 5 min before bleeding. This approach generates consistent levels of Emicizumab (0.7-19 mg/dL for 0.5-10 mg/kg dose) and both FIX and FX (85 and 101 U/dL respectively, after dosing 100 U/kg). Plasmas from these mice display FVIII-like activity in a diluted aPTT and thrombin generation assays similar to human samples containing Emicizumab. Emicizumab doses of 1.5 mg/kg and higher significantly reduced blood loss in FVIII-deficient mice. However, reduction was incomplete compared to FVIII-treated mice, and no difference in efficacy between doses was observed. FVIII-like activity of Emicizumab corresponds to a dose of 4.5 U FVIII/kg (*i.e.* 9.0 U/dL) in this model. Interestingly, combined with a low FVIII dose (5 U/kg), Emicizumab provided additive activity to allow a complete bleeding arrest. This model could be useful for further *in vivo* analysis of Emicizumab.

INTRODUCTION

Limitations hampering factor VIII (FVIII)-replacement therapy in hemophilia A have spurred the development of novel therapeutic approaches, including the bispecific antibody Emicizumab.^{1,2} Emicizumab bridges the enzyme factor IXa (FIXa) and substrate factor X (FX), thereby stimulating FX activation³⁻⁵, and markedly reduces bleeding tendency in hemophilia A-patients, with and without inhibitors.⁶⁻⁹ Unfortunately, current assays that measure Emicizumab's FVIII-like activity are unfit to predict an unbiased equivalence to true FVIII activity.^{5,10,11} Furthermore, *in vitro* assays allow only limited insight into the interaction between Emicizumab and other procoagulant drugs^{12,13}.

Since Emicizumab selectively recognizes human and primate FIX and FX, reports that explored Emicizumab in experimental models are scarce. We have therefore developed a first murine model that allows for a direct comparison between Emicizumab and FVIII *in vivo*. In this model, Emicizumab's cofactor activity mimics FVIII levels of 9 U/dL. Additionally, Emicizumab provides additional procoagulant activity at low concentrations of FVIII, resulting in enhanced hemostasis in FVIII-deficient mice.

STUDY DESIGN

Animals and ethics statement

F8-deficient mice¹⁴ were backcrossed (>10 times) on a C57Bl/6 background. Males and females were used throughout the study (8-12 weeks old, 20-25 g). Housing and experiments were performed in accordance with French regulations and the experimental guidelines of the European Community. This project was approved by the local ethical committee of Université Paris-Sud (comité d'éthique en experimentation animale n°26, protocol APAFIS#4400-2016021716431023v5).

Blood collection and plasma preparation

Blood was collected from ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) anesthetized animals by retro-orbital puncture with a glass capillary in 1/10 volume Trisodium citrate (13.8mM). Plasma was prepared by centrifugation for 20 minutes at 1,500g and stored at -80°C.

Proteins

Emicizumab (marketed under Hemlibra) was from F. Hoffman-La Roche (Basel, Switzerland). Recombinant Factor IX (FIX, Benefix) was from Pfizer Inc. (Paris, France). Recombinant murine FIX was from Sino Biologicals (Wayne, PA, USA). Plasma-derived FIX and FX were from Cryopep (Montpellier, France). Recombinant factor VIII (FVIII, Advate) was from Shire France SAS (Paris, France). Polyclonal rabbit anti-FIX antibodies and polyclonal rabbit anti-human FX antibodies were isolated from serum obtained from rabbits immunized with human FIX or FX, respectively (Eurogentec, Seraing, Belgium). Purified antibodies were labeled with peroxidase using the EZ-link activated peroxidase antibody labeling kit as instructed (ThermoFisher Scientific, Illkirch, France).

Antigen and activity assays

Human FIX antigen levels in murine plasma were determined in a FIX elisa using commercially available polyclonal sheep anti-human FIX antibodies (Cryopep) as coating antibody and peroxidase-labeled polyclonal rabbit anti-human FIX antibodies as probing antibodies. Human FX antigen levels in murine plasma were determined in a FX elisa using commercially available polyclonal goat anti-human FX antibodies (Cedarlane, Gateshead, New England, USA) as coating antibody and peroxidase-labeled polyclonal rabbit anti-human FX antibodies as probing antibodies. FVIII activity was measured in a chromogenic assay using the Biophen FVIII-assay kit (Hyphen, Neuville-sur-Oise, France) as instructed.

Determination of Emicizumab levels after infusion

Mice were given Emicizumab (0-10 mg/kg; 100 μ l volume; n=6 mice *per* dose) via retro-orbital intravenous injection. 24h after infusion, blood samples were taken under anesthesia via retro-orbital puncture from the opposite eye of where the protein was infused. Plasma was prepared and analyzed for the presence of human IgG using an immunosorbent assay. Briefly, diluted plasma samples were incubated in wells coated with a monoclonal anti-human IgG Fc antibody (Clinisciences, Nanterre, France). Bound Emicizumab was probed using a peroxidase-labeled anti-human IgG₄ Fc antibody (Abcam, Paris, France) and detected via peroxidase-mediated hydrolysis of 3,3',5,5'-tetramethylbenzidine. Murine plasma spiked with known concentrations of Emicizumab was used as reference.

Emicizumab-compatible mouse model

Mice were given Emicizumab (0-10 mg/kg; 100 μ l volume; n=6-18 per dose) via retro-orbital intravenous injection 24h before the tail amputation. A second retro-orbital injection was given 5 min before the tail amputation procedure, using a solution containing human FIX and FX (both 100 U/kg, unless indicated otherwise). Mice were then anesthetized using ketamine (100 mg/kg)/xylazine (10 mg/kg) solution before 3 mm of the distal tip of the tail was amputated. The amputated tail was immersed in prewarmed physiological saline (37°C) and blood was collected for 30 min. The mixture of collected blood and physiological saline was centrifuged at 1,500g. The red blood cell pellet was then lysed in H₂O and the amount of hemoglobin was determined by reading the absorbance at 416nm. The volume of blood loss in each sample was calculated from a standard curve, which was obtained by lysing defined volumes of mouse blood (20-40-60-80-100 μ L) in H₂O to extract hemoglobin.

In some control experiments, mice were given Emicizumab with FIX alone or FX alone. For some mice (n=3-5), blood samples were taken after infusion of the Emicizumab/FIX/FX-cocktail (with Emicizumab given 24h and FIX/FX given 5 min before blood collection), while these mice were not exposed to the tail clip assay. These samples were used to measure FIX and FX antigen levels, and to test hemostatic activity in the activated Partial Thromboplastin Time-assay (aPTT) and FXIa-induced thrombin generation assay.

FVIII-mediated correction of bleeding

Mice were given FVIII (0-10 U/kg; 100 μ l volume; n=9-18 per dose) in a solution that also contained human FIX and FX (both 100 U/kg) via retro-orbital injection. The tail clip bleeding assay was performed 5 min after infusion as described above for the Emicizumab-compatible mouse model. FVIII activity levels using the 2.5-10 U/kg dosing varied dose-dependently between 5 and 20 U/dL.

Diluted Activated Partial Thromboplastin Time (aPTT)

One stage clotting activity was determined using the STA-PTTa-assay kit (Stago, Asnières, France). Briefly, plasma samples were diluted 4-fold in STA-Owren-Koller buffer (Stago, cat# 00360) to a volume of 50 μ L. Subsequently, 25 μ L of aPTT-reagent were added. Finally, the clotting reaction was started by the addition of a 50 μ L CaCl_2 solution. Analysis was performed on a Diagnostica Stago ST4 Coagulation Analyzer.

FXIa-induced thrombin generation assay

Thrombin generation in platelet-poor murine plasma was measured in a microtiter-plate fluorometer (Fluoroskan Ascent, ThermoLabssystems, Helsinki, Finland) according to the method described by Hemker *et al.*, except that thrombin generation was initiated using phospholipids (4 μ M) and FXIa (175 nM), respectively.¹⁵ Endogenous thrombin potential (ETP; *i.e.* area under the curve) and thrombin peak as read outs for thrombin generation were determined using dedicated software (Thrombinoscope BV, Maastricht, The Netherlands). Samples of the mice that received the Emicizumab/FIX/FX- or FVIII/FIX/FX-cocktail, but did not undergo tail clip bleeding, were used for this assay.

Statistical analysis

All data are presented as mean \pm standard deviation (mean \pm SD) unless indicated otherwise. Number (*n*) refer to the number of independent experiments or animals. The statistical analysis was performed using GraphPad Prism 7 software for Mac (La Jolla, California, USA). One-way analysis of variance (1-way ANOVA) followed by Tukey's or Dunnett's multiple comparison test was performed when comparing multiple groups. $P < 0.05$ was considered as statistically significant.

RESULTS

An Emicizumab-responsive mouse model

To test Emicizumab in murine models, the presence of human FIX and FX is obligatory. Our approach therefore was to first administer Emicizumab (0.5-10 mg/kg) via intravenous infusion into FVIII-deficient mice 24h before the tail clip-procedure. Subsequently, mice received a combined dose of human FIX and FX (both 100 U/kg) 5 min before tail amputation. Emicizumab levels at 24h varied dose-dependently between 0.7 ± 0.1 for the 0.5 mg/kg dose and 18.6 ± 14.1 mg/dL for the 10 mg/kg dose (Fig. 1A). Dosing with 3 mg/kg generated plasma-concentrations of 5.7 ± 0.2 mg/dL, similar to concentrations in patients who receive weekly dosing of 1.5 mg/kg.⁶ Human FIX and FX concentrations were measured in mice that received between 1.5 and 10 mg/kg Emicizumab (n=3/dose), and pooled data revealed FIX and FX levels of 85 ± 6 U/dL (n=12) and 100 ± 11 U/dL (n=12; Fig. 1B), respectively, at the time of the tail amputation. No differences in FIX and FX levels between Emicizumab doses were observed. Circulating ternary Emicizumab/FIX/FX complexes were calculated to range between 129pM and 1270pM, based on the actual Emicizumab, human FIX and FX levels at the time of the intervention (Fig. 1C).

In vitro analysis of Emicizumab-treated FVIII-deficient mice

To investigate potential Emicizumab cofactor activity under these conditions, murine plasmas were analyzed for FVIII-like activity in a diluted aPTT-assay and in a FXIa-dependent thrombin generation assay (FXIa-TGA). Diluted aPTT-clotting times were shortened dose-dependently and clotting-times in the normal range were obtained at doses of 3 mg/kg or more (Fig. 2A). Apparently, Emicizumab fully corrects for FVIII-deficiency in the diluted aPTT, akin to what is reported previously.^{10,11,16} This activity in this clotting assay is generally considered as an overestimation of the true FVIII-equivalence of Emicizumab.^{10,11,16} As for FXIa-TGA, Emicizumab promoted thrombin generation also in this assay (Fig. 2B). Maximal endogenous thrombin potential was 511 ± 82 nM/min at 10 mg Emicizumab/kg, similar to that observed for mice that received 5 U FVIII/kg (622 ± 107 nM/min; p=0.149; Fig. 2B-C; Table 1). In contrast, peak thrombin levels were significantly lower for Emicizumab (42.2 ± 5.1 nM for 10 mg Emicizumab/kg and 58.3 ± 9.3 nM for 5 U FVIII/kg, respectively; p=0.0172; Fig. 2B-C; Table 1). Together, these data highlight that *in vitro* assays give very different FVIII-equivalent values for Emicizumab.¹⁰ Nevertheless, co-infusing Emicizumab with human FIX/FX allows for Emicizumab-enhanced hemostasis in this model.

Dose-response FVIII in a tail-clip bleeding model

To compare Emicizumab with FVIII in the tail clip-model, we first performed a dose-response study using FVIII. Mice received 2.5-10 U FVIII/kg, resulting in plasma levels of 5-20 U

FVIII/dL (not shown). To remain completely in line with mice receiving the FIX/FX/Emicizumab-cocktail, mice were given FVIII in combination with human FIX/FX. To evaluate whether the presence of additional human FIX and FX would affect the activity of FVIII, a similar dose-response was also performed in the absence of human FIX and FX. Five minutes after infusion of FVIII (with or without human FIX/FX), the tail tip was amputated. Mice receiving only human FIX/FX displayed a strong bleeding tendency (blood loss $852 \pm 139 \mu\text{L}$; mean \pm SD; $n=15$; Fig. 3A). This amount was unaffected upon infusion of the lowest FVIII dose (2.5 U/kg: $809 \pm 61 \mu\text{L}$; $n=6$; $p=0.979$). However, incremental doses of FVIII (5-7.5-10 U/kg) induced a dose-dependent decrease in blood loss (Fig. 3A). No statistical difference in blood loss was observed for the 7.5 and 10 U/kg dose ($174 \pm 219 \mu\text{L}$ and $34 \pm 34 \mu\text{L}$; $n=8$; $p=0.389$), suggesting that blood loss is maximally corrected. The half-maximal effective dose was calculated to be 5.3 U/kg (Fig. 3B). As depicted in Fig. 3B, a similar dose-response was observed in the absence of human FIX and FX, with the half-maximal effective dose being $4.6 \pm 0.5 \text{ U/kg}$ ($p=0.279$ compared to the presence of human FIX/FX).

Dose-response Emicizumab in a tail-clip bleeding model

We next analyzed mice infused with Emicizumab. Mice receiving Emicizumab with human FIX or FX alone but not both, displayed similar blood loss as mice receiving FIX/FX without Emicizumab ($862 \pm 47 \mu\text{L}$ ($n=3$; $p=1.0$) and $817 \pm 44 \mu\text{L}$ ($n=3$; $p=0.99$) versus $852 \pm 139 \mu\text{L}$ ($n=15$); Fig. 4A). A similar extent of blood loss was also observed for the lowest dose of Emicizumab tested (0.5 mg/kg; $849 \pm 221 \mu\text{L}$; $n=8$; $p=1.0$; Fig. 4B). In contrast, a significant reduction in blood loss was observed in mice receiving any Emicizumab dose between 1.5 mg/kg and 10 mg/kg (Fig. 4B). Interestingly, blood loss was not significantly different between these Emicizumab doses tested, *i.e.* 1.5, 3, 5 and 10 mg/kg ($p=0.34$). Furthermore, the bleeding profiles were also similar between these doses of Emicizumab (Supplementary Fig. S1).

Since Emicizumab activity is restricted by the concentrations of FIXa and FX available⁵, we tested the Emicizumab-mediated response at different doses of FIX and FX. Compared to the dose of 100 U/kg used throughout the study, no differences were observed with regard to doses of 50 and 200 U/kg: $700 \pm 235 \mu\text{L}$ ($p=0.091$) for 50 U/kg and $639 \pm 65 \mu\text{L}$ ($p=0.531$) for 200 U/kg, compared to $498 \pm 217 \mu\text{L}$ for 100 U/kg (Fig. 4C). In contrast, significant more blood loss was observed at a dose of 25 U/kg ($874 \pm 67 \mu\text{L}$; $p=0.0006$ compared to 100 U/kg), an amount similar to that of vehicle-treated mice ($852 \pm 139 \mu\text{L}$).

Together, these data show that this model may be used to analyze Emicizumab-mediated hemostasis in an unbiased manner, and Emicizumab shows relevant cofactor-like activity

under these *in vivo* conditions. By using the dose-response curve for FVIII as a reference, we used the average amount of blood loss from Emicizumab-treated mice (1.5-10 mg/kg) to calculate a FVIII-equivalence in this model. The combined average blood loss was 566 ± 177 μL (n=37), and interpolating this amount into the FVIII-reference curve revealed a FVIII equivalence of 4.5 ± 1.3 U/kg (which relates to a FVIII plasma concentration of 9.0 ± 0.3 U/dL).

Combining Emicizumab with FVIII

Compared to Emicizumab, FVIII has a superior affinity for FIXa⁵, and it is often assumed that FVIII will outcompete Emicizumab during coagulation when both FVIII and Emicizumab are present. We investigated therefore how hemostasis in FVIII-deficient mice is affected when FVIII and Emicizumab are applied together. As shown in Figs. 3 and 4, a single dose of 5 U FVIII/kg or 3 mg Emicizumab/kg is insufficient to fully correct bleeding. We therefore tested what would happen if both were given simultaneously (Fig. 5). Interestingly, when a FVIII dose of 5 U/kg was combined with a Emicizumab dose of 3 mg/kg, blood loss was corrected to the same extent as observed for a FVIII dose of 7.5 U/kg or higher (Fig. 5A). No further reduction in blood loss was observed when Emicizumab was combined with a 7.5 U FVIII/kg dose (Fig. 5A). A potential pitfall of this experiment, is that FVIII would be able to associate with both human and murine FIXa, whereas Emicizumab is restricted to its interaction with human FIXa. To test whether this would affect the outcome, we designed an *in vitro* FIXa-induced thrombin generation experiment using human FVIII-deficient plasma, to which we added purified recombinant murine FIX (Fig. 5B). Compared to Emicizumab (20 $\mu\text{g/mL}$) or FVIII (0.1 U/mL) alone, thrombin generation was increased when combining both proteins (Fig. 5B). The addition of murine FIX (1 U/mL) to the mixture containing FVIII and Emicizumab did not further modify thrombin generation. In conclusion, Emicizumab may provide additional hemostatic support to the tenase-complex at low FVIII concentrations. Indeed, when present in excess over FVIIIa, FIXa that is not incorporated in the tenase-complex remains available for Emicizumab, and may therefore contribute to FXa generation. Of course, the Emicizumab/FIXa/FX-complex will be less efficient than the tenase complex in doing so.

DISCUSSION

Given the impact that Emicizumab has on the management of hemophilia A, it is surprising that accessible animal models are lacking so far. This limitation is mostly due to the species-restricted interaction between Emicizumab and its ligands, FIX and FX. To fill up this gap, we here present a simple and straight-forward mouse model, which allows to test Emicizumab *in vivo*. The model is based on the intravenous infusion of Emicizumab in combination with human FIX and FX. This combination indeed results in a significant reduction in blood loss compared to vehicle-treated mice, at least when doses between 1.5 and 10 mg/kg Emicizumab are being used.

A single intravenous infusion of Emicizumab 24h before the tail clip intervention results in reproducible and dose-dependent levels of the antibody (Fig. 1). FIX and FX levels were dosed such that they were within the normal range (0.85 U/dL and 1.0 U/dL, respectively). Based on the respective Emicizumab, FIX and FX concentrations used in this study, the amount of circulating ternary complex varies between 128 pM for the lowest Emicizumab concentration and 1270 pM for the highest Emicizumab concentration (Fig. 1C). Increasing Emicizumab concentration would not lead to increased levels of ternary complex (red dots in Fig. 1C), and could even lead to reduced complex formation. This bell-shape curve is due to the fact that if the molar excess of Emicizumab is getting bigger, there will be fewer Emicizumab-molecules that will bind both FIX and FX simultaneously.

Whereas Emicizumab levels are expected to remain relatively stable during the 30-min tail clip intervention because of its long half-life, this is less so for concentrations of human FIX and FX. FIX and FX have half-lives that exceed 3-4h in mice,^{17,18} and it is thus anticipated that less than 30% of FIX and FX will be removed during the 30-min experiment. To test whether the amounts of FIX and FX that remain would be sufficient, we performed experiments in which mice were given half the amount of FIX and FX, *i.e.* 50 U/kg instead of 100 U/kg. Blood loss was similar between 50 and 100 U/kg doses (Fig. 4). This suggests that when giving a 100 U/kg dose, and even if 50% of FIX and FX would be eliminated during the 30-min observation period, there would still be sufficient amounts of FIX and FX remaining to mediate Emicizumab-dependent hemostasis.

The opposite was true when FIX and FX were given a 25 U/kg dose, with blood loss being similar to that of vehicle-treated mice (Fig. 4). Apparently, a minimal amount of FIX and FX is needed to support Emicizumab activity. By using the calculation for ternary complexes, it appears that a minimum of 260 pM ternary complex is needed to achieve a reduction in blood loss, at least in this model (Table 2). It is of interest to speculate on the consequences of these data in view of individuals with reduced FIX and FX levels, in particular newborns. Newborns have FIX and FX levels being reduced 40-60% during the first month of their

life.^{19,20} These levels restrict ternary complex formation, and will remain below 250 pM. It is possible therefore, that Emicizumab will be less efficient in these patients.

It was of interest to observe that at a low dose of FVIII, in itself too low to fully correct the bleeding, combined with Emicizumab resulted in a complete correction of blood loss. Apparently, there is an additive effect of both components under these conditions. One could argue that FVIIIa has a much higher affinity than Emicizumab for FIXa, and will thus out-compete Emicizumab. Nevertheless, it is reasonable to assume that the amount of FVIIIa available (in our case 0.03 nM, if all of the FVIII is being activated) is much less than the amount of FIXa. If 5% of all FIX, both human and murine, is activated then this would accumulate to 8.3 nM (4.5 nM murine and 3.8 nM human FIXa). Thus, even if all human FIX is occupied by FVIIIa, then this will not affect complex formation between Emicizumab and human FIXa. Indeed, we noticed that doubling the FIX concentration did not affect thrombin generation in the presence of a low dose FVIII and Emicizumab (Fig. 5B).

Our proposed model is, like many other animal models, not without limitations. First, in contrast to the human system, only FIX and FX are of human origin in this mouse model. Moreover, murine FIX and FX are still present. The murine and human proteins may behave differently during the hemostatic process. It should be noted that both human FIX and FX are able to correct the deficiencies of their murine orthologues in the respective mouse knock out-models^{21,22}, suggesting that the proteins are fully functional in the murine context. We analyzed this further in a diluted aPTT-assay, in which we compared the dose response of murine plasma containing only murine FIX with murine plasma containing equal amounts of human and murine FIX (Supplementary Fig. S2). A similar dose-response was observed, suggesting that both human and murine FIX are reacting similarly in this assay.

A second limitation is that the functionality of Emicizumab has been tested only in the tail clip-model. Emicizumab activity is driven by the amount of FIXa generated, and this amount may be different dependent on the severity of the injury and the location of the injury. We are currently exploring the hemostatic potential of Emicizumab in other models with injuries at different locations and with different severities.

Finally, as mentioned above in this Discussion, levels of human FIX and FX are transient, and do not allow long-term follow up. However, there are strategies to obtain stable levels over a longer period of time. Attempts to generate knock in-mice that express human FIX and FX have only been partially successful, as these mice express strongly reduced levels of both proteins (10-20%).²³ These levels are too low to form sufficient amounts of ternary Emicizumab complexes. Alternatively, FIX and FX can be produced over a longer period of time following hydrodynamic injection (approximately 2 weeks stable production) or by using viral vectors (e.g. Adeno-associated virus or lentivirus). The issue here is that the protein expression levels are often highly variable, and difficult to predict. However, our data using

different doses of FIX and FX indicate that levels of FIX (85 U/dL) and FX (100 U/dL) can be halved or doubled without any major consequences.

In conclusion, we provide a relatively straightforward and robust mouse model that can be used to assess the *in vivo* action of Emicizumab (or variants thereof) alone or in combination with other procoagulant agents.

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Conflict of interest:

PJL received honoraria/speakers fee from Biotest, Chugai, Roche, Sobi and Takeda. The other authors have no relevant conflict of interest to declare.

Author contributions:

SF, VM, CK performed experiments.

SF, IP, CC, ODC, PJL and CVD analyzed data.

ODC, AN, PJL and CVD conceived the study.

AK, ODC, PJL and CVD supervised the study.

PJL and CVD wrote the manuscript.

All authors contributed to the editing of the final manuscript.

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Figure 1: Basic properties of Emicizumab-based mouse model

Panel A: Mice received Emicizumab (0-10 mg/kg) via retro-orbital infusion. Plasma samples obtained 24h after infusion were used to determine circulating Emicizumab levels, expressed as mg/dL. Each data point represents an individual mouse. *Panel B:* FVIII-deficient mice previously infused with Emicizumab (1.5-10 mg/kg), received a cocktail containing human FIX and FX (both 100U/kg) via retro-orbital infusion. Plasma samples obtained 5 min after infusion were used to determine circulating levels of human FIX (left Y-axis) and FX (right Y-axis) antigen, expressed as U/dL. Each data point represents an individual mouse, and pooled data from the various Emicizumab doses are shown. *Panel C:* Based on the reported affinity of Emicizumab for FIX (1.85 μ M) and FX (1.58 μ M) and the respective concentrations of Emicizumab (0.7-18.6 mg/dL), FIX (85 U/dL) and FX (100 U/dL) in our model (see *panels A-B*), the concentration of the ternary Emicizumab/FIX/FX complex for each Emicizumab dose used in this study (blue symbols) was calculated as described.⁴ Higher doses of Emicizumab do not further increase ternary complex formation, and may even lead to reduced ternary complex formation (red symbols). In contrast, increasing FIX and/or FX concentrations would allow for higher concentrations of ternary complexes (e.g. black symbol: 9.6 mg/dL Emicizumab, 200 U/kg FIX, 100 U/kg FX)

Figure 2: *In vitro* analysis of Emicizumab activity in murine plasma samples

Panel A: Plasma samples obtained from FVIII-deficient mice receiving Emicizumab combined with human FIX/FX were analyzed for procoagulant activity in a diluted aPTT-assay. Clotting times for each Emicizumab-dose are presented. Data represent mean \pm SD of 3-9 samples. Clotting times of murine FVIII-deficient plasma spiked with known FVIII concentrations are indicated as dotted blue lines for comparison. *Panels B-C:* Plasma samples obtained from FVIII-deficient mice receiving Emicizumab combined with human FIX/FX (*panel B*) or FVIII combined with human FIX/FX (*panel C*) were analyzed in a FXIa-induced thrombin generation assay. Data represent mean of 3-5 mice. Thrombin generation in the absence of Emicizumab or FVIII (black dotted line) is the same for both panels. Data on lag-time, endogenous thrombin potential and thrombin peak are presented in Table 1.

Figure 3: *In vivo* dose-response of FVIII in FVIII-deficient mice

Panel A: FVIII-deficient mice received a mixture of FVIII (0-10 U/kg), human FIX (100 U/kg) and human FX (100 U/kg) via retro-orbital infusion. Five minutes after infusion, the terminal 3mm of the tail was amputated, and shed blood was collected for 30 min. Presented is blood loss over 30 min *versus* FVIII dose. Each data-point represents an individual mouse. Statistical analysis was performed via 1-way Anova with Dunnett's multiple comparison.

Panel B: Data presented in panel A but depicted as mean±SD (n=6-9; blue symbols). A similar dose-response curve was generated in the absence of human FIX and FX (n=6 per dose; red symbols).

Figure 4: Emicizumab activity in FVIII-deficient mice

Panel A: FVIII-deficient mice were given incomplete mixtures of Emicizumab (3 mg/kg given 24h before tail amputation) and FIX/FX (both 100 U/kg given 5 min before tail amputation), in which one of these proteins was missing. The tail bleeding was executed as described in the legend of Fig. 3. Data represent individual mice. *Panel B*: FVIII-deficient mice were given Emicizumab (0-10 mg/kg given 24h before tail amputation) and FIX/FX (both 100 U/kg given 5 min before tail amputation). Presented is blood loss over 30 min *versus* Emicizumab dose. Data represent individual mice. Data for 0 mg/kg Emicizumab are similar as those presented in panel 3A. *Panel C*: Emicizumab (5 mg/kg) was given to FVIII-deficient mice in combination with FX (100 U/kg) and FIX (100 or 200 U/kg) prior to tail bleeding as described for *panel B*. Data for 0 mg/kg Emicizumab and for 100 U FIX-FX/kg data are similar to those presented in *panel B*. Statistical analysis was performed via 1-way Anova with Dunnett's multiple comparison.

Figure 5: Combined activity of FVIII and Emicizumab

Panel A: Emicizumab (3 mg/kg given 24h before tail bleeding) and FVIII (5 or 7.5 U/kg given 5 min before tail bleeding) were given separately or in combination together with human FIX/FX (100 U/kg) to FVIII-deficient mice as described for Figures 3-4. Each data-point represents an individual mouse. Data for FVIII without Emicizumab are similar to those presented in Fig. 3A, and data for Emicizumab without FVIII are similar to those presented in Fig. 4B. Statistical analysis was performed via 1-way Anova with Tukey's multiple comparison. Data of mice receiving only FIX/FX (but no Emicizumab or FVIII) are similar as those presented in Fig. 3A & 4B. *Panel B*: FXIa-induced thrombin generation of human FVIII-deficient plasma (orange), which was spiked with Emicizumab (20 µg/mL; blue), FVIII (10 U/dL; green) or both in the absence (red) or presence (black dotted line) of recombinant murine FIX (100 U/dL). Thrombogram is representative for three independent experiments.

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Table 1: Thrombin generation parameters

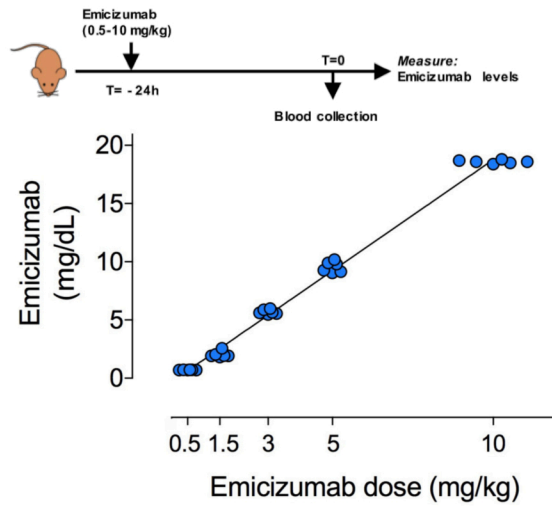
Protein	Dose (mg/kg)	n	Lagtime (min)	ETP (nM/min)	Thrombin peak (nM)
--	N/A	8	2.3±0.3	73±19	7.6±1.2
FVIII	2.5	3	2.7±0.2	371±106	32.7±5.5
	5.0	3	2.0±0.3	622±107	58.7±9.1
	7.5	3	2.0±0.4	692±41	68.3±2.9
	10	3	2.2±0.3	827±113	85.7±11.0
Emicizumab	1.5	3	3.6±1.2	146±78	10.8±2.8
	3.0	4	2.5±0.4	374±107	27.5±9.3
	5.0	3	2.5±0.4	349±111	33.0±9.2
	10	5	1.9±0.2	511±82	42.2±5.1

N/A: not applicable; ETP: endogenous thrombin potential. Thrombograms are depicted in Fig. 2B-C

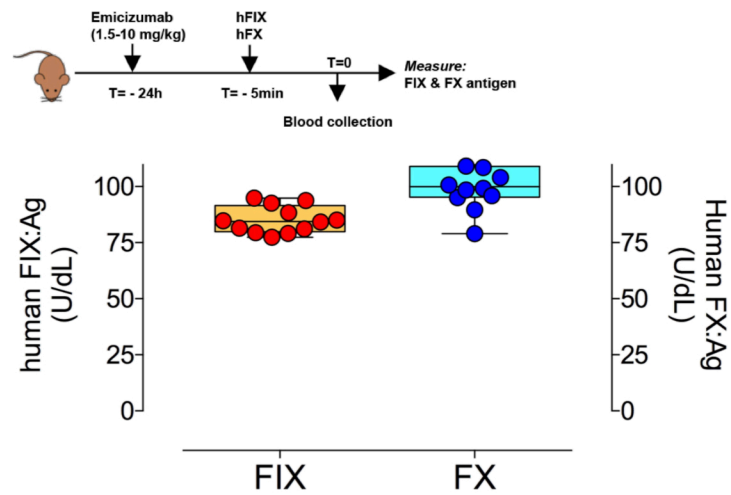
Table 2: Relation ternary complex formation and hemostatic potential

Emicizumab (mg/kg)	FIX (U/kg)	FX (U/kg)	Estimated Ternary complex (pM)	Reduced blood loss?
0	100	100	0	No
5	0	100	0	No
5	100	0	0	No
0.5	100	100	129	No
1	100	100	333	Yes
3	100	100	748	Yes
5	100	100	1001	Yes
10	100	100	1270	Yes
5	25	25	66	No
5	50	50	258	Yes
5	200	100	1961	Yes

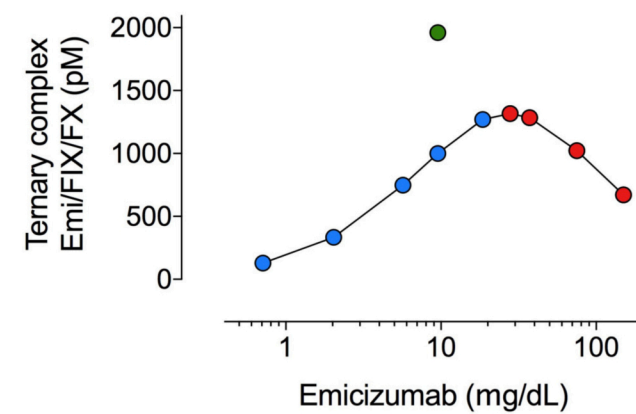
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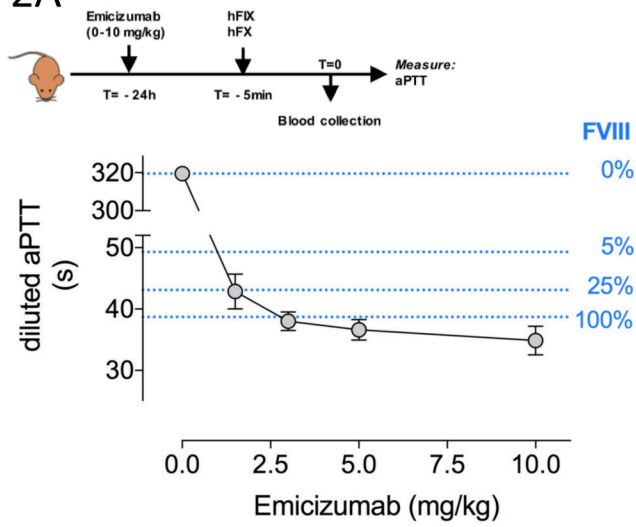
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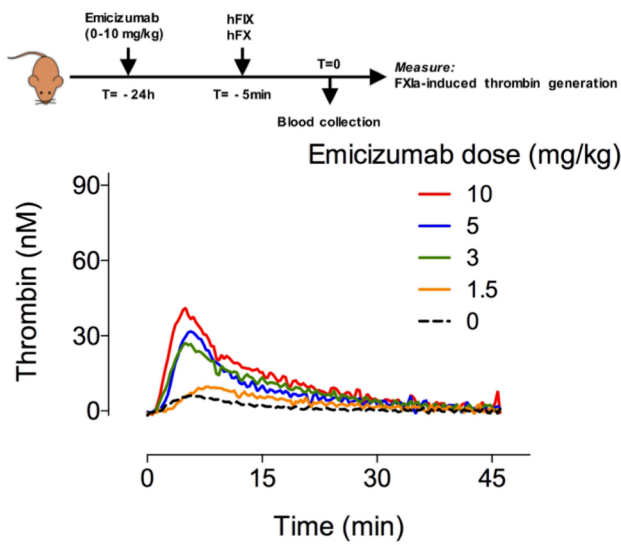
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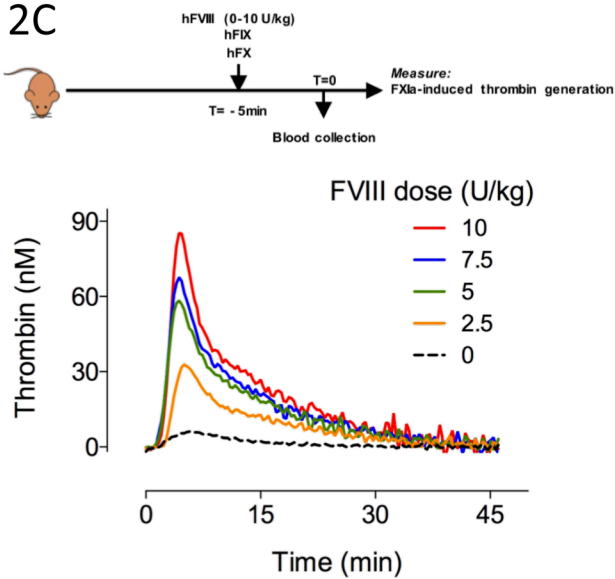
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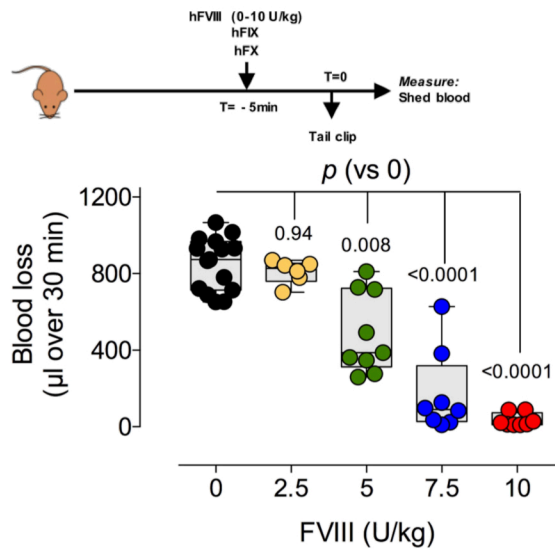
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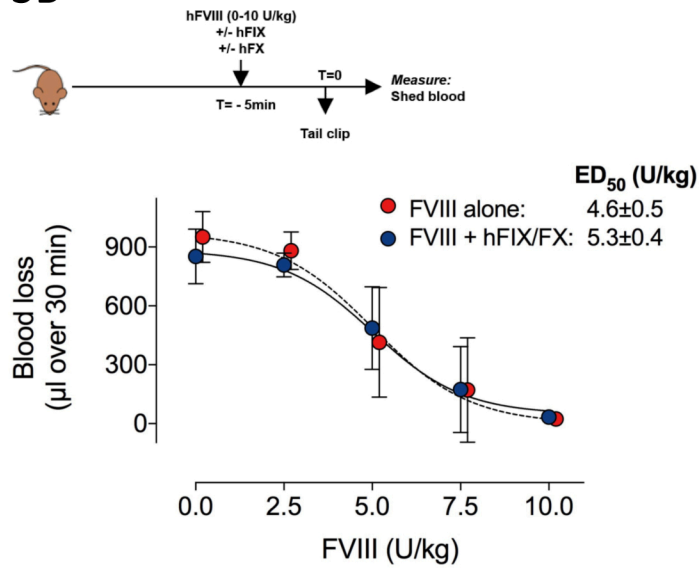
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3A



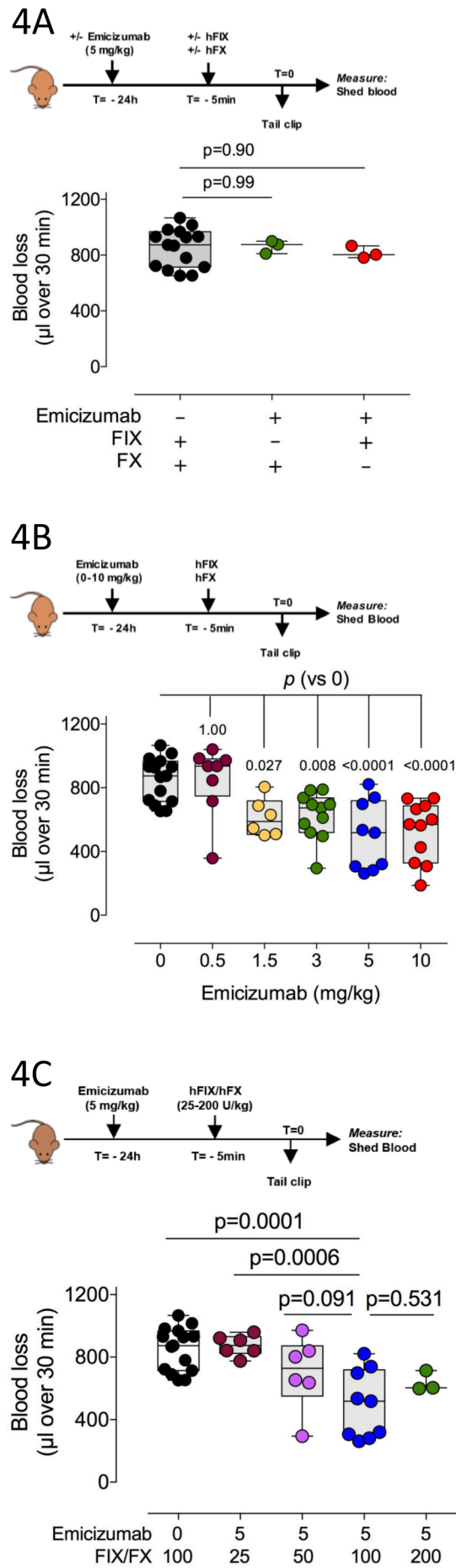
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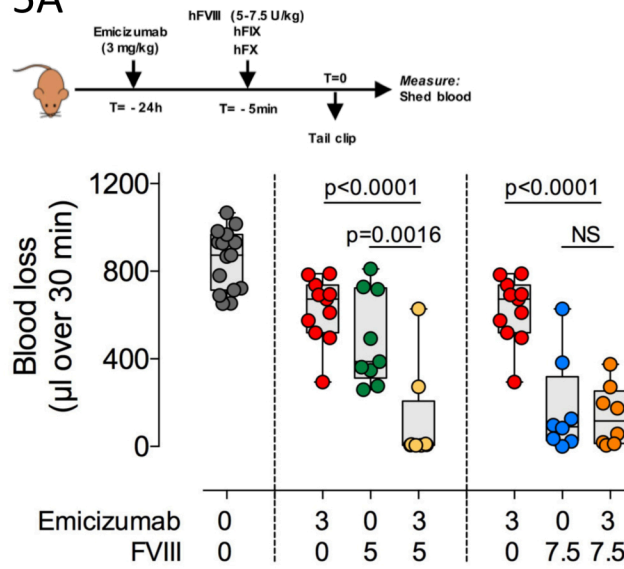
Emicizumab cofactor activity in FVIII-deficient mice

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Figure 4



5A



5B

