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ORIGINAL ARTICLE

VhH anti-thrombomodulin clone 1 inhibits TAFI activation and enhances fibrinolysis in human whole blood under flow

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Abstract

Background: Thrombomodulin on endothelial cells can form a complex with thrombin. This complex has both anticoagulant properties, by activating protein C, and clot-protective properties, by activating thrombin-activatable fibrinolysis inhibitor (TAFI). Activated TAFI (TAFIa) inhibits plasmin-mediated fibrinolysis.

Objectives: TAFIa inhibition is considered a potential antithrombotic strategy. So far, this goal has been pursued by developing compounds that directly inhibit TAFIa. In contrast, we here describe variable domain of heavy-chain-only antibody (VhH) clone 1 that inhibits TAFI activation by targeting human thrombomodulin.

Methods: Two llamas (*Lama Glama*) were immunized, and phage display was used to select VhH anti-thrombomodulin (TM) clone 1. Affinity was determined with surface plasmon resonance and binding to native TM was confirmed with flow cytometry. Clone 1 was functionally assessed by competition, clot lysis, and thrombin generation assays. Last, the effect of clone 1 on tPA-mediated fibrinolysis in human whole blood was investigated in a microfluidic fibrinolysis model.

Results: VhH anti-TM clone 1 bound recombinant TM with a binding affinity of 1.7 ± 0.4 nM and showed binding to native TM. Clone 1 competed with thrombin for binding to TM and attenuated TAFI activation in clot lysis assays and protein C activation in thrombin generation experiments. In a microfluidic fibrinolysis model, inhibition of TM with clone 1 fully prevented TAFI activation.

Discussion: We have developed VhH anti-TM clone 1, which inhibits TAFI activation and enhances tPA-mediated fibrinolysis under flow. Different from agents that directly target TAFIa, our strategy should preserve direct TAFI activation via thrombin.

1 | INTRODUCTION

To prevent excessive fibrin accumulation, thrombin formation is controlled by several anticoagulant pathways. The transmembrane receptor thrombomodulin plays an essential role in maintaining the hemostatic balance. Thrombomodulin is expressed by vascular endothelial cells and is continuously exposed to circulating blood. Thrombomodulin captures circulating thrombin, forming the thrombomodulin-thrombin complex, thereby altering thrombin's substrate specificity.[1] When in complex with thrombomodulin, thrombin loses its ability to convert fibrinogen into fibrin. Instead, it activates protein C, which then inhibits the formation of thrombin by inactivating the coagulation cofactors Va and VIIIa.[2] From this perspective, the thrombomodulin-thrombin complex has an anticoagulant function. However, the complex also has clot-protective properties, as it can activate thrombin-activatable fibrinolysis inhibitor (TAFI).[3] TAFI is produced in the liver as a zymogen and acts as an inhibitor of fibrinolysis upon activation.[4] Fibrinolysis is mediated by the enzyme plasmin. During fibrinolysis, plasmin exposes carboxy-terminal lysine residues in fibrin fibers through limited proteolysis.[5] These lysine residues act as binding scaffolds for plasminogen and enhance plasmin generation up to 1000-fold. Activated TAFI (TAFIa) removes these lysine residues and consequently abrogates this feedback loop.[6] From this perspective, the thrombomodulin-thrombin complex slows down fibrinolysis and has a procoagulant function. Whether the anti- or procoagulant properties of thrombomodulin prevail is directly related to its concentration.[7]

Thrombomodulin consists of various domains: a C-type lectin like domain, six epidermal growth factor (EGF)-like domains, a serine-threonine-rich region, a transmembrane region, and a short cytoplasmic tail. The divergent properties of thrombomodulin involve distinct structural regions on the EGF domains. Thrombin binds to EGF-like domains 5 and 6[8]; protein C and TAFI both bind regions on EGF domain 4,[9] and TAFI also binds EGF domain 3.[10-12] Inhibiting TAFI activation is considered a potential antithrombotic strategy as it improves physiological fibrinolysis:[5,13] first because elevated TAFIa levels have been associated with an increased risk for deep vein thrombosis, angina pectoris, and coronary artery disease;[14,15] and second because inhibiting TAFI activation could reduce dosing of the conventional therapeutic plasminogen activator tPA, which bears an inevitable risk for therapy-related bleeding.[16]

On a mechanistic level, TAFI inhibition can be performed either by directly blocking TAFIa or by blocking thrombomodulin-mediated TAFI activation.[5] Several strategies have been reported for direct inhibition of TAFIa, involving antibodies,[17,18] small molecules[19] and variable domain of heavy-chain-only antibodies (VhHs).[13] VhHs are specifically interesting because they have a

ESSENTIALS

- The thrombomodulin-thrombin complex inhibits plasminogen activation by activating TAFI.
- Blocking TAFI activation is considered an attractive strategy to enhance fibrinolytic therapies.
- We developed VhH anti-thrombomodulin clone 1 that inhibits activation of TAFI.
- Clone 1 enhances fibrinolysis in human whole blood under flow.

high affinity for conformational epitopes, their small size allows for improved penetration of target tissue[5] and they lack the Fc-domain of conventional antibodies, limiting the risk for triggering an endogenous immune response.[20] Earlier, a VhH anti-rat TAFI has been described that inhibits fibrin deposition in murine lungs in a thromboembolism model and, in addition, enhances fibrinolytic therapy with tPA.[16] To our knowledge, however, there have not been strategies that inhibit TAFI activation by targeting the thrombomodulin-thrombin complex. It can be argued that interfering early in the activation cascade increases therapeutic effectivity.[21,22] However, it might simultaneously also interfere with protein C activation.[23]

We here describe the development of a VhH targeting human thrombomodulin that prevents TM-mediated TAFI activation, thereby enhancing fibrinolysis in human whole blood.

2 | MATERIALS AND METHODS

2.1 | Subjects

Citrated human pooled normal plasma (PNP) was prepared from fresh venous blood of 170 healthy volunteers. All donors provided written informed consent in accordance with the Declaration of Helsinki. Approval was obtained from the local ethics committee. Fresh whole blood for flow studies was obtained from healthy volunteers through venipuncture, collected in BD Vacutainer trisodium citrate (105 mM) tubes and used within 4 h after collection.

2.2 | Biochemicals

The entire extracellular region of thrombomodulin, spanning from ala19 to his516 and containing the C-type lectin domain, six EGF-like domains, and serine-threonine–rich region, was expressed

with a N-terminal tobacco etch virus -cleavable His$_6$-tag and purified by U-Protein Express BV (Utrecht, the Netherlands). VhHs targeting GP1b (clone 17), fibrin (clone B12), and azo-dye RR6 (clone R2, negative control)[24] were produced in *Escherichia coli* and purified through sequential immobilized metal affinity chromatography and size exclusion chromatography as described.[25] According to instructions of the manufacturers, VhH anti-GP1bα clone 17 was labeled with AlexaFluor488-NHS-ester (Thermo Fisher, Roskilde, Denmark). VhH anti-fibrin clone B12 was labelled with Gly$_3$-azide (IRIS Biotech GmbH, Marktredwitz, Germany) through sortagging and clicked to DBCO-AlexaFluor-647 as described.[20]

2.3 | VhH selection

Two llamas (*Lama Glama*) received four rounds of subcutaneous immunizations with the recombinant extracellular domain of human thrombomodulin at weekly intervals. A bacteriophage library containing the heavy chain only-antibody repertoire of these llamas was created as described.[25] A transformation efficiency of 10^7 was achieved. Subsequently, phage display was used to select VhH anti-TM clones. Hereto, thrombomodulin was biotinylated with EZ-Link Sulfo-NHS-LC-Biotin according to the instructions of the manufacturer (Life Technologies, Carlsbad, CA) and immobilized on a neutravidin-coated PolySorp flat bottom plate (0.5 µg/ml) in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 9.2 mM Na$_2$HPO$_4$, 1.76 mM KH$_2$PO$_4$, pH 7.4) at 4°C overnight. Phages were then panned on thrombomodulin, eluted with 0.1 M triethanolamine, pH 11.5, and allowed to infect *E coli* (TG1), as described.[25] TG1 were plated on yeast tryptone-agar plates containing glucose (2% w/v) and ampicillin (100 µg/ml) and incubated overnight at 37°C. The following day, colonies representing individual VhHs were picked and grown. VhH production was initiated with 0.1 M isopropyl β-d-1-thiogalactopyranoside in 2xYT medium with 0.2% glucose and 100 µg/ml ampicillin and culture supernatants were used for subsequent selection of VhH anti-TM clones. Thrombomodulin was immobilized on Nunc MaxiSorp 96-well microtiter plates (0.5 µg/ml) in PBS at 4°C overnight, after which plates were blocked with 2% skimmed milk (Sigma Aldrich, St Louis, MO, USA). Culture supernatants were incubated on immobilized thrombomodulin. After thorough washing with PBS containing 0.1% Tween-20, plates were incubated with mouse anti-c-myc antibody (clone 9E10, 1 µg/ml) in PBS containing 2% skimmed milk for 1 h at room temperature (RT), washed thoroughly again and incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako Cytomation, Glostrup, Denmark) for 1 h at RT. Plates were washed a final time and incubated with 100 µl/well 3,3′,5,5′tetramethylbenzidine (Tebu-bio, Heerhugowaard, the Netherlands) per well. Colorimetric reactions were stopped with 50 µl 0.1 M H$_2$SO$_4$ and plates were read in a SpectraMax iD3 at 450 nm. VhH clones binding thrombomodulin were sequenced.

2.4 | VhH production and purification

VhH anti-TM with a unique complementarity-determining region 3 were codon optimized and ordered as gBlocks at Integrated DNA Technologies (Coralville, USA). gBlocks were ligated in pJET1.2 vector, cut with restriction enzymes BamHI and NotI (New England Biolabs; Leiden, The Netherlands) and ligated into the pTH4. Click production vector.[20] One-shot Bl21 pLysS (*E coli*) were transformed with VhHs and grown in 2xYT media with 0.2% glucose, 100 µg/ml ampicillin, and 34 µg/ml chloramphenicol. VhH production was initiated with 1 mM isopropyl β-d-1-thiogalactopyranoside, followed by overnight production at 37°C. The following day, bacteria were collected through centrifugation at 5000*g* for 15 min, resuspended in 25 mM HEPES, 500 mM NaCl, pH 7.8, 10 mM MgCl$_2$, and 1 µg/ml DNaseI, after which they were lysed in three sequential freeze-thaw cycles. VhHs were purified from supernatant with immobilized metal affinity chromatography with Talon Superflow Sepharose (Cytiva, Marlborough, USA). Purified VhHs were then subjected to size exclusion chromatography on a Superdex 200 pg column (Cytiva). VhH preparations were >95% pure as confirmed with SDS-PAGE and Coomassie blue staining. Extinction coefficients were calculated for each VhH with the online ProtParam Tool (http://web.expasy.org/protparam/) and concentrations were determined by measuring absorbance at 280 nm.

2.5 | Surface plasmon resonance experiments

Surface plasmon resonance (SPR) experiments were performed on a Biacore T100 system (Cytiva). Then, 400RU of thrombomodulin was immobilized on a CM5 Series S sensor chip with standard amine-coupling chemistry according to the instructions of the manufacturer. Flow channel 1 received a blank immobilization and was used as a reference. VhH anti-TM clones were injected at various concentrations as indicated at a flowrate of 30 µl/min in 10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4, for 2 min, followed by a 10-min dissociation phase. Thrombomodulin surfaces were regenerated with 10 mM glycine, pH 2.0, for 1 min at a flowrate of 30 µl/min. Signals obtained in the reference channel were subtracted from the sensorgrams of the channel that contained thrombomodulin. Reference-corrected sensorgrams were fitted with a 1:1 Langmuir binding model with Biacore T100 evaluation software to determine association (K_a), dissociation, and affinity (K_D) constants. Experiments were repeated three times.

2.6 | Binding of VhHs to endothelial cells

A total of 100 µl VhH anti-TM clone 1 and VhH R2 (functioning as isotype control) was incubated with 900 µl of Alexa Fluor 647 NHS Ester (Thermo Fisher Scientific, Waltham, USA) and 100 µl NaHCO$_3$ (0.1 M, pH 8.3) for 1 h at RT in dark (molar ratio VhH:Dye of 1:40).

Free label was removed by ZebaSpin (Thermo Scientific). Human umbilical vein endothelial cells were isolated from umbilical cords by trypsinization as described[26] and cultured in Endothelial Cell Growth Medium-2, supplemented with Supplement Mix (C-39216) and penicillin-streptomycin, in a T-75 cell culture flask at 37°C, 5% CO_2. Medium was refreshed every 2 to 3 days. Flow cytometry experiments were performed when cells were in passages 2 through 4. Then, cells were washed with PBS (2×) and detached with 20 mM EDTA. Subsequently, cells were centrifuged at $300g$ for 5 min and resuspended in fresh medium. Either 1, 3, or 5 µg of VhH anti-TM clone 1 or VhH R2 was added to 10^5 cells in 100 µl and incubated for 45 min at RT. Unbound VhHs were removed by spinning down cells, removing the supernatant, and resuspending cells in Dulbecco PBS (Sigma Aldrich). Cells were diluted (1:6) with fixative solution (137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH_2PO_4, 1.15 mM KH_2PO_4, 10.2 mM Na_2HPO_4, 4 mM EDTA, 1.11% formaldehyde, pH 6.8). Flow cytometry experiments were performed on a BD FACS Canto II (BD Biosciences, San Jose, CA, USA). Cells were gated based on forward and side scatter. Binding of VhHs was expressed as median fluorescent intensity measured in the APC channel. Signals obtained with VhH R2 isotype control were subtracted to correct for background fluorescence.

2.7 | Competition assay

Thrombomodulin (50 µl of 0.5 µg/ml) was coated on a MaxiSorp microtiter plate in PBS at 4°C overnight. Plates were blocked with 1% bovine serum albumin (BSA) (w/v) in PBS for 1 h at RT and then washed three times with 0.1% Tween-20 in PBS. To test competition with thrombin for binding to TM, human α-thrombin (15 nM; ERL, South Bend, IN, USA) was incubated with unlabeled VhH anti-TM clone 1 or VhH R2 (control) at indicated concentrations for 1 h at RT. Plates were washed and incubated with goat anti-human thrombin (1:1000; Affinity Biologicals, Lancaster, Canada) for 1 h at RT, washed again, and incubated with horseradish peroxidase-conjugated rabbit anti-sheep IgG (1:2000; Dako Cytomation, Glostrup, Denmark) for 1 h at RT. Plates received a final washing step and were incubated with 3,3′,5,5′tetramethylbenzidine and H_2SO_4 as indicated earlier. Absorbance was measured in a SpectraMax iD3 at 450 nm. Data were presented as residual binding of either thrombin, VhH anti-TM clones 1 or 3, or VhH R2 (control). Inhibition data were analyzed with nonlinear regression in GraphPad Prism 8.0.1.

2.8 | Clot lysis assay

Clot lysis assays were performed in PNP in a microtiter plate with or without thrombomodulin. A titration range of VhH anti-TM clone 1 or VhH R2 (control) was added at final concentrations as indicated. To induce coagulation 50 µl PNP was incubated with 50 µl coagulation buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 20 mM $CaCl_2$, pH 7.4) with 0.1% BSA, containing 10 nM thrombomodulin, 0.5 pM tissue factor (TF, Dade Innovin; Siemens, Marburg, Germany), 4µM PS:PC:PE phospholipids vesicles in 30:20:50 ratio (Coag reagent I; Avanti polar lipids, Alabaster, USA), 2 nM tPA (Actilyse; Boehringer Ingelheim, Alkmaar, the Netherlands), and VhHs (at indicated concentrations) or 25 µg/ml PCPI (Sigma Aldrich, Zwijndrecht, the Netherlands) where indicated. Changes in absorbance at 405 nm were measured in a SpectraMax iD3 at 37°C for 2 h at 20-s intervals. Clot lysis times were determined from the clot-lysis turbidity profile and defined as the time from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing the lysis of the clot.[27] Inhibition data were analyzed with nonlinear regression in GraphPad prism 8.0.1. Where indicated, data were presented as relative (%) inhibition of TAFI activation, in which 0% inhibition was equal to clot lysis times (CLTs) in the presence of thrombomodulin and 100% inhibition was equal to the CLT in the absence of thrombomodulin in control samples without VhHs.

2.9 | Thrombin generation assay

Thrombin generation was measured with the Calibrated Automated Thrombogram method as described,[28] with a few modifications. In short, 60 µl of PNP with or without 25 nM thrombomodulin was incubated with 38 µl of VhH anti-TM clones or VhH R2 (control) at indicated concentrations in Immulon 2 HB microtiter plates with 7 µl of reagent mix containing 0.5 pM TF and 4 µM phospholipids in HBS with 0.1% BSA for 10 min at 37°C. Next, thrombin generation was initiated with 15 µl of FluCa (100 mM $CaCl_2$, 2.5 mM z-Gly-Gly-Arg-AMC; Bachem, Bubendorf, Switzerland) in HBS with 0.1% BSA. Thrombin-α2-macroglobulin (20 µl) complexes (Thrombinoscope BV, Maastricht, the Netherlands) and 25 µl HBS were added to calibrator wells. Fluorescence was measured in a SpectraMax iD3 with excitation at 390 nm, emission at 475 nm at 15-s intervals for 1 h. Thrombin generation parameters were deduced from fluorescence data as described[29] and adjusted for calibrator activity. Inhibition data were analyzed with nonlinear regression in GraphPad Prism 8.0.1.

2.10 | Flow studies in human whole blood

Coverslips (20 × 50 mm) were functionalized with aminopropyltriethoxysilane (Sigma Aldrich, Zwijndrecht, the Netherlands) as described,[30] thoroughly washed and dried, coated with a mixture of horm collagen (100 µg/ml; Takeda, Linz, Austria), recombinant human TF (10 pM) and thrombomodulin (10 nM) in HT buffer (145 mM NaCl, 5 mM KCl, 500 nM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM HEPES, pH 7.4) for 1.5 h at RT and subsequently blocked with 1% HSA (w/v) in HT-buffer at 4°C overnight. Next, coverslips were attached to a parallel plate flow chamber with vacuum

as described[31] and mounted in a Zeiss Observer Z1 microscope, equipped with Zeiss Fluar 40×/1.30 objective and Zeiss Filtersets 10 and 50 in combination with LED 470 nm and LED 625 nm, respectively. A DIC filter block in combination with a halogen light bulb was used to take brightfield images. Before the experiment, citrated whole blood was supplemented with tPA (5.7 nM), AlexaFluor488-conjugated VhH anti-GPIbα (clone 17; 190 ng/ml), AlexaFluor647-conjugated VhH anti-fibrin (clone B12; 830 ng/ml), and either VhH anti-TM clone 1 (514 nM), VhH R2 (negative control; 514 nM) or TAFIa inhibitor PCPI (25 µg/ml) and was recalcified with 3.1 mM $MgCl_2$ and 6.6 mM $CaCl_2$. Then, blood was pulled through the flow chamber with a Harvard apparatus syringe pump at a shear rate of 800 s^{-1}. Snap shots were taken at a 40 times magnification with an interval of 20 s to monitor platelet adhesion and fibrin deposition in real time. After 7 min, noncalcified blood samples with same supplements entered the flow chamber, allowing tPA-mediated fibrinolysis to predominate and, consequently, to investigate fibrinolysis without continuous clotting. Fluorescent intensities were analyzed with ZEN Pro software (version 2.0). To correct for inter-donor variability, the maximal absolute fibrin signal from VhH R2 runs (control) for each donor was set at 100% and the maximal signals from VhH anti-TM clone 1 and PCPI (positive control) were presented as percentage thereof.

2.11 | Statistical analysis

Differences between runs in flow studies after supplementing whole blood with VhH anti-TM clone 1, PCPI, or VhH R2 were analyzed with Kruskal-Wallis analysis with multiple comparisons from mean values with GraphPad Prism software 8.0.1. Results were considered statistically significant at $p \leq .05$.

3 | RESULTS

3.1 | Selection and characterization of a high affinity VhH targeting TM

Phage display yielded 49 clones with affinity for thrombomodulin, derived from eight distinct families based on sequence variation. VhH anti-TM clone 1 showed high binding in solid-phase binding assays during the panning procedure (not shown). Subsequently, clone 1 was codon-optimized for production in E coli, produced, and purified. The biophysical properties of clone 1 are listed in Table 1. Binding characteristics were analyzed with SPR (Figure 1A), which indicated that clone 1 has an affinity of 1.7 ± 0.4 nM (K_D) for recombinant thrombomodulin. To confirm reactivity towards native human thrombomodulin, binding of fluorophore-labeled clone 1 to quiescent human umbilical vein endothelial cells was assessed with FACS, showing specific and dose-dependent binding (Figure 1B). In addition, clone 1 fully attenuated thrombin binding to thrombomodulin (IC_{50} 148.6 nM) (Figure 1C).

TABLE 1 Biophysical characteristics

	VhH anti-TM clone 1
Target	Extracellular thrombomodulin domains
Full-sequence amino acids	155
CDR1	GFTFSSYWMY
CDR2	VSKISNGGRDTYYADS
CDR3	SKDSNGLER
MW (Da)	16523.06
Isoelectric point	6.77
Ext. coeff. (280 nm)	1.630
Thrombomodulin binding	
K_D	1.68 ± 0.40 nM
K_a (1/Ms)	$6.11E^5 \pm 3.25E^5$
K_d (1/s)	$9.76E^{-4} \pm 3.78E^{-4}$

CDR, complementarity-determining region; K_a, association constant; K_d, dissociation constant; K_D, affinity constant.

3.2 | Clone 1 interferes with thrombomodulin activity and inhibits both TAFI activation and protein C activation

To determine whether clone 1 influences the procoagulant properties of thrombomodulin, clot lysis experiments were performed in the presence and absence of 10 nM thrombomodulin. Addition of thrombomodulin caused an approximately two-fold lengthening of the CLT. The effect of thrombomodulin on CLT was completely attenuated in the presence of TAFIa inhibitor PCPI, confirming that the thrombomodulin-induced lengthening of the clot lysis time was TAFI dependent (Figure 2A). Clone 1 induced a dose-dependent shortening of the CLT in the presence of thrombomodulin, leading to full inhibition of TAFI activation by targeting thrombomodulin (IC_{50}: 6.1µM) (Figure 2B). In contrast, isotype control VhH R2 had no effect on the CLT. Clot lysis times in the absence of thrombomodulin at the highest concentration of clone 1 showed no significant reduction of the clot lysis time (Figure 2C), indicating that inhibition of TAFI activation was thrombomodulin dependent.

However, the observation that clone 1 interferes with binding of thrombin to recombinant thrombomodulin suggests that it potentially interferes with protein C activation. This hypothesis was assessed with TF initiated thrombin generation assays. Addition of 25 nM thrombomodulin to plasma caused a two-fold reduction in endogenous thrombin potential (ETP), consistent with protein C activation (Figure 2D). Indeed, addition of clone 1 also inhibited activation of protein C (IC_{50}: 4.7 µM) (Figure 2E).

3.3 | Clone 1 enhances fibrinolysis under flow

To investigate whether clone 1 had fibrinolytic properties in flowing blood, we developed a microfluidic fibrinolysis model. Sodium
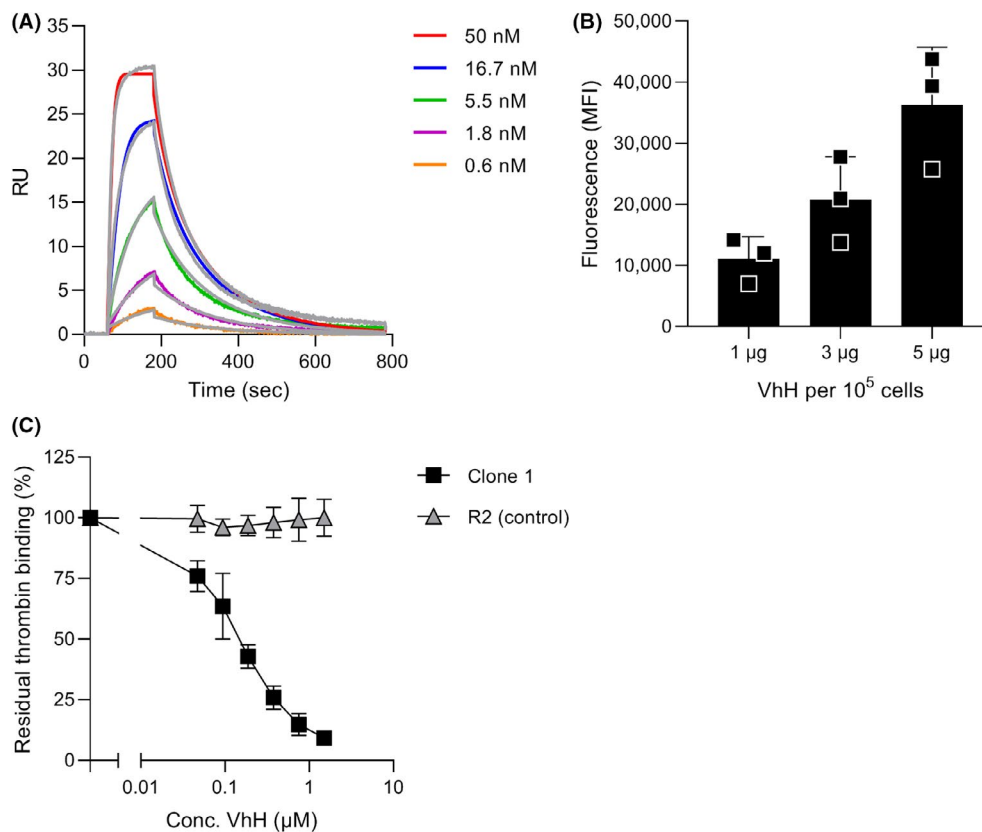
FIGURE 1 Selection and characterization of VhH anti-TM clone 1. (A) Clone 1 was injected over a thrombomodulin-immobilized surface at the indicated concentrations for SPR analysis. Association was measured for 2 min, followed by 10 min of dissociation and full regeneration of the surface. Reference-corrected sensorgrams are shown in color and the fit on the data is shown in gray. (B) Quiescent human umbilical vein endothelial cells (10^5 cells in 100 μL) were incubated with fluorescently labelled VhH anti-TM clone 1 or VhH R2 isotype control (1, 3, or 5 μg). Fluorescence was measured with FACS. Mean fluorescent intensity (MFI) was corrected for VhH R2 isotype control for each concentration separately. (C) A total of 15 nM human α-thrombin was incubated on thrombomodulin-coated wells in the presence of indicated concentrations of unlabeled clone 1 or VhH R2 (control). Thrombin binding in the absence of VhH was set at 100%. All experiments were performed in triplicate.

citrate anticoagulated whole blood was recalcified with $CaCl_2$, spiked with tPA, and perfused over a surface coated with collagen, TF, and thrombomodulin at an arterial shear rate. To prevent clogging of the flow chamber, we allowed nonrecalcified (but for the remainder identical) whole blood to perfuse after 7 min, and we stopped each run after 15 min. Under these conditions, platelet adhesion starts immediately, whereas fibrin formation starts with a delay of approximately 2 min (Figure 3A). Both platelet adhesion and fibrin formation increased over time. Fibrin formation reached a maximum at 9.6 ± 0.9 min, which was followed by a rapid disintegration of the fibrin network (Figure 3B,D). In contrast, platelet adhesion remained stable despite fibrinolysis (Figure 3B,C).

To confirm TAFI activation in this fibrinolysis model, whole blood was spiked with TAFIa inhibitor PCPI. Whereas kinetics of platelet aggregation was similar (Figure 3C), fibrin deposition was reduced (Figure 3D). Spiking with PCPI resulted in a $40.2 \pm 19.0\%$ reduction in fibrin deposition at $t = 7$ min ($p = .01$) compared with perfusions in the presence of VhH R2 (control) (Figure 3E). Like inhibition of TAFIa with PCPI, blocking thrombomodulin-mediated TAFI activation with clone 1 had no effects on platelet aggregation kinetics (Figure 3C)

and similarly reduced fibrin deposition compared with VhH R2 (control) ($33.6 \pm 13.0\%$ reduction, $p = .015$) at $t = 7$ min (Figure 3E). Combined, these data suggest that blocking thrombomodulin with clone 1 effectively prevents TAFI activation.

4 | DISCUSSION

We developed a VhH targeting human thrombomodulin (VhH anti-TM clone 1) that interferes with thrombin binding to thrombomodulin and, consequently, activation of protein C and TAFI by the thrombin-thrombomodulin complex. In addition, clone 1 completely attenuated TAFI activation in an *in vitro* fibrinolysis model in flowing human whole blood and enhanced tPA-induced fibrinolysis.

The thrombomodulin-thrombin complex has both antifibrinolytic effects by activating TAFI and anticoagulant effects by activating protein C. Whether thrombomodulin has net antifibrinolytic or anticoagulant effects depends on its concentration: low concentrations favor TAFI activation and high concentrations favor protein C activation.[7] Clone 1 interferes with thrombin binding to
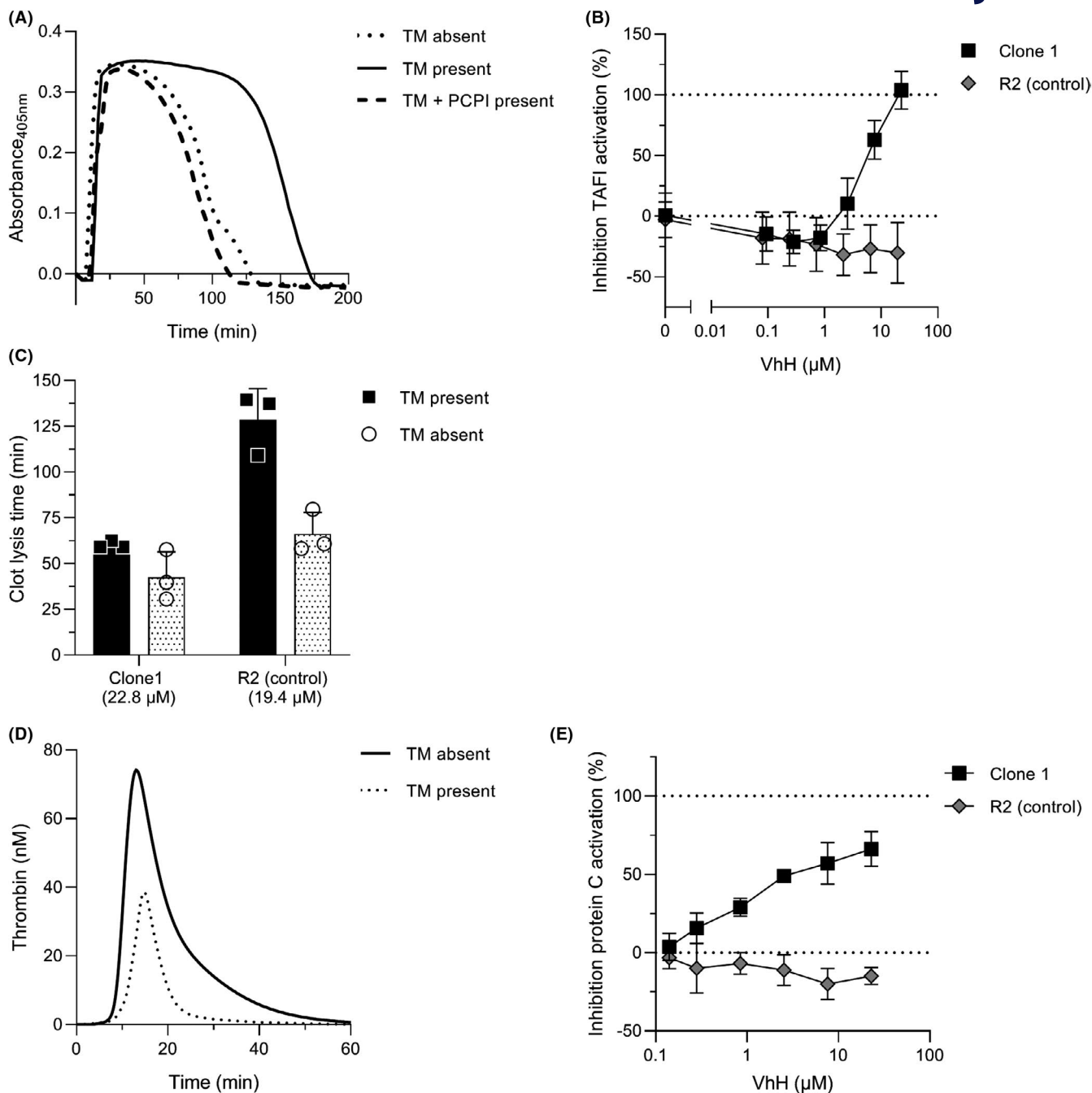
FIGURE 2 Inhibition of TAFI and protein C activation by VhH anti-TM clone 1. (A) To measure TAFI activation, clot lysis experiments were performed. Plasma was spiked with tPA and coagulation was initiated with 0.5 pM TF and 4 μM phospholipids (30:20:50 ratio PS:PC:PE), in the presence or absence of 10 nM thrombomodulin and PCPI (5 μg/ml) as indicated. Clot formation and clot lysis were measured as an increase and decrease in absorbance at 405 nm. (B) Clot lysis times (CLTs) were determined in the presence of thrombomodulin and clone 1 or negative control VhH R2 at the indicated concentrations. Data are presented as relative (%) inhibition of TAFI activation, in which 0% inhibition was equal to CLT in the presence of thrombomodulin and 100% inhibition was equal to the CLT in the absence of thrombomodulin in control samples without VhHs. (C) CLTs in the absence of thrombomodulin were determined at highest concentrations of clone 1 or negative control VhH R2 and measured in a SpectraMax iD3 (absorbance at 405 nm). (D) Representative thrombograms obtained in plasma after initiation of coagulation with 0.5 pM TF and 4 μM phospholipids (30:20:50 ratio PS:PC:PE) in the presence or absence of 25 nM thrombomodulin. (E) Relative inhibition of protein C activation in PNP in the presence of clone 1 or VhH R2 (control) at indicated concentrations. Data are presented as relative (%) inhibition of protein C, in which 0% inhibition was equal to the ETP in the presence of thrombomodulin and 100% inhibition was equal to the ETP in the absence of thrombomodulin in control samples without VhHs. All experiments were performed in triplicate. (B, C, E) Data represent normalized means and SDs of each individual run.

(A)
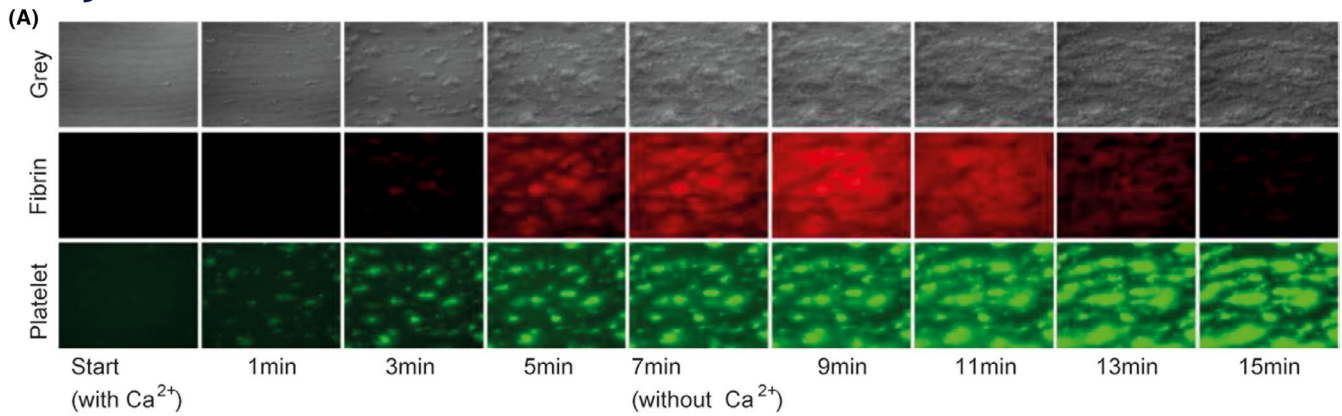
(B) All runs (n=16)
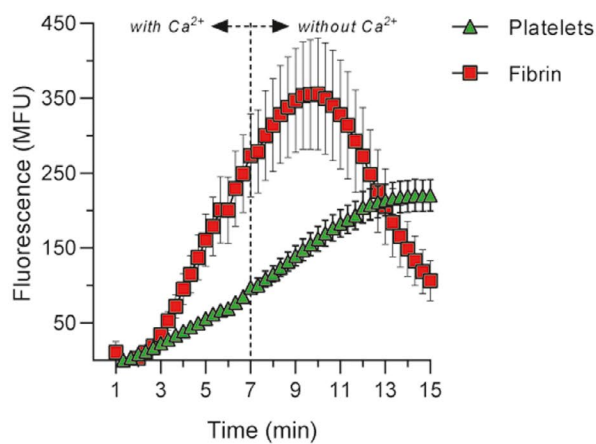
(C) Platelet adhesion
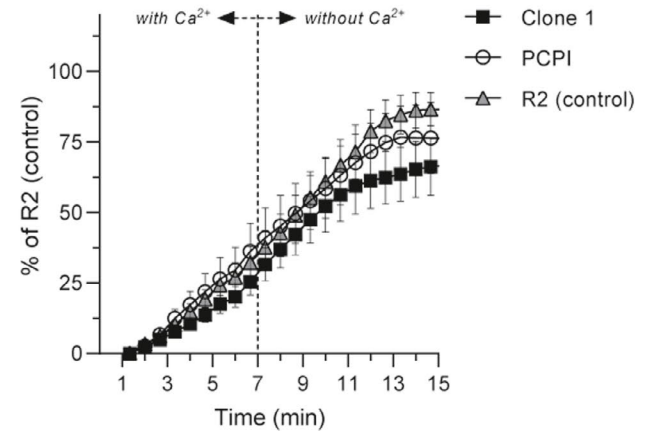
(D) Fibrin deposition
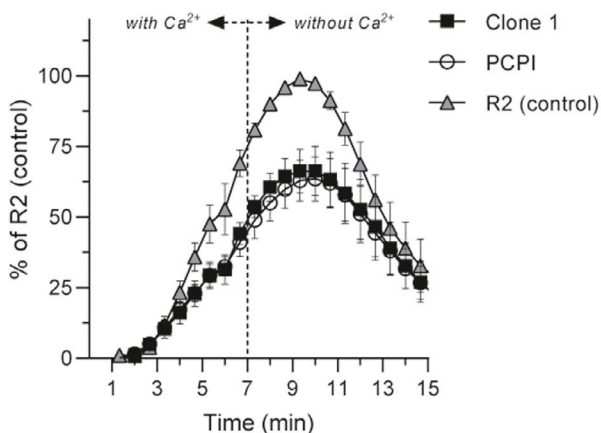
(E) Fibrin deposition T=7 min
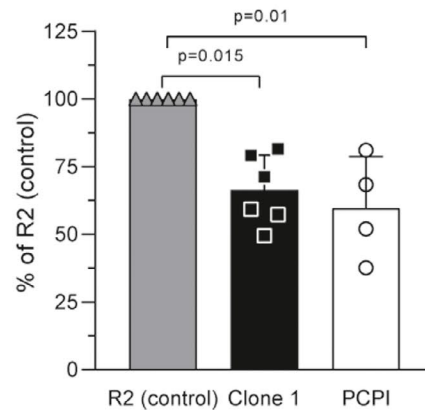
thrombomodulin and, consequently, activation of protein C and TAFI by the thrombin-thrombomodulin complex. We therefore cannot exclude dual effects of the VhH on protein C and TAFI activation in our fibrinolytic flow model; however, it is unlikely that inhibition of protein C activation was of significant influence on fibrin formation in the flow model because this would have resulted in increased fibrin formation. Instead, our data clearly demonstrate reduced fibrin deposition in the presence of clone 1 and to a similar extent as TAFI

inhibition with PCPI. This corresponds with a low concentration of immobilized thrombomodulin on the glass surface, generating a net antifibrinolytic environment.

TAFI can be activated by plasmin[32] and both the thrombin-thrombomodulin complex or by thrombin itself. Direct thrombin-dependent TAFI activation requires a high local thrombin concentration, which is thought to be achieved by feedback amplification of coagulation through thrombin mediated FXI activation

FIGURE 3 Blocking thrombomodulin-dependent TAFI activation with clone 1 enhances fibrinolysis in a microfluidic flow model. A total of 16 runs were performed with blood from 6 healthy donors. From four of these donors, separate runs were performed with subsequent PCPI (25μg/mL), clone 1 (514 nM), or VhH R2 (control) (514 nM). From two of these donors, separate runs were performed with either clone 1 (514 nM) or VhH R2 (control) (514 nM). (A) Citrated whole blood samples were recalcified and spiked with tPA and perfused at a shear rate of 800 s^{-1} over a coverslip coated with human TF, horm collagen, and thrombomodulin. After 7 min, noncalcified blood samples with same supplements entered the flow chamber, allowing tPA-mediated fibrinolysis to predominate. Platelets were stained with AlexaFluor488-conjugated anti-GPIbα VhH clone 17 (shown in green) and fibrin was stained with AlexaFluor647-conjugated anti-fibrin VhH clone B12 (shown in red). Images were captured every 20 s at 40× magnification. Images represent a single run. (B) Platelet adhesion and fibrin deposition were quantified with ZEN Pro software and plotted over time. Graphs represent mean ± SEM values of all runs ($N = 16$). (C-D) Whole blood was supplemented with tPA and either VhH R2 (control, $N = 6$), clone 1 ($N = 6$), or PCPI ($N = 4$), followed by perfusion in the microfluidic fibrinolysis model. Platelet adhesion and fibrinolysis were evaluated over time. Data represent mean ± SEM values. (E) Maximum fibrin deposition in the presence of calcium (at $t = 7$ min) was derived from biological replicates and is expressed as percentages of the run supplemented with VhH R2 (control) within the same donor. Kruskal-Wallis analysis with multiple comparisons was performed with GraphPad Prism 8.0.1.

under low TF conditions.[33-35] Although high thrombin concentrations are likely to occur under static conditions, rheological conditions in a growing thrombus favor flushing out coagulation factors that lack surface binding capacity, such as factor XI.[33,36] Although thrombin itself is retained in the fibrin network, it loses substrate specificity because of the involvement of both its exosites in fibrin binding.[37] This makes the thrombomodulin-thrombin complex the most likely source of TAFI activation under high shear stress conditions in flowing blood.

Data obtained in our fibrinolysis model support a dominant role for this complex in TAFI activation in flowing blood. Nevertheless, the bleeding pattern in patients with congenital FXI deficiency indicate a predilection for tissue with high fibrinolytic activity,[38] offering strong indirect evidence of a role for FXI in inhibition of fibrinolysis.[35] Our fibrinolysis model incorporated arterial flow conditions (shear rate 800 s^{-1}), favoring the flush-out of FXIa. We cannot exclude that FXI contributes to inhibition of fibrinolysis under venous conditions.

In addition, our model incorporates exogenous tPA to induce fibrinolysis, whereas no exogenous tPA was added in the *in vivo* models described by others.[16,17,19,39] Together with presumable low concentration of thrombomodulin, this tips the balance toward profibrinolytic conditions in our model, which might explain the difference in these observations. Patients with acute ischemic stroke will often be treated with tPA to restore blood flow to ischemic tissue, resulting in a similar profibrinolytic environment. It has been suggested to target TAFI itself to enhance fibrinolysis under these conditions,[16] potentially leading to enhanced thrombus resolution and preservation of brain tissue. However, because a failure to properly activate TAFI is thought to be the main determinant in the bleeding tendency observed in factor XI-deficient patients, this strategy is not without bleeding risk.[38] By targeting TAFI activation by the thrombomodulin-thrombin complex only, direct TAFI activation by thrombin can be preserved, potentially reducing bleeding risk. It should be noted that this strategy only holds clinical potential when protein C activation remains largely unaffected. Whether inhibition of TAFI activation by targeting thrombomodulin will enhance fibrinolysis in the setting of a pre-existent thrombus remains to be determined.

AUTHOR CONTRIBUTIONS

Joost C. M. Meijers, Raymond M. Schiffelers, Coen Maas, and Rolf T. Urbanus conceived/designed the study. Marc V. A. van Moorsel, Geke C. Poolen, Cornelis A. Koekman, Sandra Verhoef, Steven de Maat, Arjan Barendrecht and Nadine D. van Kleef performed experiments. Marc V. A. van Moorsel, Geke C. Poolen, and Rolf T. Urbanus wrote the paper.

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