Targeting shear gradient activated von Willebrand factor by the novel single-chain antibody A1 reduces occlusive thrombus formation *in vitro*

Thomas Hoefer,[1*] Akshita Rana,[2*] Be'eri Niego,[2] Shweta Jagdale,[1,2] Hugo J. Albers,[3,4] Elizabeth E. Gardiner,[5] Robert K. Andrews,[2] Andries D. van der Meer,[3] Christoph E. Hagemeyer[1,2#] and Erik Westein[1,2#]

[1]Baker Heart and Diabetes Institute, Melbourne, Victoria, Australia; [2]Australian Center for Blood Diseases, Monash University, Melbourne, Victoria, Australia; [3]Applied Stem Cell Technologies, University of Twente, Enschede, the Netherlands; [4]BIOS Lab-on-a-Chip, University of Twente, Enschede, the Netherlands and [5]ACRF Department of Cancer Biology and Therapeutics, John Curtin School of Medical Research, Australian National University, Canberra, New South Wales, Australia

*TH and AR contributed equally as co-first authors.
#CEH and EW contributed equally as co-senior authors.

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Correspondence: *ERIK WESTEIN* - erik.westein@monash.edu

Supplementary method and results

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Thomas Hoefer[1*], Akshita Rana[2*], Be'eri Niego[2], Shweta Jagdale[1,2], Hugo J. Albers[3,4], Elizabeth E. Gardiner[5], Robert K. Andrews[2], Andries D. van der Meer[3], Christoph E. Hagemeyer[1,2**]& Erik Westein[1,2**]

[1]Baker Heart and Diabetes Institute, Melbourne, Australia;
[2]Australian Centre for Blood Diseases, Monash University, Melbourne, Australia;
[3]Applied Stem Cell Technologies, University of Twente, Enschede, The Netherlands;
[4]BIOS Lab-on-a-Chip, University of Twente, Enschede, The Netherlands;
[5]ACRF Department of Cancer Biology and Therapeutics, John Curtin School of Medical Research, Australian National University, Canberra, Australia

Methods and materials:

Single-chain antibody generation

A panel of IgG antibodies was previously raised in mice against a 34/39 kDa fragment incorporating the human VWF A1 domain and a sequence N-terminal to that (1). The sequence of the light and heavy chains was determined as described before(2). Briefly, mRNA was prepared and reverse transcribed using an oligo-dT primer. The variable regions of the antibody's heavy and light chains were amplified by polymerase chain reaction (PCR) using primers that anneal to conserved regions at the 5' and 3' ends of the variable regions and sequenced. The variable regions of the light and heavy chains of 2 antibodies from this panel were combined with a standard linker sequence and various tags added for purification and bioconjugation purposes (FLAG-tag, a HIS-tag and a sortase reaction site). The derived DNA-sequences were ordered from a commercial supplier (Geneart, Invitrogen) optimized for the final host expression system, cloned into a pSec-tag vector, expressed using a HEK293 cell line and purified as described before(3, 4). In brief, after confirmation of the final clone, DNA was transfected with polyethylenimine (Polyscience Inc.) into human HEK293 kidney cells. After transfection, cells were cultured for 7 days at 37°C, with 5% CO_2, shaking at 140 rpm. The supernatant was collected and purified with a nickel-based metal affinity chromatography column, Ni-NTA column (Qiagen), according to the manufacturer's instructions. Purified scFv was dialysed against PBS at 4°C overnight. The cyclic peptide, OS-1 which is an allosteric

inhibitor of GPIbα/VWF interactions with sequence CTERMALHNLC was obtained from Auspep (Parkville, Australia)

Blood sampling

The study was approved by the Alfred Hospital's (Melbourne, Australia) ethics committee no 67/15 and all volunteers gave written consent. The volunteers abstained from aspirin, non-steroidal anti-inflammatory drugs (NSAIDs) and any other anti-platelet therapy for 10 days before blood was taken from the median cubital vein using a 21G butterfly needle and collected into vacuette tubes (Sarstedt AG & Co, Nuembrecht, Germany) containing trisodium citrate (0.32% w/v final). Blood samples were immediately processed for further use.

Microfluidic chips

In-house designed PDMS microfluidic chips (Figure 1A) contained channels of 52 µm height with a footprint of 300 µm x 46 mm (used in figure 1) or 300 µm x 17 mm. Selected channels incorporated a semi-circular stenotic section of various lengths (600 µm, 1000 µm or 2000 µm diameter), creating 80% lumen reduction. Chips were manufactured as previously reported(5). The produced PDMS chips were sealed with cover slips by hydrophobic interaction and coated with collagen by perfusion of collagen solution followed by static incubation at RT for at least 1 hr. Channels were rinsed with PBS and blocked with 2% BSA. Wall shear rates of the input flow for these microchannels were approximated from volumetric flow rates by using the equation, $\gamma = 6Q/wh^2$. Herein, γ is the wall shear rate (s^{-1}), Q the volumetric flow rate (mls^{-1}), w the channel with (cm) and h the channel height (cm).

Computational fluid dynamics and calculations

Fluid dynamics were simulated with a COMSOL Multiphysics 4.2 laminar flow module applied to three-dimensional meshes. For all simulations, no-slip boundary conditions and Newtonian fluids were assumed. To obtain meshes from the plain channel geometries, channel dimensions were taken from the original computer-aided design (CAD) files and reconstructed in COMSOL. To obtain meshes from platelet aggregate geometries, confocal fluorescence microscopy image z-stacks of labelled platelets were processed in ImageJ. Each image in a z-stack was converted into a binary image by thresholding, and an x-y heightmap was constructed by summing all binary data in a z-stack followed by multiplying the result with the height of the original z-stack as documented by the confocal microscope. These heightmaps were imported in COMSOL to construct three-dimensional meshes. Input flow parameters

were set based on pump settings as used in the experiments, and were adjusted to account for the reduced width of the fluorescence aggregate meshes compared to the full channel geometries. Both the computational fluid dynamical simulation and the calculations of fluid dynamical and geometrical parameters were carried out with the assumption that the channels and vessels were filled with Newtonian fluids. This assumption is valid because the shear rates are in a regime in which the non-Newtonian behaviour of blood is limited (6)(6).

***In vitro* flow perfusions**

Citrated whole blood was incubated with $DiOC_6$ (0.5 µg ml^{-1}) (Sigma-Aldrich Co, St. Louis, MO, USA) and drawn through microfluidic channels by a programmable syringe pump (Legato 130, KD Scientific, USA). Time-lapse z-stack images of platelet deposition were captured in multiple track, resonant scanning mode, using a Nikon A1R confocal microscope system. Platelet deposition was recorded over time (2 frames per minute) for up to 20 minutes and quantified in ImageJ using a Z-projection of the acquired signal.

For experiments measuring rolling velocities, anti IIb3 antibody (abciximab; 20 µg ml^{-1})-treated whole blood containing 0.5 µg ml^{-1} $DiOC_6$ was drawn over a collagen type III-coated surface. Where indicated, blood was incubated with 30 µM aspirin and 300 µM 2-MeSAMP (Sigma-Aldrich) before blood perfusion. 10-second video clips of rolling platelets were recorded in the green fluorescent channel using an Olympus IX81 microscope system. Platelet rolling velocities were calculated using Diatrack v3.04 large scale particle tracking software(7).

Platelet agglutination

Platelet rich plasma (PRP) was obtained from citrated whole blood by centrifugation at 300 x g for 10 min and further centrifugation at 1700 x g for 7 min to obtain platelet-poor plasma (PPP; source of plasma VWF). Isolated platelets (3 x 10^8/ml) suspended in platelet wash buffer (4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 113 mM NaCl, 5.5 mM D-glucose, and 10 mM theophylline, pH 6.5) were centrifuged at 1500 x g for 7 min. The washed platelets were subsequently resuspended at the same concentration in modified Tyrode's buffer (10 mM Hepes, 12 mM $NaHCO_3$, 137 mM NaCl, 2.7 mM KCl and 5 mM glucose, pH 7.3) containing calcium (1 mM), magnesium (1 mM) and apyrase (0.02 U ml^{-1}) (ADPase activity). Isolated platelets (150 or 300 µL at 3 x 10^8/ml) and platelet poor plasma (50 µl) were incubated with 2, 2.5, 4, 5, 40 and 80 µg ml^{-1} scFv A1 and the corresponding concentration of control scFv or

Tyrode's buffer. αIIbβ3 (GPIIb/IIIa) blocker, Eptifibatide (Integrilin) was used to neutralise any other aggregation mechanisms besides the VWF A1-GPIbα interaction. Platelet agglutination in response to 0.75 mg mL^{-1} ristocetin (Helena Biosciences, Gateshead, UK) was performed for 10 min on an AggRam system from Helena Laboratories, USA

Western Blot

Denatured full-length VWF (Mybiosource, USA) was electrophoresed at 0.1, 10, 100 ng and 1 µg using 5% SDS-Polyacrylamide gel at 125 V for 90 min. The protein was transferred onto PVDF membrane at 90V for 90 min followed by incubation with 1 µg ml^{-1} scFv A1 overnight and the membrane was probed with horse-radish peroxidase (HRP)-conjugated detection antibody (1:2500, Sigma). Later, the membrane was stripped and reprobed with 1 µg ml^{-1} polyclonal sheep anti-VWF antibody (Abcam), followed by donkey anti-sheep HRP-conjugated secondary antibody (1:2500, R&D Systems, USA). Chemiluminescent detection of protein bands was done using ECL Western Blotting Substrate (ThermoFisher) and visualisation using a ChemiDoc Touch Imaging system (Bio-Rad, UK).

ELISA

ELISA wells (Thermo Fisher Scientific) were coated with purified full-length VWF or isolated A1 domain at 0.1, 1 and 10 µg ml^{-1} in the presence of ristocetin (0.75 mg ml^{-1}) and blocked with 2% BSA. Next, the coated wells were incubated with 5 µg ml^{-1} of scFv A1 or sheep anti-VWF antibody followed by incubation with an HRP-conjugated secondary antibody (Anti-his HRP for scFv A1, Donkey anti-sheep HRP antibody for sheep anti-VWF antibody) (1:4,000). Readings were measured at 450 nm on a BMG Optima multi-well reader.

BLItz

Bio-layer Interferometry was performed on a BLItz System (Pall Forte Bio, USA). Protein-A biosensors were hydrated for 30 minutes in PBS-Tween (0.05%) and loaded with Fc-tagged VWF-A1 domain (130 µg ml^{-1}). Remaining binding sites were blocked by exposure to a polyclonal mouse IgG for 5 min. Sequential adhesion of scFv-A1 at 0, 20, 40 and 100 µg ml^{-1} was monitored for 5 min per concentration. Real-time visualisation of binding interactions between scFv A1 and full-length VWF/A1 domain was performed using the BLItz Pro software. Traces were manually baseline aligned and corrected for control binding (0 µg ml^{-1} scFv-A1).

Supplementary Figures

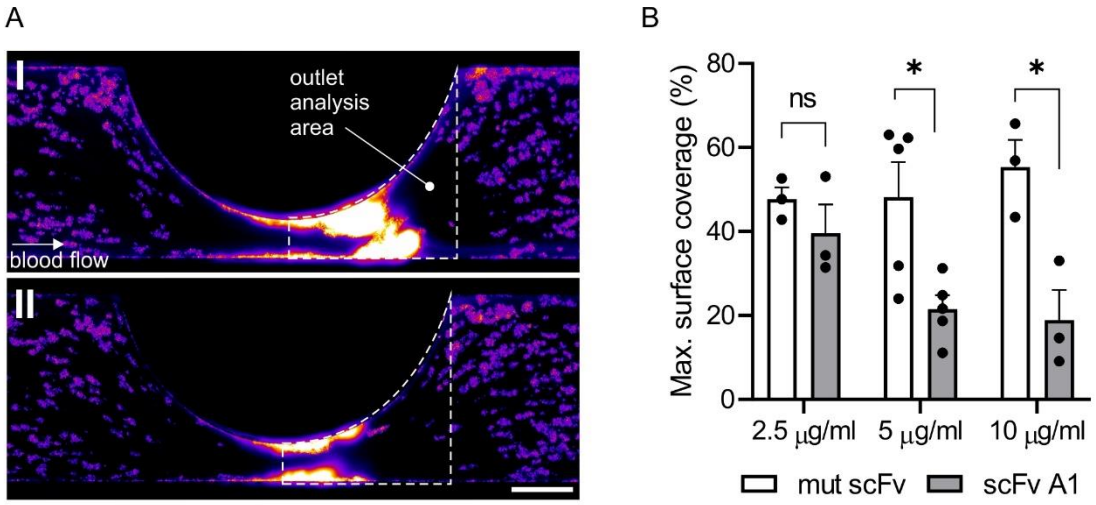

Figure S1. ScFv A1 dose dependently inhibits platelet aggregation at stenotic sites. (A) representative false colour images of platelet aggregate formation in the stenosis outlet region in the presence of (I) control mut-scFV and (II) 10 μg ml^{-1} scFv A1 after 10 minutes of flow at 250s^{-1} input shear rate. (B) Maximum surface coverage measured in the outlet analysis area as shown in (A). Data represented as mean±SEM; n=3-5; *p<0.05 (multiple t-tests).

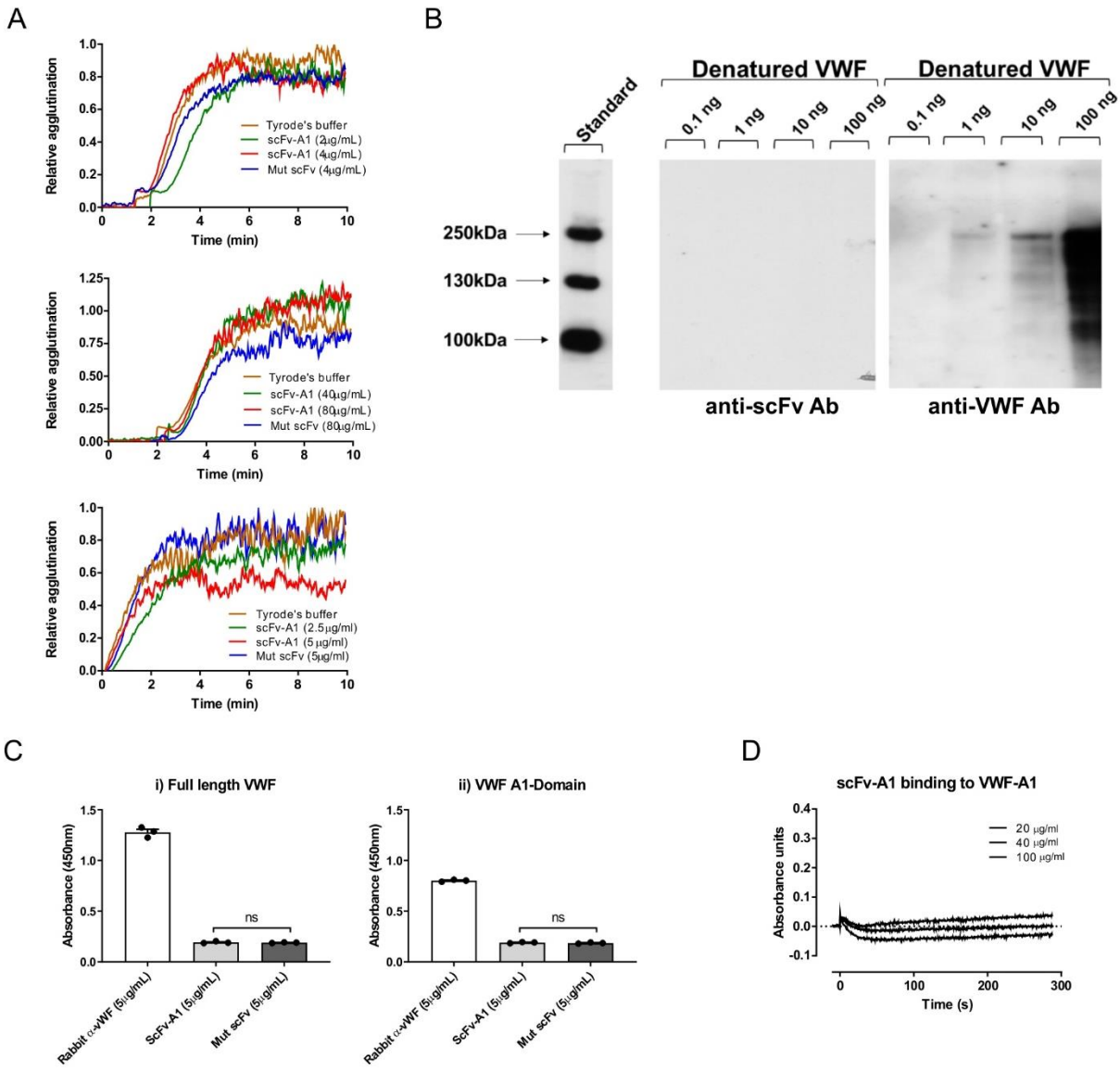

Figure S2. Static assays to characterize the binding of ScFv A1 to VWF. (A) Ristocetin (0.75 mg ml^{-1}) induced platelet agglutination traces. i) 300 µL washed platelets and 50 µL of platelet poor plasma (PPP) treated with 2, 4 µg ml^{-1} scFv A1, Mutated scFv or Tyrode's buffer. ii) 150 µL of isolated platelets, 50 µL of platelet poor plasma (PPP) treated with 40, 80 µg ml^{-1} scFv A1, Mutated scFv or Tyrode's buffer. iii) 50 µL of platelet poor plasma (PPP) preincubated with ristocetin for 30 min before mixing with 150 µL of isolated platelets, treated with 2.5 or 5 µg ml^{-1} scFv A1, control scFv or Tyrode's buffer. Agglutination was recorded for 10 minutes and normalized to Tyrode's buffer sample. (B) Western blot analysis of VWF protein (at 0.1, 10, 100 ng µl^{-1} and 1 µg µl^{-1}) denatured via SDS-PAGE, probed with i) scFv-A1(1 µg ml^{-1}) followed by anti-his HRP antibody (1:2500), ii) sheep α-VWF (1 µg ml^{-1}) antibody followed by donkey α-sheep HRP antibody (1:2500). (C) ELISA absorbance values for binding of 5 µg ml^{-1} scFv-1 A1 or control mut-scFV to i) full-length VWF (10 µg ml^{-1}) in the presence of 0.75 mg ml^{-1} ristocetin , and ii) purified A1 domain (10 µg ml^{-1}). Data are mean±SEM; n=3 (t-test). (D) BLItz association traces for scFv-A1 (20, 40 and 100 µg ml^{-1}) binding to VWF-A1 domain immobilized onto a protein-A BLItz probe. Shown traces are background subtracted using a PBS only sample.

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