SUPPLEMENTARY APPENDIX

The von Willebrand factor A1 domain mediates thromboinflammation, aggravating ischemic stroke outcome in mice

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The von Willebrand Factor A1 domain mediates thromboinflammation, aggravating ischemic stroke outcome in mice

Running head: VWF-mediated thromboinflammation in stroke

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Supplemental Materials and Methods

Animals & Nanobodies

For this study, 10-week-old VWF knockout (KO)[1] and littermate wild-type (WT) C57BL/6 mice were used. All animal experiments were approved by the local ethical committee (P050/2017 KU Leuven, Leuven, Belgium) and were performed following the ARRIVE guidelines (www.nc3rs.org.uk), including randomization of treatment as well as surgery and analysis blind to the treatment. Mice were treated with a well-characterized nanobody targeting the VWF A1 domain (KB-VWF-006 bv; 10 mg/kg) or a control nanobody (KB-VWF-004 bv; 10 mg/kg).[2]

Cerebral ischemia and reperfusion injury model

Transient middle cerebral artery occlusion (tMCAO) was performed as described previously.[3] Anesthesia was induced and maintained by inhalation of 5% and 2% isoflurane in medical oxygen respectively. After a midline incision in the neck, the proximal common carotid artery and the external carotid artery were ligated and a standardized silicon rubber–coated 6.0 nylon monofilament (6021; Doccol Corp, Redlands, CA) was inserted and advanced via the right internal carotid artery to occlude the origin of the right MCA. The intraluminal suture was left in situ for 60 minutes. Next, animals were re-anesthetized, and the occluding monofilament was withdrawn to allow reperfusion. Surgery time did not exceed 10 minutes per animal. Immediatly after the start of reperfusion, nanobodies were administered via intravenous injection.

The following criteria excluded mice from endpoint analyses: death within 24 hours after tMCAO or the occurrence of surgical complications. Of the 55 mice subjected to tMCAO, four were excluded because they died during the time of the experiment and six mice were excluded due to surgical complications. No difference in mortality rate was observed between the different experimental groups. Researchers and operators were blinded to the experimental groups for all readout parameters.

Neurological tests

Twenty-four hours after induction of tMCAO; mice were subjected to the modified Bederson test[4] and the grip test[5] to assess global neurological and motor function respectively, as described.[6]

Cerebral lesion quantification and assessment of bleeding

To measure cerebral infarct volumes, mice were euthanized 24 hours after induction of tMCAO. Brains were quickly isolated and cut into 2-mm-thick coronal sections using a mouse brain slice matrix. The slices were stained with 2% 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma-Aldrich, St Louis, MO) to distinguish healthy tissue from unstained infarctions. Stained slices were photographed with a digital Nikon D70 camera and infarct areas (white) were measured using Image J software (National Institutes of Health, Bethesda, MD) by an operator blinded to the experimental conditions. Edema corrected infarct sizes were calculated by use of the following equation: $V_{corrected} = V_{uncorrected}$ X (1 - (V_I - V_C) / Vc) where V_I - V_C represents the volume difference between the ischemic hemisphere and the contralateral hemisphere and (V_I - V_C) / Vc expresses this difference as a fraction of the contralateral hemisphere. The presence of cerebral hemorrhages was macroscopically assessed upon brain isolation as well as via careful visual analysis of the coronal brain sections after cutting.

Flow cytometry

Twenty-four hours after stroke, mice were euthanized and perfused with 20mL of phosphate-buffered saline. After dissecting the brain and removing the cerebellum, the two hemispheres were separated into the right ischemic (ipsilateral) and left non-ischemic (contralateral) one. The hemispheres were mechanically disrupted using a scalpel and subsequently incubated for 30 minutes at 37°C with a digestion buffer (2.5 mg/mL collagenase D (17104-019, Invitrogen, Carlsbad, CA), 5 U/mL DNase I (EN0521, Thermo

Scientific, Waltham, MA) in RPMI 1640 + 10% inactivated fetal calf serum (FCS, 10500-064, Invitrogen)) and pressed through a cell strainer (40 μm). Next, cells were separated from myelin and debris by Percoll (17-0891-02, GE Healthcare, Chicago, IL) gradient centrifugation and incubated with appropriate antibody cocktails (described below) containing an Fc-blocker (30 minutes at room temperature) in PBS + 5% FBS. Live cells were stained with Live/Death violet cell viability staining (L34963; ThermoFisher; Waltham, MA) after which they were fixed in 200 μL 0.5% paraformaldehyde and analyzed with a FACSVerse flow cytometer (BD, Franklin Lakes, NJ) and BD Facs Suite software. Sample acquisition was performed until 10000 live cells were analyzed, and thereafter the total cell count per hemisphere was calculated.

Two antibody cocktails were used to identify different leukocyte subsets (Table 1). The first antibody cocktail allowed determination of overall leukocyte ($CD45^{high}$), myeloid leukocyte ($CD45^{high}$ and $CD11b^{+}$), neutrophil ($CD45^{high}$, $CD11b^{+}$ and $Ly6G^{+}$) and monocyte ($CD45^{high}$, $CD11b^{+}$, $Ly6C^{+}$ and $Ly6G^{-}$) count. With the second antibody cocktail, lymphocyte ($CD45^{high}$, $CD11b^{-}$ and $CD11c^{-}$), T-cell ($CD45^{high}$, $CD11b^{-}$, $CD11c^{-}$, $CD3e^{+}$) and $CD3^{neg}$ lymphocytes ($CD45^{high}$, $CD11b^{-}$, $CD11c^{-}$, $CD3e^{-}$) count were determined.

Immunofluorescence

Twenty-four hours after stroke, mice were euthanized and the brains were disected. Brains were cut into 2mm-thick coronal sections, immediately placed in O.C.T. compound and frozen in liquid nitrogen for sectioning. Nine μm thick sections were stained for the presence of neutrophils (rat anti-mouse Ly6G, 1/500, eBioscience, San Diego, CA), T-cells (Armenian hamster anti-mouse CD3e, 1/500, Biolegend, San Diego, CA), VWF (rabbit anti-human VWF, 1/1500, Dako, Santa Clara, CA) or platelets (rat anti-mouse GPIX, 1/100, emfret, Würzburg, Germany). For staining of the microvasculature, a lectin dye (FITC conjugated lectin from Lycopersicon esculentum, 1/500, Sigma-Aldrich) was used. Briefly, sections were washed in TBS; blocked with 1% normal rat, Armenian hamster, or rabbit serum; 3% BSA and 0.1% Triton X-100 in TBS for one hour and incubated overnight at 4°C with various combinations of primary antibodies. After

washing, slides were incubated for one hour at room temperature with the corresponding secondary antibodies: Alexa Fluor 647 goat anti-Armenian hamster IgG (1,5 µg/ml, 405510; BioLegend), Alexa Fluor Plus 555 goat anti-rat IgG (1,5 µg/ml, A21434, Invitrogen) or Alexa Fluor 647 donkey anti-sheep IgG (1,5 µg/ml, A21488, Invitrogen). Prolong Gold Antifade mounting solution with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) was used to stain DNA. As negative control, the primary antibody was omitted. Images were acquired using a laser scanning confocal microscope (LSM710, Zeiss, Oberkochen, Germany). Images were processed by Zen 2012 (blue edition, version 2.3, Zeiss) software. Neutrophils were counted in 6 brain sections per mouse for 3 WT mice, for quantification of their localization.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism Version 8.1.2. The number of experimental animals in each group was based on power calculations with infarct volume as primary parameter and with mean differences and standard deviations taken from available data from the same tMCAO model (power of 80% and α of 0.05). Prior to statistical analysis, a D' Agostino and Pearson normality test was used to check data distribution. One-way ANOVA with Dunnett's post hoc test or a Mann-Whitney test was used for statistical comparison of infarct size and immune cell infiltration when applicable. In the case of non-parametric data (Bederson and grip-test score) a Kruskal–Wallis test with post hoc Dunn correction was performed. Infarct size is represented as mean $\pm$ standard deviation. Bederson and grip-test score are shown as scatter plot with median. Immune cell recruitment is shown as a min-max box plot, with the median.

Supplemental Table 1. Comparison of effect of genetic VWF deficiency or anti-VWF A1 nanobody treatment on cerebral leukocyte recruitment after stroke. Data is represented as mean ± standard error of the mean; * $p < 0.05$; **, $p<0.01$; ***: $p < 0.005$.

	VWF WT	VWF KO	fold reduction	p-value	KB-VWF-004 bv	KB-VWF-006 bv	fold reduction	p-value
WBCs	12435 ± 2083	5596 ± 1644	2,2	*	16310 ± 3980	7095 ± 2550	2,3	**
Myeloid WBCs	10162 ± 2842	3680 ± 1638	2,8	*	11978 ± 3322	5195 ± 2259	2,3	*
Neutrophils	2017 ± 733	512 ± 203	3,9	*	1557 ± 317	304 ± 94	5,1	***
Monocytes	6750 ± 1414	2466 ± 955	2,7	*	8260 ± 2651	3726 ± 1824	2,2	*
Lymfoid WBCs	3300 ± 330	1412 ± 203	2,3	*	3017 ± 514	1529 ± 269	2,0	**
T-cells	973 ± 184	243 ± 44	4,0	**	1111 ± 248	487 ± 93	2,3	**
CD3- cells	2324 ± 245	1168 ± 188	2,0	ns	1633 ± 284	1057 ± 203	1,5	ns

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