SUPPLEMENTARY APPENDIX

Macrophage scavenger receptor SR-AI contributes to the clearance of von Willebrand factor

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Macrophage scavenger-receptor SR-AI contributes to the clearance of von Willebrand factor

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Supplementary Methods

Proteins:

Plasma-derived (pd)-VWF was purified from VWF concentrates (Wilfactin) via gel-filtration chromatography to homogeneity. Recombinant VWF (wt-VWF, VWF/p.R1205H, VWF/p.V1316M, and VWF/p.S2179F) were produced in stably transfected BHK-furin cells using serum-free medium. All variants displayed a similar distribution of multimers (not shown). Non-purified cell culture supernatants were used for binding experiments. Bovine serum albumin (BSA) was from Sigma Aldrich (Saint-Quentin Fallavier, France). Human recombinant soluble SR-AI receptor was purchased from R&D Systems Europe (Lille, France). Polyclonal rabbit anti-human VWF antibodies (unlabeled and peroxidase-conjugated) were from Dako (Trappes, France). Peroxidase-labeled anti-human IgG1 antibodies were from Sanquin (Amsterdam, the Netherlands). Anti-human SR-AI biotinylated polyclonal- and anti-human SR-AI monoclonal antibodies were from R&D Systems Europe (Lille, France). Anti-mouse SR-AI monoclonal IgG2b antibody was from Thermo Fisher Scientific (Rockford, IL, USA). Anti-human early endosome antigen-1 (EEA-1) polyclonal IgG antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). Duolink Reagent Kits (anti-mouse plus, anti-goat plus and anti-rabbit minus) were from Olink Bioscience, Uppsala, Sweden. Anti-human propeptide antibodies (unlabeled and peroxidase-conjugated) were from Sanquin (Amsterdam, the Netherlands).

Cell culture:

Human macrophages were differentiated from the THP1 acute monocytic leukemia cell line cultured in RPMI-1640 (Life Technologies, Grand Island, New-York) supplemented with 10% Fetal Bovine Serum (FBS; Dutscher, Brumath, France), 1%

ZellShield, 1% non-essential amino acids, 1% sodium pyruvate, 1% HEPES-buffer and 0.02mM 2-Mercaptoethanol.[1, 2] Briefly, 10^5 THP1-cells were induced with 100nM of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Saint-Louis, Missouri) overnight to allow cells to seed on a glass coverslip and differentiated for 6 days with 10ng/mL of human Macrophage Colony Stimulating Factor (hM-CSF) and 1ng/mL of human Granulocyte Macrophage Colony Stimulating Factor (hGM-CSF) (R&D System, Minneapolis, Minnesota).

A stable cell line of HEK293-cells expressing human SR-AI in pcDNA6/V5-His vector[1] was cultured in Dulbecco's Modified Eagle Medium: Nutrient mixture F12 (DMEM:F12; Life Technologies) supplemented with 10% FBS and 1% ZellShield in the presence of 5µg/mL blasticidin. Non-transfected HEK293-cells were cultured in the same medium without blasticidin. 10^5 cells were seeded on glass coverslips overnight, before the VWF binding assay.

Murine macrophages were obtained from $CD115^+$ cells [3]. Femur and humerus from C57BL/6 and SR-AI-deficient mice were harvested and bone-marrow was flushed in PBS/BSA (0.1%)/EDTA (5mM) to collect bone-marrow cell progenitors. CD115+ cells were then isolated by positive selection using the $CD115^+$ sorting kit (Miletnyi Biotec) according to manufacturer's recommendations. 10^5 cells were resuspended in modified RPMI-1640 supplemented with 20ng/ml of murine M-CSF and 2ng/mL of murine GM-CSF (Miltenyi Biotec) and differentiated on glass coverslips over 6 days with the medium being changed every 2 days.

Human-VWF binding assay:

Cells seeded and differentiated on 9-mm glass coverslips as described in the previous paragraph were incubated with DMEM:F12/ 0.5% lipid-rich bovine serum albumin (AlbuMax II, Gibco, ThermoFisher Scientific) for 1 hour at 37°C. Cells were then incubated with or without 10µg/ml purified pd-VWF for 1 hour at 37°C in a humid chamber. Where indicated, culture medium containing recombinant wt-VWF, VWF/p.R1205H, VWF/p.V1316M or VWF/p.S2179F was used. In other experiments, pd-VWF was pre-incubated with monoclonal antibodies to VWF (MAb723 and MAb540, 167µg/ml) for 30 min at room temperature.

Cells were gently washed twice and then fixed for 15 minutes with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at room

temperature. Fixed cells were stored at 4°C in Phosphate Buffered Saline until immunofluorescence was performed.

Microscopy analyses and immunofluorescence based quantification:

Coverslips were incubated in Phosphate Buffered Saline with 5% Bovine Serum Albumin (PBS/BSA 5%) 30min at room temperature (RT) to saturate non-specific binding sites. Primary antibodies were probed 2h at RT in PBS/BSA 1% followed by a 1h incubation of the secondary antibodies. Nuclei were counterstained with 1µg/mL of 4',6'-diamidino-2-phenylindole (DAPI) and AlexaFluor-647 or AlexaFluor-488 labeled Phalloïdin (Life Technologies) was used to determine cell boundaries. Coverslips were mounted in Prolong Gold antifade reagent (Life Technologies) and stored at -20°C.

For Duolink-Proximity Ligation Assay (Duolink-PLA) experiments to detect close proximity between different proteins, double immunostaining were performed as described above with the second antibodies replaced by PLA probes (Sigma-Aldrich). The remainder of the protocol was conducted according to the manufacturer's recommendations and the 550nm wavelength detection kit was used. Hybridization between the two PLA probes leading to fluorescent signal only occurs when the distance between the two detected antigens is less than 40nm.

Widefield microscopy images were acquired on an AxioImager A1 (Carl Zeiss, OberKocher, Germany) or an Olympus IX73 (Tokyo, Japan) equipped with a Hamamatsu C11440-22CU ORCA-Flash4.0 V2 digital camera and further analyzed using ImageJ software for quantification of fluorescence by measuring the total pixel intensity per cell. Duolink-PLA experiments were analyzed using BlobFinder software (Uppsala University, Sweden) to quantify the number of fluorescent spot per cells. All images were assembled using ImageJ software.

Immunosorbent Binding Assay:

Binding of purified pd-VWF or recombinant fragments thereof to the soluble extracellular domain of SR-AI was assessed in an immunosorbent assay. Where indicated, non-purified recombinant wt-VWF, VWF/p.R1205H, VWF/p.V1316M or VWF/p.S2179F present in culture medium was used. Microtiter plates (Greiner Bio-one, Kremsmünster, Austria) were coated with SR-AI or BSA (1 µg/well) for 2h at 37°C in 50mM Na_2CO_3 and post-coated 1h at 37°C in working buffer (50nM Tris

pH7.4, 150nM NaCl, 0.1% Tween20, 0.1% PolyVinylPyrrolidone) to saturate non-specific binding sites. Increasing concentrations of VWF (0-5 μg/ml) were incubated 2h at 37°C in working buffer with 2mM $CaCl_2$. Unbound proteins were washed away and bound VWF was probed by incubation of a peroxidase labeled anti-VWF antibody (DAKO) or peroxidase-labeled anti-human Fc antibodies followed by chromogenic conversion of Tetramethylbenzidine and reading at 450nM. In alternative experiments, VWF fragments were immobilized (1 μg/ml) and incubated with soluble SR-AI (sSR-AI; 0-5μg/ml). Bound sSR-AI was probed with peroxidase-labeled polyclonal goat anti-SR-AI antibodies. Half-maximal binding was calculated using a non-linear regression model in GraphPad Prism software.

Statistical analysis:

All analyses were performed using GraphPad Prism software (La Jolla, Ca, USA). Pair-wise analysis was performed using unpaired Student t-test or Mann-Whitney test where indicated, while multiple comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test.

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