Factor VIII and von Willebrand factor are ligands for the carbohydrate-receptor Siglec-5

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Online Supplementary Design and Methods

Non-equilibrium binding assays

FVIII, VWF or BSA (2.5 μg/mL) were adsorbed to microtiter wells. After blocking with polyvinylpyrrolidone (PVP, 0.5 %) for 2 h at 37°C, the immobilized proteins were incubated with various concentrations of sSiglec-5/HPC4 in incubation-buffer (0.1% Tween-20, 0.5% PVP, 5 mM $CaCl_2$, 150 mM NaCl, 25 mM Tris-HCl (pH 7.4)) for 3 h at 37°C. After washing, bound sSiglec-5/HPC4 was probed with peroxidase-labeled antibody HPC4 for 2 h at 37°C and detected by peroxidase hydrolysis of the substrate tetramethylbenzidine (TMB). Where indicated, FVIII, VWF or BSA were incubated in the presence of polyclonal sheep-anti-human FVIII antibodies (Kordia Life Sciences, Leiden, The Netherlands), polyclonal goat-anti-human VWF antibodies (Dakocytomation, Glostrup, Denmark) or polyclonal goat-anti-human Siglec-5 antibodies (R&D Systems). In some cases, immobilized proteins were incubated in the absence or presence of sialidase (0.1 U/ml) for 16 h at 37°C in incubation-buffer before blocking with PVP (0.5%). In an alternative approach, wells were coated with an in-house monoclonal anti-FVIII antibody D4H1 (2 μg/mL). After blocking of unoccupied sites with PVP (0.5%), wells were incubated in the absence or presence of plasma-derived FVIII or recombinant B-domainless FVIII (both 0.2 μg/mL) for 2 h at 37°C in incubation-buffer. This incubation appeared sufficient to saturate antibody D4H1 with either type of FVIII, both of which are recognized similarly by this antibody (*data not shown*). After washing, wells were incubated with various concentrations of sSiglec-5/Fc in incubation-buffer for 3 h at 37°C. After washing, bound sSiglec-5/Fc was probed with peroxidase-labeled anti-human Fc antibody (Sanquin, Amsterdam, The Netherlands) for 1 h at 37°C and detected by peroxidase hydrolysis of TMB.

Equilibrium binding assays

Equilibrium binding was performed via biolayer-interferometry (BLI)-analysis using Octet-QK-equipment (ForteBio, Reading, UK). Protein A-coated biosensors were incubated with total human IgG (Sanquin, Amsterdam, The Netherlands) or sSiglec-5/Fc (both 20 μg/mL for 10 min in BLI-buffer (5 mM $CaCl_2$, 125 mM NaCl, 25 mM Hepes (pH 7.4)). Biosensors were incubated for 5 min in BLI-buffer to achieve stable baseline, and subsequently incubated with various concentrations of plasma-derived FVIII or VWF in BLI-buffer for 10 min to allow association. Finally, biosensors were put in BLI-buffer for 10 min to initiate dissociation. All incubations were performed at room temperature under continuous shaking (1,000 rpm). Data were analyzed using Octet Software version 4.0.

Cellular binding of FVIII and VWF

Cells (non-transfected HEK293 cells or HEK293-Siglec-5 cells) were seeded on glass cover slips in 24-well culture plates in DMEM/Ham F-12 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin. One day after seeding, cells were washed with PBS and transferred into DMEM/Ham's F-12 medium without supplements for 12 h at 37°C. After refreshing the medium, cells were incubated for a further hour at 37°C in the absence or presence of sialidase (0.1 U/mL) and washed three times with PBS and once with DMEM/Ham's F-12 medium and then put at 4°C. Then, cells were incubated with FVIII or VWF (both 10 μg/mL) in DMEM/Ham's F-12 medium for 1 h at 4°C to avoid internalization of the proteins. Subsequently, excess of unbound protein was removed by washing two times with ice-cold PBS and cells were fixed by the addition of methanol (30 min at room temperature). Fixed cells were stored at 4°C in PBS until further use.

Hydrodynamic injection

Full-length Siglec-5 was subcloned into pLIVE-expression plasmid (Mirus Bio, Madison, WI, USA), in which a mouse albumin promotor drives hepatocyte-specific expression. pLIVE-Siglec-5 or empty pLIVE (each 100 μg) were applied to wild-type C57B6 mice (20-25 gram) with the use of the hydrodynamic injection method as described previously.[1] Blood samples were taken four days after injection via retro-orbital puncture for the analysis of FVIII, VWF and FX plasma levels. Expression of Siglec-5 in the hepatocytes was verified via immunohistological staining of livers taken four days after injection (see below).

Immunostaining

Cellular bound VWF or FVIII was detected via immunostaining of the methanol-fixed cells. To this end, fixed cells were first incubated for 30 min at 37°C in immunostain buffer (PBS/2% ovalbumin/0.01% azide). After washing with PBS, cells were incubated with a pool of mouse monoclonal anti-human VWF antibodies or mouse monoclonal anti-FVIII antibody D4H1 (both 2 μg/mL) in immunostain buffer for 30 min at 37°C. After washing with PBS, bound antibodies were probed with Alexa Fluor 488-conjugated F(Ab') 2 fragments of goat anti-mouse IgG (Invitrogen, Cergy Pontoise, France) for 30 min at 37°C. After washing with PBS, glass cover slips were embedded in VectaShield+DAPI mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). To prepare liver sections, murine livers were explanted, rinsed with physiological serum and snap frozen in Tissue-Tek OCT-compound (Sakura Finetek, Zouterwoude, The Netherlands) in liquid nitrogen vapors. Three micrometer cryosections were prepared, washed twice and fixed in 3.7% formaldehyde for 20 min at room temperature. Rinsed slides

were blocked via incubation with PBS supplemented with 4% BSA and 2.5 μg/mL purified mouse IgG (Invitrogen, Saint-Aubin, France). Subsequently, slides were incubated with polyclonal goat-anti-human Siglec-5 antibodies, which were probed using TRITC-labeled rabbit-anti-goat IgG (SouthernBiotech, Birmingham, AL, USA). Slides were finally washed, dried and mounted with Fluorescence Mounting Medium (Dako, Carpinteria, CA, USA).

Duolink-proximity ligation assay (Duolink-PLA)

Fixed cells were first incubated for 30 min at 37°C in immunostain buffer (PBS/2% ovalbumin/0.01% azide). After washing with PBS, cells were incubated simultaneously with a pool of mouse monoclonal anti-human VWF antibodies or mouse monoclonal anti-FVIII antibody D4H1 in combination with polyclonal rabbit antibodies against the early endosomal marker EEA1 (Abcam, Cambridge, UK) or with polyclonal goat anti-human Siglec-5 antibodies (all 2 μg/mL) in immunostain buffer for 30 min at 37°C. The Duolink-PLA assay was subsequently performed as instructed (Olink Bioscience, Uppsala, Sweden). Briefly, this assay relies on the incubation with secondary antibodies coupled to complementary oligonucleotides that can hybridize when within a range of 40 nm. Following hybridization, this DNA template is amplified and highlighted using fluorescent-labeled oligonucleotides, generating one discrete red spot for each protein complex.

Microscopic imaging

Images were visualized using an AxioImager A1 microscope (Carl Zeiss, Göttingen, Germany) using a Plan-Apochromat 63x/NA 1.4-oil immersion objective or an EC Plan-Neofluor 40x/0.75-air objective. Confocal images were obtained using an Axiovert 200M microscope using a Plan-Apochromat 63x/NA 1.4-oil immersion objective and a Zeiss LSM510-meta confocal system (Carl Zeiss). AxioVision LE 7.7.1 (Carl Zeiss) acquisition software was used. To quantify the number of positive cells, between 11 and 25 microscopic fields of two independent staining procedures that contained at least 15 cells/field were analyzed. Positively-stained cells were defined as cells that contained at least 3 green spots. Quantitative analysis of fluorescence intensity/cell was determined using BlobFinder software[2] and is expressed in relative units (RU).

References

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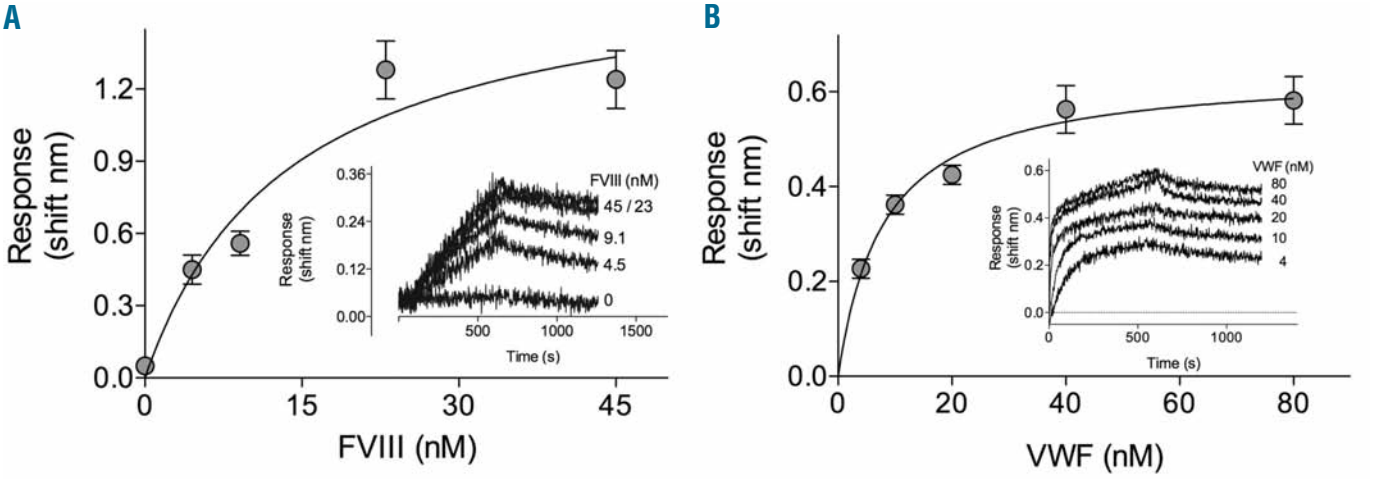

Online Supplementary Figure S1. Binding of FVIII and VWF to immobilized sSiglec-5/Fc. Binding of FVIII (A) or VWF (B) to sSiglec-5/Fc was examined via BLI-analysis using Octet-QK equipment. Various concentrations of FVIII (0-45 nM) or VWF (0-80 nM) were incubated with dimeric sSiglec-5/Fc adsorbed onto Protein A-coated biosensors. Plotted are the responses at equilibrium (shift nm) *versus* protein concentration (nM). Responses at equilibrium were calculated by fitting the association phase of the sensorgrams to a 1:1 Langmuir association. The drawn lines were obtained by fitting the data to an equation describing the binding of soluble Siglec-5 to a single class of binding sites, and were used to calculate half-maximal binding. Data represent mean±SEM of 3 independent analyses. Insets show representative examples of original sensorgrams showing association and dissociation.