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AUTHOR(S)

Soracha Enright Ward, Jamie O'Sullivan, Alan B Moran, Daniel IR Spencer, Richard A Gardner, Jyotika Sharma, Judicael Frazavana, Marco Monopoli, Thomas AJ McKinnon, Alain Chion, Sandra Haberichter, James O'Donnell

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Sialylation on O-linked glycans protects von Willebrand factor from macrophage galactose lectin-mediated clearance

Soracha E. Ward,[1] Jamie M. O'Sullivan,[1] Alan B. Moran,[2,3] Daniel I. R. Spencer,[2] Richard A. Gardner,[2] Jyotika Sharma,[4] Judicael Fazavana,[1] Marco Monopoli,[5] Thomas A. J. McKinnon,[6] Alain Chion,[1] Sandra Haberichter[7] and James S. O'Donnell[1,8,9]

[1]Irish Centre for Vascular Biology, School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland, Dublin, Ireland; [2]Ludger, Ltd., Culham Science Centre, Abingdon, Oxfordshire, UK; [3]Leiden University Medical Centre, Centre for Proteomics and Metabolomics, Leiden, the Netherlands; [4]Department of Basic Biomedical Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND, USA; [5]Department of Chemistry, Royal College of Surgeons in Ireland, Dublin, Ireland; [6]Faculty of Medicine, Imperial College, Hammersmith Hospital, London, UK; [7]Versiti, Blood Research Institute, Milwaukee, WI, USA; [8]National Children's Research Centre, Our Lady's Children's Hospital, Dublin, Ireland and [9]National Coagulation Centre, St James's Hospital, Dublin, Ireland

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ABSTRACT

Terminal sialylation determines the plasma half-life of von Willebrand factor (VWF). A role for macrophage galactose lectin (MGL) in regulating hyposialylated VWF clearance has recently been proposed. In this study, we showed that MGL influences physiological plasma VWF clearance. MGL inhibition was associated with a significantly extended mean residence time and 3-fold increase in endogenous plasma VWF antigen levels ($P<0.05$). Using a series of VWF truncations, we further demonstrated that the A1 domain of VWF is predominantly responsible for enabling the MGL interaction. Binding of both full-length and VWF-A1-A2-A3 to MGL was significantly enhanced in the presence of ristocetin ($P<0.05$), suggesting that the MGL-binding site in A1 is not fully accessible in globular VWF. Additional studies using different VWF glycoforms demonstrated that VWF O-linked glycans, clustered at either end of the A1 domain, play a key role in protecting VWF against MGL-mediated clearance. Reduced sialylation has been associated with pathological, increased clearance of VWF in patients with von Willebrand disease. Herein, we demonstrate that specific loss of α2-3 linked sialylation from O-glycans results in markedly increased MGL-binding *in vitro*, and markedly enhanced MGL-mediated clearance of VWF *in vivo*. Our data further show that the asialoglycoprotein receptor (ASGPR) does not have a significant role in mediating the increased clearance of VWF following loss of O-sialylation. Conversely however, we observed that loss of N-linked sialylation from VWF drives enhanced circulatory clearance predominantly via the ASGPR. Collectively, our data support the hypothesis that in addition to regulating physiological VWF clearance, the MGL receptor works in tandem with ASGPR to modulate enhanced clearance of aberrantly sialylated VWF in the pathogenesis of von Willebrand disease.

Correspondence:

JAMES O'DONNELL
jamesodonnell@rcsi.ie

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Introduction

von Willebrand disease (VWD) is the commonest inherited human bleeding disorder and is caused by either quantitative or qualitative deficiency of plasma von Willebrand factor (VWF).[1,2] Increased plasma clearance of VWF constitutes an important mechanism in the pathogenesis of VWD.[3] The MCMDM-1VWD European study, US Zimmerman Program and Willebrand in the Netherlands (WIN) study have all found pathological, enhanced VWF clearance in approximately 45% of patients with type 1 VWD, leading to the proposal that patients

with a shortened VWF half-life should be considered as a distinct type 1C (1-Clearance) subgroup.[4-8] Interestingly, subsequent studies have highlighted that enhanced VWF clearance also contributes to pathogenesis in patients with low VWF, as well as type 2 and type 3 VWD.[7,9-12]

Given the importance of enhanced clearance in the pathogenesis of VWD, significant research has focused on defining the cellular and molecular clearance pathways involved. Potential roles for macrophages, liver sinusoidal endothelial cells and hepatocytes have been proposed.[13-17] A number of specific clearance receptors have also been described.[3] These include the low-density lipoprotein receptor-related protein-1 (LRP1), the scavenger receptor class A member I (SR-A1), sialic-acid-binding-immunoglobulin-like-lectins 5 (Siglec 5) and the macrophage galactose-type lectin (MGL) which are all expressed on macrophages.[18-21] On liver sinusoidal endothelial cells, receptors that may play a role in VWF clearance include stabilin-2 (STAB2), scavenger receptor class A member 5 (SCARA 5) and C-type lectin domain family 4 member M (CLEC4M).[17,22,23] Finally, the asialoglycoprotein receptor (ASGPR), predominantly expressed on hepatocytes and macrophages, has also been implicated.[24]

More than 30 different VWF sequence variations have been reported in patients with increased VWF clearance.[3,25] The archetypal type 1C mutation is the VWD Vicenza variant which is characterized by an R1205H substitution in the D3 domain of VWF.[15,26] VWF glycosylation also plays a critical role in determining the rate of clearance of the protein.[27-30] For example, plasma VWF:antigen (VWF:Ag) levels are 20-30% lower in blood group O individuals compared to non-O subjects due to a significant reduction in plasma half-life.[31,32] Enzymatic removal of terminal sialic acid residues from VWF also markedly enhances clearance.[27,28] Moreover, genetic inactivation of the ST3Gal-IV sialyltransferase was associated with a significant reduction in plasma VWF half-life.[33] These data are important from a clinical perspective because the majority of both the N- and O-linked glycans of VWF are normally capped by sialic acid residues.[34-37] As glycoproteins age in plasma, there is a stepwise elimination of saccharides from the termini of complex glycan chains.[38] Glycan remodeling begins with loss of capping sialic acid, catalyzed by plasma neuraminadases 1 (Neu1) and 3 (Neu3) respectively. This time-dependent desialylation is important in triggering clearance of senescent glycoproteins.[38] Significantly reduced VWF sialylation levels have also been observed in a number of pathological conditions including sepsis, pulmonary hypertension and liver cirrhosis.[39-41] Importantly, several groups have reported reduced VWF sialylation in patients with type 1 VWD.[10,33,40,42] Together, these data suggest that quantitative sialylation plays a critical role in regulating both physiological and pathological clearance of VWF *in vivo*.

Grewal *et al.* originally described a role for the ASGPR in regulating enhanced clearance of desialylated VWF (particularly in the context of sepsis).[24] More recently, we identified MGL as another receptor involved in regulating hyposialylated VWF clearance.[21] Critically, however, important questions remain unanswered regarding the roles played by VWF sialylation in regulating physiological and/or pathological clearance. These include: (i) the relative importance of N- *versus* O-linked sialylation in regulating VWF clearance; (ii) the relative contributions of the ASGPR and MGL clearance receptors; and (iii) the molecular mechanisms through which hyposialylated VWF interacts with its clearance receptors.

Methods

A detailed description of the materials and methods can be found in the associated *Online Supplementary Material.*

Isolation and purification of human plasma-derived von Willebrand factor

Plasma-derived VWF (pdVWF) was purified from the VWF-containing concentrate Fandhi® (Grifols, Barcelona, Spain) as previously described.[21] Platelet-VWF was purified from lysed platelets as described elsewhere.[43] Eluate fractions were then assessed for VWF antigen, multimer distribution, and purity.

Glycosidase digestion and quantitative analysis of glycan expression

To generate VWF glycoforms, pdVWF was treated with α2-3 neuraminidase, α2-3,6,8,9 neuraminidase, β1-3 galactosidase, peptide N-glycosidase F (PNGase F) and/or O-glycosidase under non-denaturing conditions overnight at 37°C.[29,44] Following glycosidase digestion, changes in VWF glycans were assessed using specific lectin enzyme-linked immunosorbent assays (ELISA) as previously described.[10]

Expression and purification of recombinant von Willebrand factor variants

The expression vectors pcDNA-VWF encoding full length recombinant VWF, VWF-A1A2A3, VWF-A1, VWF-A2, VWF-A3, VWF-D'A3 or VWF-A3-CK fragments have previously been described.[16] Additional VWF-A1 constructs containing either of the two O-linked glycan (OLG) clusters were also included; A1-OLG cluster 1 (T1248A, T1255A, T1256A, S1263A), and A1-OLG cluster 2 (T1468A, T1477A, S1486A, T1487A). All recombinant VWF variants were transiently expressed in HEK293T cells. Conditioned serum-free medium was harvested 72 h after transfection and concentrated via anion exchange chromatography as described before.[16]

In vitro von Willebrand factor binding studies

Solid phase plate-binding assays were used to evaluate VWF binding to MGL. Briefly, recombinant human MGL (Stratech, UK) was immobilized on a PolySorp™ 96-well plate (Nunc, Thermo Scientific™), the wells were blocked, and VWF was incubated at 37°C for 1 h. Bound VWF was detected using horseradish peroxidase (HRP)-conjugated polyclonal anti-VWF (Dako, Agilent Technologies), high-sensitivity streptavidin-HRP (ThermoScientific, UK) or anti-His-HRP antibody (Qiagen, UK) (see *Online Supplementary Material* for details).

von Willebrand factor clearance studies in *MGL1*$^{-/-}$, *VWF*$^{-/-}$ and *VWF*$^{-/-}$/*Asgr1*$^{-/-}$ mice

All clearance experiments were performed on mice 6 to 8 weeks old. All animal studies were approved by the Health Product Regulatory Authority, Ireland and an internal ethics committee. *VWF*$^{-/-}$ and *Asgr1*$^{-/-}$ mice, both on a C57BL/6J background, were obtained from the Jackson Laboratory (Sacremento, CA, USA) and crossbred to obtain a dual *VWF*$^{-/-}$/*Asgr1*$^{-/-}$ knockout model as previously described.[21] MGL specific clearance studies were also performed after inhibition of murine MGL1/2 using a commercial polyclonal goat anti-mouse MGL1/2 antibody (2 mg/kg) (R&D Systems, UK) as previously described.[21] For endogenous clearance studies, murine VWF was labeled with N-hydrox-

ysuccinimide-biotin (10 mg/kg; Thermo-Scientific), residual biotinylated murine VWF was quantified using a modified VWF ELISA. All clearance data were fitted to monoexponential equations, based on analysis of the Akaike information criterion. The slope and intercept of the equation of the line were used to calculate pharmacokinetic parameters including mean residence time (MRT) and half-life ($t_{1/2}$).

Data presentation and statistical analysis

Experimental data were analyzed with GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean values ± standard error of the mean. Data were analyzed with the Student unpaired two-tailed *t*-test and *P*-values <0.05 were considered to be statistically significant.

Results

Physiological importance of MGL in regulating von Willebrand factor clearance *in vivo*

Mice have two distinct MGL homologs - murine MGL1 (mMGL1) and murine MGL2 (mMGL2).[45] To gain insight into the biological importance of MGL, we first investigated murine MGL1 and MGL2 binding to VWF *in vitro*. Similar to human MGL, dose-dependent binding of both mMGL1 and mMGL2 to VWF was observed (Figure 1A). VWF binding to MGL2 was significantly greater than to mMGL1 (P<0.001). Murine plasma VWF:Ag levels are significantly elevated (~ 1.5 fold) in *MGL1*$^{-/-}$ mice. Since both mMGL1 and mMGL2 bind VWF, we hypothesized that knocking down mMGL1 alone may underestimate the biological importance of MGL-mediated clearance. Dual *mMGL1*$^{-/-}$/*mMGL2*$^{-/-}$ mice are not commercially available; thus, to address this hypothesis, *in vivo* clearance studies were repeated in *mMGL1*$^{-/-}$ mice in the presence or absence of dual anti-MGL1/2 inhibitory antibodies. Following treatment with anti-MGL2, murine VWF:Ag levels were significantly increased compared to those in *mMGL1*$^{-/-}$ controls (2.78±04 U/mL *vs.* 1.5±0.5 U/mL respectively; P<0.05) (Figure 1B). Thus, complete murine MGL inhibition was associated with an almost 3-fold increase in endogenous plasma VWF:Ag levels compared to the levels in wild-type (mMGL1$^{+/+}$mMGL2$^{+/+}$) controls. In the presence of combined mMGL1 and mMGL2 inhibition, endogenous VWF clearance was significantly attenuated compared to that in controls (P<0.05) (Figure 1C) and murine VWF MRT was increased 2.4-fold (Figure 1D). These data confirm that the observed increase in murine VWF levels associated with

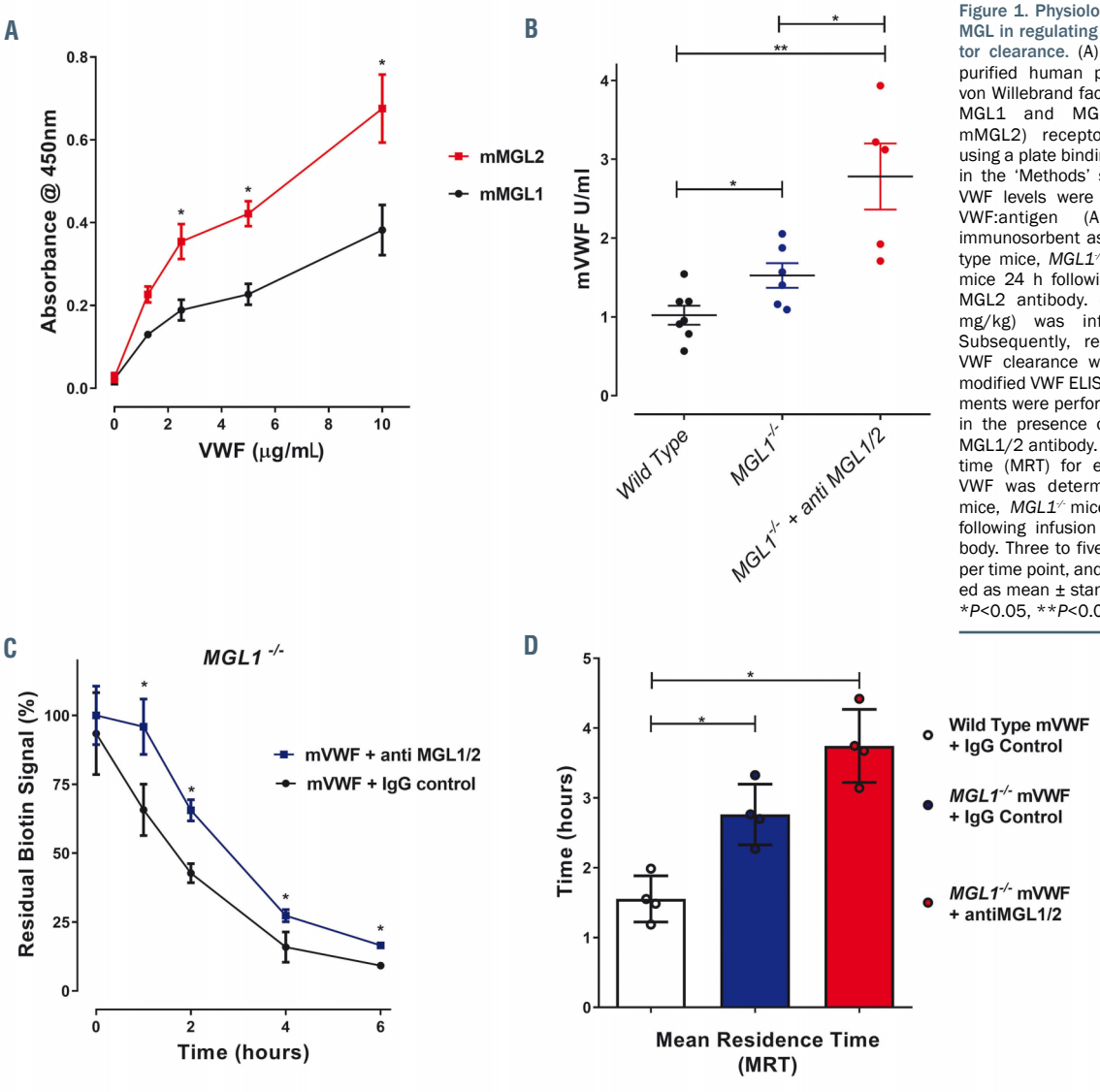


Figure 1. Physiological importance of MGL in regulating von Willebrand factor clearance. (A) *In vitro* binding of purified human plasma-derived (pd) von Willebrand factor (VWF) to murine MGL1 and MGL2 (mMGL1 and mMGL2) receptors was assessed using a plate binding assay as detailed in the 'Methods' section. (B) Plasma VWF levels were measured using a VWF:antigen (Ag) enzyme-linked immunosorbent assay (ELISA) in wild-type mice, *MGL1*$^{-/-}$ mice, and *MGL1*$^{-/-}$ mice 24 h following infusion of anti-MGL2 antibody. (C) NHS-biotin (10 mg/kg) was infused at t=0 h. Subsequently, residual biotinylated VWF clearance was quantified by a modified VWF ELISA. Clearance experiments were performed in *MGL1*$^{-/-}$ mice in the presence or absence of anti-MGL1/2 antibody. (D) Mean residence time (MRT) for endogenous murine VWF was determined for wild-type mice, *MGL1*$^{-/-}$ mice and *MGL1*$^{-/-}$ mice following infusion of anti-MGL2 antibody. Three to five mice were studied per time point, and data are represented as mean ± standard error of mean. **P*<0.05, ***P*<0.01.

inhibition of MGL-mediated clearance is attributable to an increase in VWF half-life. Importantly the magnitude of this effect of MGL on plasma VWF levels is also greater than that previously reported in *Asgr1*$^{-/-}$ mice (VWF:Ag levels increased ~1.5 fold).[24] Collectively, these data demonstrate that mMGL2 constitutes another novel macrophage clearance receptor for VWF in mice. More importantly, the findings further suggest that MGL has a greater effect than ASGPR in regulating physiological VWF clearance.

The A domains of von Willebrand factor play a critical role in regulating MGL binding

Previous studies have demonstrated that macrophages play a key role in VWF clearance, and have further shown that macrophage receptor-recognition site(s) are present within the VWF-A1A2A3 domains.[13,16] To assess the specific role of VWF domains in modulating MGL binding a series of variants were expressed (Figure 2A) (*Online Supplementary Table S1*). Since MGL is expressed on macrophages, we investigated whether the A domains of VWF influence MGL binding. Dose-dependent binding of pdVWF to recombinant human MGL was observed (Figure 2B). In keeping with the fact that MGL is a C-type lectin, this binding was ablated in the presence of EDTA (Figure 2B). Conversely, VWF-MGL binding was significantly enhanced in the presence of ristocetin (1 mg/mL) (Figure 2B). No significant effect of VWF multimer distribution on MGL-binding was observed (*Online Supplementary Figure S1*). Binding studies confirmed dose-dependent binding of VWF-A1A2A3 to MGL, which was again ristocetin- and calcium-dependent (Figure 2C). Finally, the relative importance of the individual domains within A1A2A3 in determining MGL binding was assessed. Although significant binding of the VWF-A1 domain to MGL was seen, no binding for either VWF-A2 or VWF-A3 was observed (Figure 2D). Together, these data support the hypothesis that the A1 domain of VWF plays a critical role in determining VWF binding to the MGL surface receptor on macrophages.

O-linked glycans on von Willebrand factor modulate the MGL interaction

Each VWF monomer contains 13 N-linked and ten O-linked glycan structures (Figure 3A).[34,35] Whereas N-glycans are distributed across the VWF monomer, eight of the ten O-glycans are clustered in two groups around the VWF-A1 domain.[35,36] To study the importance of specific N- and O-glycans in regulating MGL binding, pdVWF was treated with PNGase F and/or O-glycosidase respectively. Following each digestion, residual VWF glycan expression was assessed using lectin-binding ELISA (*Online Supplementary Figure S2A, B*). Although treatment with PNGase F successfully removed N-linked glycans from full-length pdVWF, it did not have a significant effect on MGL binding (Figure 3B). Conversely, digestion with O-glycosidase was associated with a marked reduction in pdVWF binding to MGL (P<0.001) (Figure 3B). The VWF-A1A2A3 truncation contains two N-linked glycans and eight O-linked glycans (Figure 3A). In keeping with full-length VWF, PNGase treatment of A1A2A3-VWF (*Online Supplementary Figure S2B, C*) had no impact on the MGL interaction, whereas removal of O-glycans markedly attenuated binding (P<0.01) (Figure 3C). Digestion of isolated A1 domain with O-glycosidase (*Online Supplementary Figure S2E*) was also associated with a significant reduction in MGL binding (P<0.001) (Figure 3D). Finally, to investigate the relative importance of the two OLG clusters at either side of the A1 domain, isolated A1-OLG cluster 1 and A1-OLG cluster 2 were expressed (Figure 2A). Although MGL-binding was observed for both of these VWF A1 domain truncations, significantly enhanced binding was observed for A1-OLG cluster 2 (Figure 3E). Cumulatively, these findings demonstrate that VWF OLG, particularly those clustered either side of the A1 domain, play a major role in regulating MGL interaction.

α2-3 sialylation on O-glycans protects von Willebrand factor against MGL-mediated clearance

Recent mass spectrometry studies have characterized the O-glycan structures expressed on human pdVWF and highlighted significant heterogeneity (Figure 4A).[35-37] Critically, however, a consistent feature of these O-glycan chains is that they generally terminate with sialic acid, which may be present in either α2-3 or α2-6 linkage.[35,36] In contrast, most N-linked sialic acids are α2-6 linked.[34] To further investigate the role of VWF O-glycans in determining MGL-mediated clearance, pdVWF was digested with a series of exoglycosidases to generate specific VWF glycoforms (Figure 4A). Treatment with α2-3 neuraminidase to remove α2-3 linked sialylation from O-glycans (*Online Supplementary Figure S2F*) significantly enhanced pdVWF binding to MGL (P=0.017) (Figure 4B). Similarly, digestion with α2-3,6,8,9 neuraminidase (which removes α2-3 linked sialylation from O-glycans and α2-6 linked sialylation from both N- and O-glycans) (*Online Supplementary Figure S2G*) was also associated with significantly increased MGL binding (P=0.006). Despite the fact that an estimated 80% of total sialylation on VWF is α2-6 linked, α2-3,6,8,9 Neu-VWF binding to MGL was not different to that observed following α2-3 neuraminidase digestion alone (Figure 4B). Significantly enhanced binding was observed for PNG-VWF following additional removal of α2-3 linked sialylation and exposure of the O-linked T antigen structure (Figure 4C). Finally, PNG-VWF was sequentially treated with α2-3 neuraminidase and β1-3 galactosidase to remove both terminal sialic acid and subterminal galactose (Gal) residues from VWF O-glycan chains (*Online Supplementary Figure S2H*). This combined digestion ablated the enhanced binding observed following α2-3 neuraminidase digestion alone (Figure 4C). These data demonstrate that α2-3 linked sialylation on VWF O-glycans specifically protects VWF against MGL-mediated clearance. Loss of this capping sialic acid results in Gal residue exposure on VWF O-glycans, which then triggers clearance through the MGL receptor. In order to consider whether other VWF domains/glycans may contribute to MGL-interaction, we compared binding for N-terminal D'A3-VWF and C-terminal A3-CK-VWF fragments. In keeping with a key role for the A1 domain, significant binding of D'A3-VWF to MGL was observed (Figure 4D). Interestingly, however, some A3-CK-VWF binding was also seen, suggesting that O-glycans (T1679 and/or T2298) downstream of the A1 domain may also play a role.

Role of MGL and ASGPR in modulating pathological enhanced clearance of desialylated von Willebrand factor

Previous studies have demonstrated altered VWF sialylation in patients with VWD as well as in a number of other conditions.[41] To investigate the role of MGL in mediating the enhanced clearance of pathologically desia-

lylated VWF, pdVWF was treated *ex vivo* with α2-3 neuraminidase to remove α2-3 linked sialylation from O-glycans. *In vivo* clearance studies were then performed in *VWF*$^{-/-}$ mice in the presence or absence of combined mMGL1 and mMGL2 inhibition. Removal of α2-3 linked sialylation was associated with a marked reduction in VWF half-life compared to that of the wild-type control (Figure 5A). Importantly, however, this enhanced clearance was attenuated in the presence of MGL inhibition (Figure 5A). To assess the relative roles of MGL and ASGPR in modulating the pathological, enhanced clearance following removal of α2-3 sialylation, *in vivo* clearance studies were also performed in dual *VWF*$^{-/-}$*Asgr1*$^{-/-}$ knockout mice in the presence or absence of combined mMGL1 and mMGL2 inhibition (Figure 5B). Critically, we observed that MGL inhibition was also able to block enhanced clearance of pdVWF after loss of α2-3 sialylation equally effectively in the presence or absence of ASGPR (Figure 5B).

Terminal sialylation on VWF O-glycans can be either α2-3 or α2-6 linked. In contrast, sialylation on VWF N-glycan chains is predominantly α2-6 linked (Figure

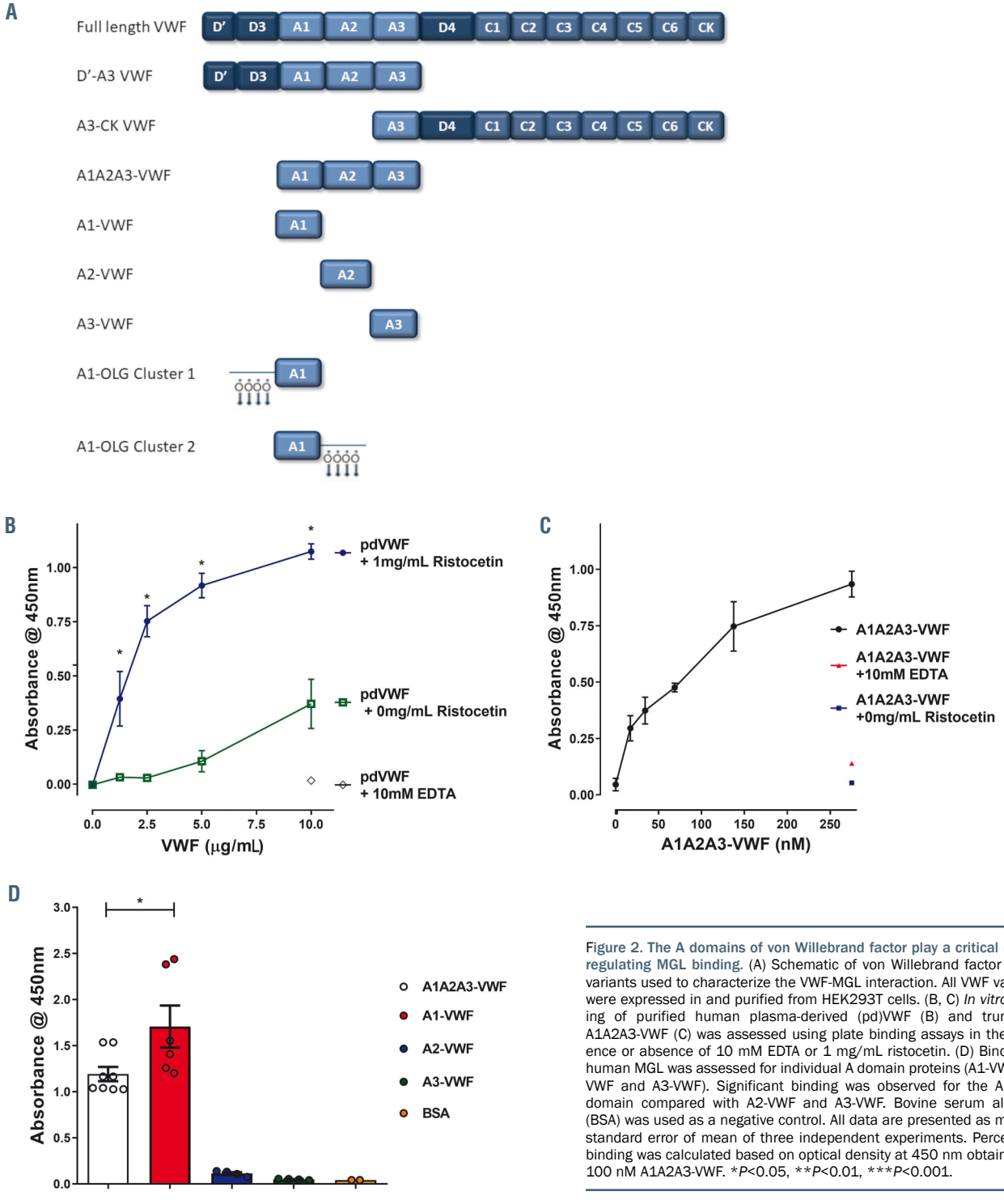
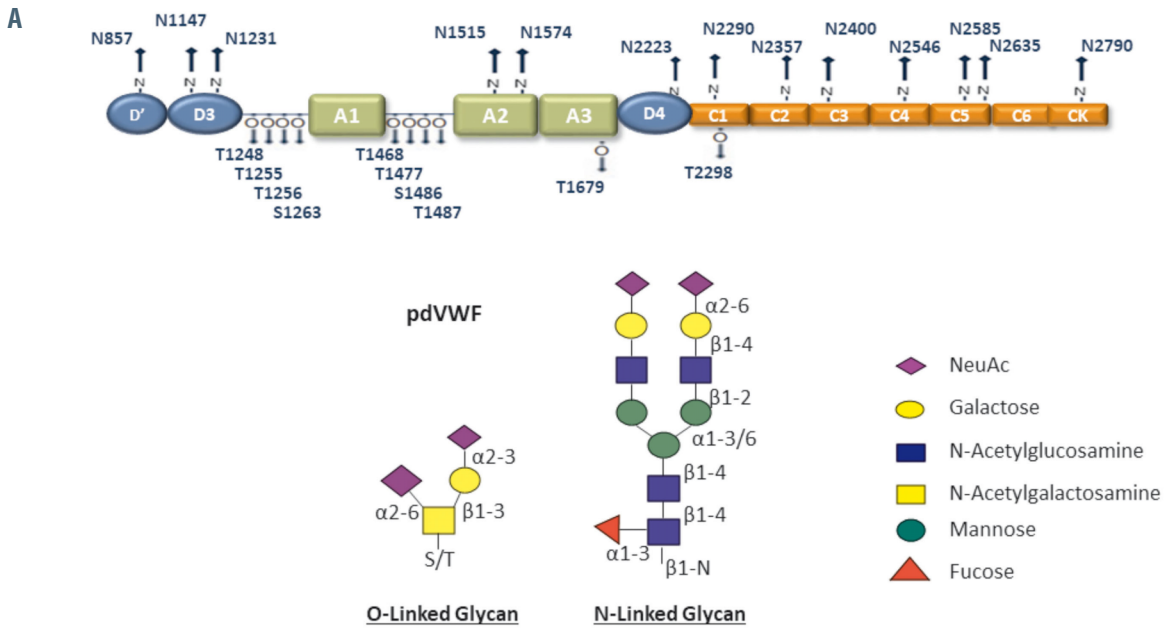


Figure 2. The A domains of von Willebrand factor play a critical role in regulating MGL binding. (A) Schematic of von Willebrand factor (VWF) variants used to characterize the VWF-MGL interaction. All VWF variants were expressed in and purified from HEK293T cells. (B, C) *In vitro* binding of purified human plasma-derived (pd)VWF (B) and truncated A1A2A3-VWF (C) was assessed using plate binding assays in the presence or absence of 10 mM EDTA or 1 mg/mL ristocetin. (D) Binding to human MGL was assessed for individual A domain proteins (A1-VWF, A2-VWF and A3-VWF). Significant binding was observed for the A1-VWF domain compared with A2-VWF and A3-VWF. Bovine serum albumin (BSA) was used as a negative control. All data are presented as mean ± standard error of mean of three independent experiments. Percentage binding was calculated based on optical density at 450 nm obtained for 100 nM A1A2A3-VWF. **P*<0.05, ***P*<0.01, ****P*<0.001.



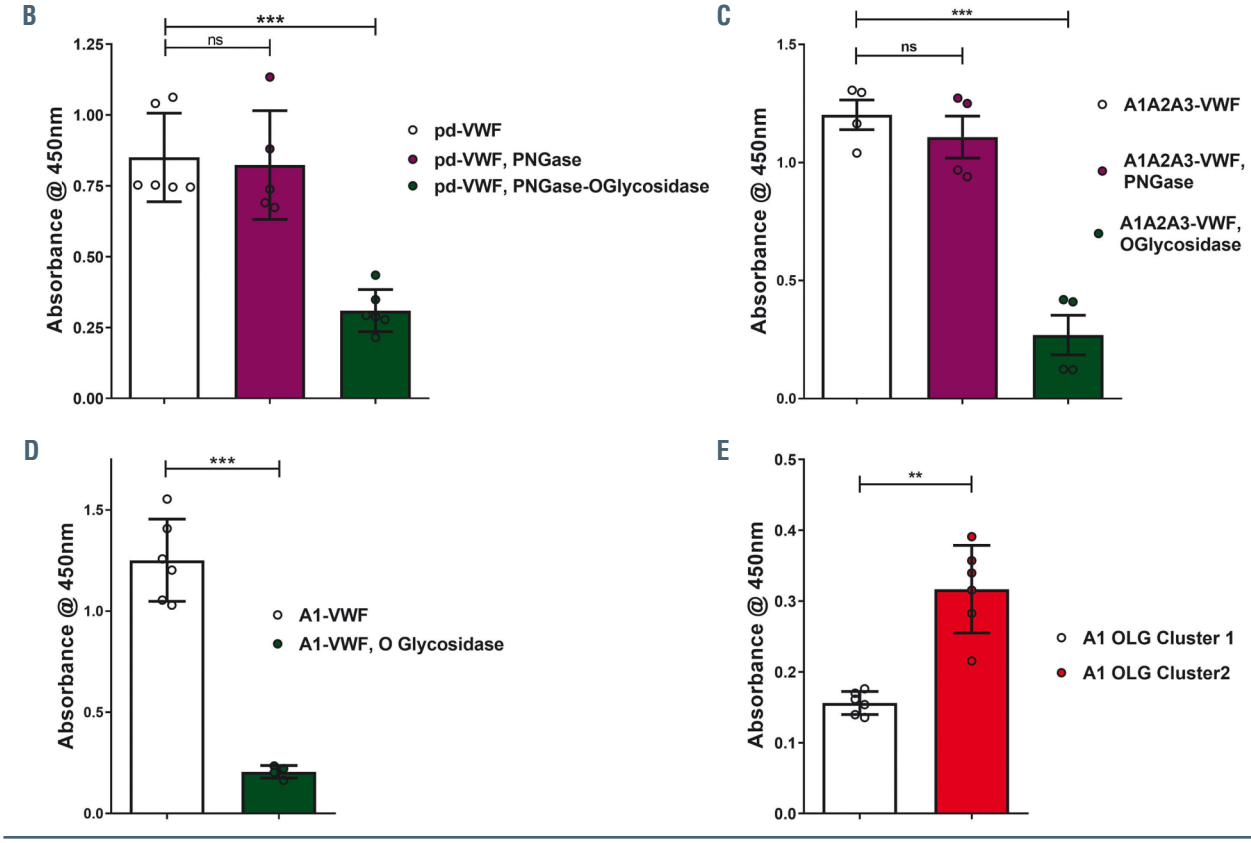


Figure 3. O-linked glycans on von Willebrand factor modulate the interaction with MGL. (A) Each von Willebrand factor (VWF) monomer contains 13 N-linked and ten O-linked glycan structures. The diagram also shows the most common VWF N-linked carbohydrate structure (a monosialylated, biantennary, core fucosylated complex glycan) and O-linked carbohydrate structure (core 1 sialylated T-antigen). (B) To investigate the role of VWF carbohydrate determinants in modulating the interaction with MGL, plasma-derived (pd)VWF (10 μg/mL) was treated with either PNGase F (PNGase VWF) to remove N-glycans or PNGase F and O glycosidase (PNGase-OGly VWF) to remove both N- and O-glycans. Binding of the pdVWF glycoforms to human MGL was then compared to binding to untreated pd-VWF as before (100% binding = OD450 obtained for 10 μg/mL pdVWF). (C) To study a potential role for glycans in the A domains of VWF in regulating MGL binding, A1A2A3-VWF (150 nM VWF) was treated with either PNGase F or O-glycosidase. Binding to human MGL was then assessed compared to to the binding to WT A1A2A3-VWF (100% binding = OD450 obtained for 150 nM A1A2A3-VWF). (D) Since A1-VWF does not contain any N-linked glycan determinants, MGL binding studies were examined for WT-A1-VWF compared to O-glycosidase-treated VWF-A1 (100% binding = OD450 obtained for 150 nM A1-VWF). (E) Eight O-linked glycans are located in two clusters of four either side of the VWF A1 domain. To investigate the importance of these O-glycans in modulating the MGL interaction, two A1-VWF variants were generated each of which contained only one O-glycan cluster (A1-OLG cluster 1 contained T1248A, T1255A, T1256A, S1263A, while A1-OLG cluster 2 contained T1468A, T1477A, S1486A, T1487A). MGL-binding studies were compared for these two cluster variants as previously described (100% binding = OD450 obtained for A1-OLG cluster 1). All data are represented as mean ± standard error of mean of three independent experiments. *P<0.05, **P<0.01, ***P<0.001. PNGase F: peptide N-glycosidase F; OLG: O-linked glycans; OD450: absorbance at 450 nm.

4A).[34,35] Since sepsis-related neuraminidases may target both the N- and O-glycans of VWF, we further investigated the role of MGL in clearing VWF from which both the N- and O-sialylation had been removed following digestion with α2-3,6,8,9 neuraminidase. *In vivo* clearance studies in *VWF*$^{-/-}$ mice demonstrated that combined mMGL1 and mMGL2 inhibition was not able to significantly reduce the pathological, enhanced clearance observed following loss of N-linked sialylation (Figure 6A). Interestingly, however, in mice deficient for the ASGPR clearance receptor, mMGL1/2 inhibition was associated with attenuation of the enhanced clearance of α2-3,6,8,9 Neu-VWF (Figure 6B). Collectively, these findings further support the hypothesis that O-linked α2-3 sialylation on VWF plays a critical role in protecting against MGL-mediated clearance. Moreover, the data also suggest that loss of α2-6 sialylation (predominantly N-linked) on VWF drives enhanced clearance in a predominantly MGL-independent manner, mediated through the ASGPR.

Increased VWF clearance plays a key role in the pathogenesis of both type 1 and type 2B VWD.[3,11,46] Previous studies have implicated macrophages, and in particular the LRP1 and SR-A1 receptors, in regulating this enanced

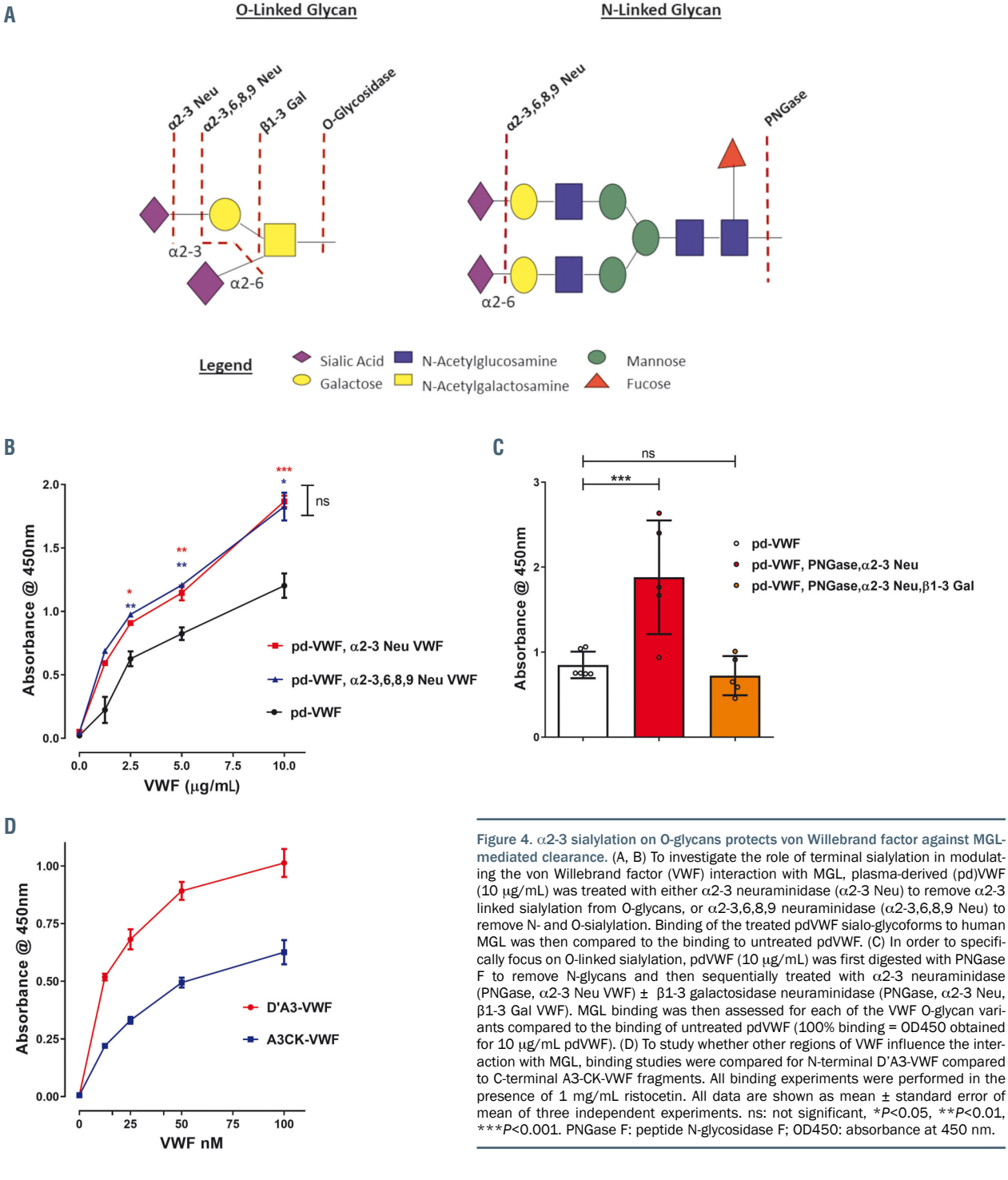


Figure 4. α2-3 sialylation on O-glycans protects von Willebrand factor against MGL-mediated clearance. (A, B) To investigate the role of terminal sialylation in modulating the von Willebrand factor (VWF) interaction with MGL, plasma-derived (pd)VWF (10 μg/mL) was treated with either α2-3 neuraminidase (α2-3 Neu) to remove α2-3 linked sialylation from O-glycans, or α2-3,6,8,9 neuraminidase (α2-3,6,8,9 Neu) to remove N- and O-sialylation. Binding of the treated pdVWF sialo-glycoforms to human MGL was then compared to the binding to untreated pdVWF. (C) In order to specifically focus on O-linked sialylation, pdVWF (10 μg/mL) was first digested with PNGase F to remove N-glycans and then sequentially treated with α2-3 neuraminidase (PNGase, α2-3 Neu VWF) ± β1-3 galactosidase neuraminidase (PNGase, α2-3 Neu, β1-3 Gal VWF). MGL binding was then assessed for each of the VWF O-glycan variants compared to the binding of untreated pdVWF (100% binding = OD450 obtained for 10 μg/mL pdVWF). (D) To study whether other regions of VWF influence the interaction with MGL, binding studies were compared for N-terminal D'A3-VWF compared to C-terminal A3-CK-VWF fragments. All binding experiments were performed in the presence of 1 mg/mL ristocetin. All data are shown as mean ± standard error of mean of three independent experiments. ns: not significant, **P*<0.05, ***P*<0.01, ****P*<0.001. PNGase F: peptide N-glycosidase F; OD450: absorbance at 450 nm.

clearance.[14,15,19,20] To examine whether MGL may also play a role, we investigated binding for a number of type 1C (VWF-R1205H, R1205C, R1205S, S2179F) and type 2B (VWF-V1316M and -R1450E) variants. No evidence of enhanced MGL binding was observed for VWF-V1316M or any of the type 1C variants (*Online Supplementary Figure S3*). Interestingly, significantly reduced MGL binding was seen for VWF-R1450E compared to wild-type recombinant VWF. We hypothesise that this change in binding is due to conformational effects within the A1 domain affecting O-linked glycosylation during post-translational modification and/or accessibility of specific OLG for the MGL interaction.

Platelet-von Willebrand factor sialylation and MGL interaction

Platelet α-granules contain approximatey 20% of the total VWF present in platelet-rich plasma.[47,48] Previous studies have demonstrated that platelet-derived (plt)-VWF has altered glycosylation compared to pdVWF.[48] In particular, plt-VWF does not express ABO blood group determinants and is hypo-sialylated.[49,50] Importantly, these glycosylation differences influence susceptibility to ADAMTS-13 cleavage.[43] Using lectin-binding ELISA, we confirmed that the quantitative reduction in plt-VWF sialylation was predominantly attributable to a specific reduction in N-linked sialylation (Figure 7A, B). As a result of this decreased N-sialylation, terminal galactose expression was significantly increased on plt-VWF compared to pd-VWF (Figure 7C). Critically, despite the significant reduction in N-linked sialylation, we observed no increase in MGL binding for plt-VWF (Figure 7D). Moreover, *in vivo* clearance of plt-VWF in *VWF*$^{-/-}$ mice was similar to that of pd-VWF (Figure 7E). Cumulatively, these novel data further support our hypothesis that O-linked sialylation on VWF plays a key role in protecting VWF against MGL-mediated clearance.

Discussion

Recent studies have demonstrated that complex glycan structures, which account for 20% of total VWF monomeric mass, play a key role in regulating the half-life of VWF *in vivo*.[3,25,51] In addition, a number of lectin receptors have been shown to bind VWF.[3] Critically, however, the relative importance of these receptors in modulating physiological and pathological VWF clearance has not been defined. Moreover, the particular VWF glycan determinants involved in modulating interactions with specific lectin receptors remain unclear. In this study, using a series of *in vivo* and *in vitro* methodologies, we demonstrated that both murine homologs of the MGL receptor bind to VWF and contribute to the physiological clearance of endogenous murine VWF. Consequently, combined inhibition of both mMGL1 and

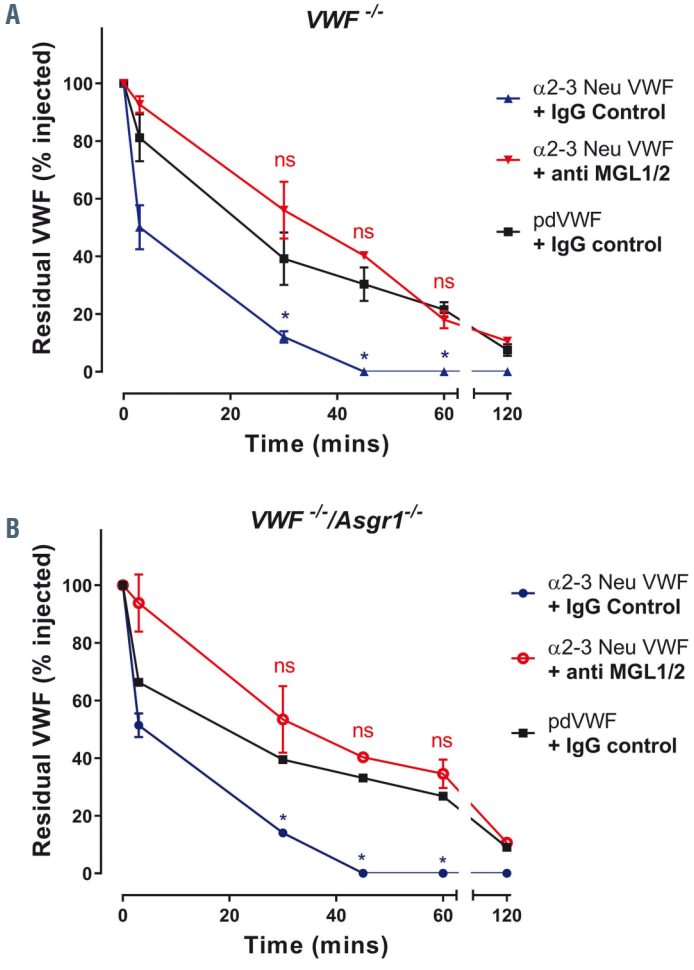


Figure 5. Role of MGL in modulating pathological enhanced clearance of α2-3 Neu-VWF. (A) To investigate the importance of MGL in regulating the enhanced clearance of von Willebrand factor (VWF) with reduced O-linked sialylation, purified human plasma-derived (pd)VWF was treated with α2-3 neuraminidase (α2-3 Neu-VWF). *In vivo* clearance was then assessed in *VWF*$^{-/-}$ mice for α2-3 Neu-VWF in the presence or absence of combined mMGL1 and mMGL2 inhibition and compared to that of wild type pdVWF. At each time point, residual circulating VWF concentration was determined by an enzyme-linked immunosorbent assay for VWF:antigen (VWF:Ag). All results are plotted as percentage residual VWF:Ag levels relative to the amount injected. Data are represented as mean ± standard error of mean (SEM). In some cases, the SEM cannot be seen because of its small size. (B) To assess the relative roles of MGL and ASGPR in modulating the pathological increased clearance following removal of α2-3 sialylation, *in vivo* clearance studies were also performed in dual *VWF*$^{-/-}$*Asgr1*$^{-/-}$ knockout mice in the presence or absence of combined mMGL1 and mMGL2 inhibition.

mMGL2 resulted in a 3-fold increase in murine plasma VWF levels which was attributable to a significant decrease in clearance rate. Importantly, the magnitude of the increased *in vivo* VWF levels associated with combined MGL inhibition was greater than that reported following inhibition of other VWF clearance receptors in mice (~2.5 fold *vs.* ~1.5 fold), suggesting that MGL plays an important role in regulating physiological clearance of VWF.

To further investigate how MGL interacts with VWF, we first investigated the roles of specific VWF domains. Our data demonstrate that the A1A2A3 domains of VWF are predominantly responsible for modulating MGL binding. Furthermore, studies using isolated A domains showed that the A1 domain plays a critical role in regulating the MGL interaction. Interestingly, the binding of both full-length and A1A2A3-VWF to MGL was markedly enhanced in the presence of ristocetin, suggesting that the MGL-binding site in A1 may not be fully accessible in normal globular VWF. This concept is in keeping with findings of previous studies that showed significantly increased VWF binding to macrophages in the presence of ristocetin, botrecetin or shear stress.[14,16] From a biological perspective, these data suggest that any VWF circulating in an 'active' GpIb binding conformation will be cleared rapidly by macrophage MGL, which may be important in minimizing thrombotic risk. Importantly, our data further show that C-terminal A3-CK-VWF also binds MGL. Although the binding was less than that observed with N-terminal D'A3-VWF, this observation suggests that additional MGL-recognition sites beyond the A1 domain may contribute to the MGL interaction.

Mass spectrometry studies have demonstrated significant and site-specific heterogeneity in the carbohydrate structures expressed on human pdVWF.[34-37] Nevertheless, the majority of both the N- and O-linked glycans are capped with negatively-charged sialic acid residues. In this study, we demonstrated that specific loss of α2-3 linked sialylation from the O-linked glycans of VWF causes enhanced MGL binding *in vitro*, and causes markedly enhanced MGL-mediated clearance *in vivo*. In contrast, removal of α2-6 linked sialylation, which constitutes most of the total sialic acid expressed on human VWF and, in particular, the vast majority of the sialylation on N-glycans, has minimal effect on MGL binding and/or clearance. Our data further suggest that the two O-linked glycan clusters located either side of the A1 domain play a key role in regulating binding to MGL. Previous studies have demonstrated that these O-glycan clusters have significant effects on local VWF conformation.[52,53] Further studies will be required to determine the molecular mechanisms through which these specific O-glycans regulate MGL-mediated VWF binding and clearance. Nevertheless, our findings demonstrate that MGL contributes to physiological VWF clearance by binding to exposed Gal residues on O-linked carbohydrate struc-

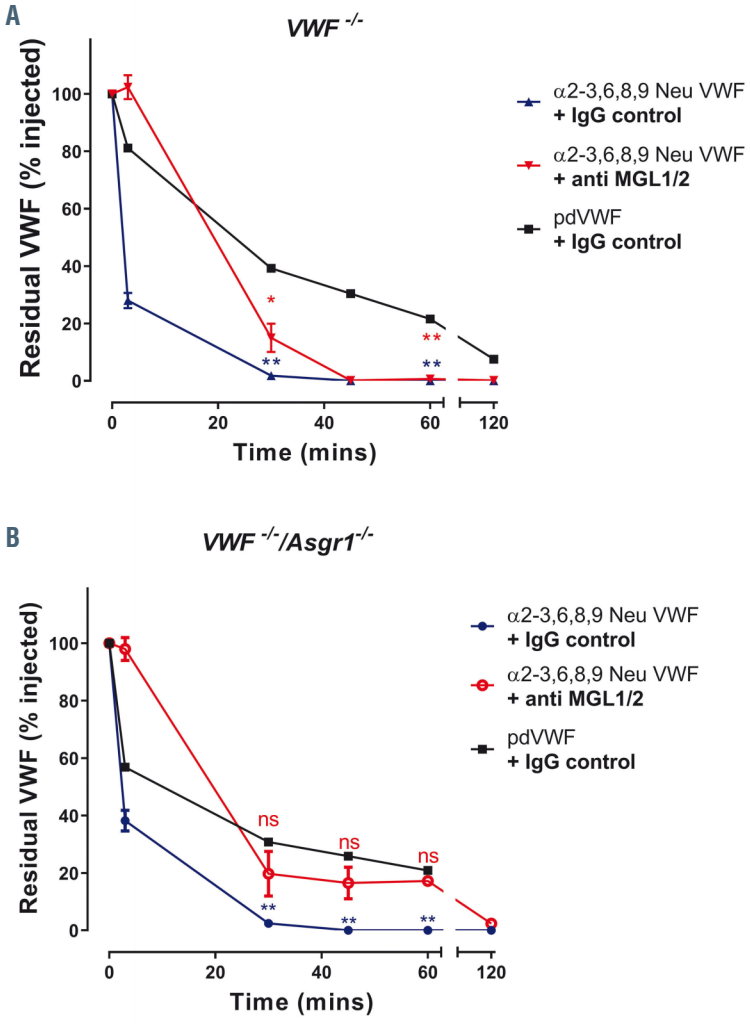


Figure 6. ASGPR in combination with MGL modulates the increased clearance α2-3,6,8,9 Neu-VWF. (A) To investigate whether MGL plays a role in the enhanced clearance of von Willebrand factor (VWF) from which both the N- and O-sialylation had been removed purified human plasma-derived (pd)VWF was treated with α2-3,6,8,9 neuraminidase (α2-3,6,8,9 Neu-VWF). *In vivo* clearance of α2-3,6,8,9 Neu-VWF was then assessed in *VWF*$^{-/-}$ mice in the presence or absence of combined mMGL1 and mMGL2 inhibition and compared to that of wild-type pdVWF. At each time point, residual circulating VWF concentration was determined by an enzyme-linked immunosorbent assay for VWF:antigen (VWF:Ag). All results are plotted as percentage residual VWF:Ag levels relative to the amount injected. Data are represented as mean ± standard error of mean (SEM). In some cases, the SEM cannot be seen because of its small size. (B) To assess the relative roles of MGL and ASGPR in modulating the pathological, increased clearance following removal of α2-3,6,8,9 sialylation, *in vivo* clearance studies were also performed in dual *VWF*$^{-/-}$ *Asgr1*$^{-/-}$ knockout mice in the presence or absence of combined mMGL1 and mMGL2 inhibition. **P*<0.05, ***P*<0.01, ns: not significant.

tures. Importantly, glycoprotein aging in plasma is associated with progressive loss of capping sialic acid, and thus increased exposure of these sub-terminal Gal residues.[38]

There are previous reports of significantly increased binding of RCA-I lectin to plasma VWF in patients with VWD.[10,33,40,42] This lectin binds preferentially to Gal or GalNAc sugars which are typically present as sub-terminal residues on the O- and N-glycans of pdVWF, but become exposed following loss of capping sialic acid. Increased RCA-I binding has also been correlated with enhanced VWF clearance in VWD patients.[10,33,40] Our data suggest that the shortened half-life associated with increased Gal exposure (and hence RCA-I binding) in VWD patients is mediated in large part through enhanced MGL-mediated clearance. Importantly, van Schooten *et al.* previously reported significantly increased binding of peanut agglutinin (PNA) lectin to VWF in a cohort of VWD patients.[40] This lectin preferentially binds to the T antigen structure which is exposed following loss of O-linked sialylation. The authors further showed that increased PNA-binding (T antigen exposure) was associated with a significant increase in the VWFpp/VWF:Ag ratio, consistent with enhanced VWF clearance.[40] In keeping with these results, we have demonstrated that α2-3 linked sialylation on O-linked glycan structures plays a particular role in protecting VWF against MGL-mediated clearance. Consequently, our findings suggest that the enhanced clearance associated with T antigen exposure on VWF previously reported by van Schooten *et al.* is attributable to enhanced clearance via MGL.

Besides VWD, abnormal VWF glycosylation has also been reported in a number of other disease states.[24,39-41] For example, reduced PNA-binding to VWF has been reported in patients with liver cirrhosis who have significantly elevated plasma VWF:Ag levels. The biological mechanisms underlying reduced T antigen exposure on VWF in patients with cirrhosis have not been defined. Nonetheless, our findings build upon these previous observations and in particular suggest that the altered O-glycosylation associated with cirrhosis will cause increased plasma VWF levels as a result of decreased MGL-mediated clearance. Conversely, a number of different pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* express neuraminidase enzymes that can cause desialylation of host glycoproteins.[24,54] VWF desialylation associated with pathological, enhanced clearance has been observed in mice infected with *S. pneumoniae*.[24] Our data further suggest that increased MGL-mediated clearance

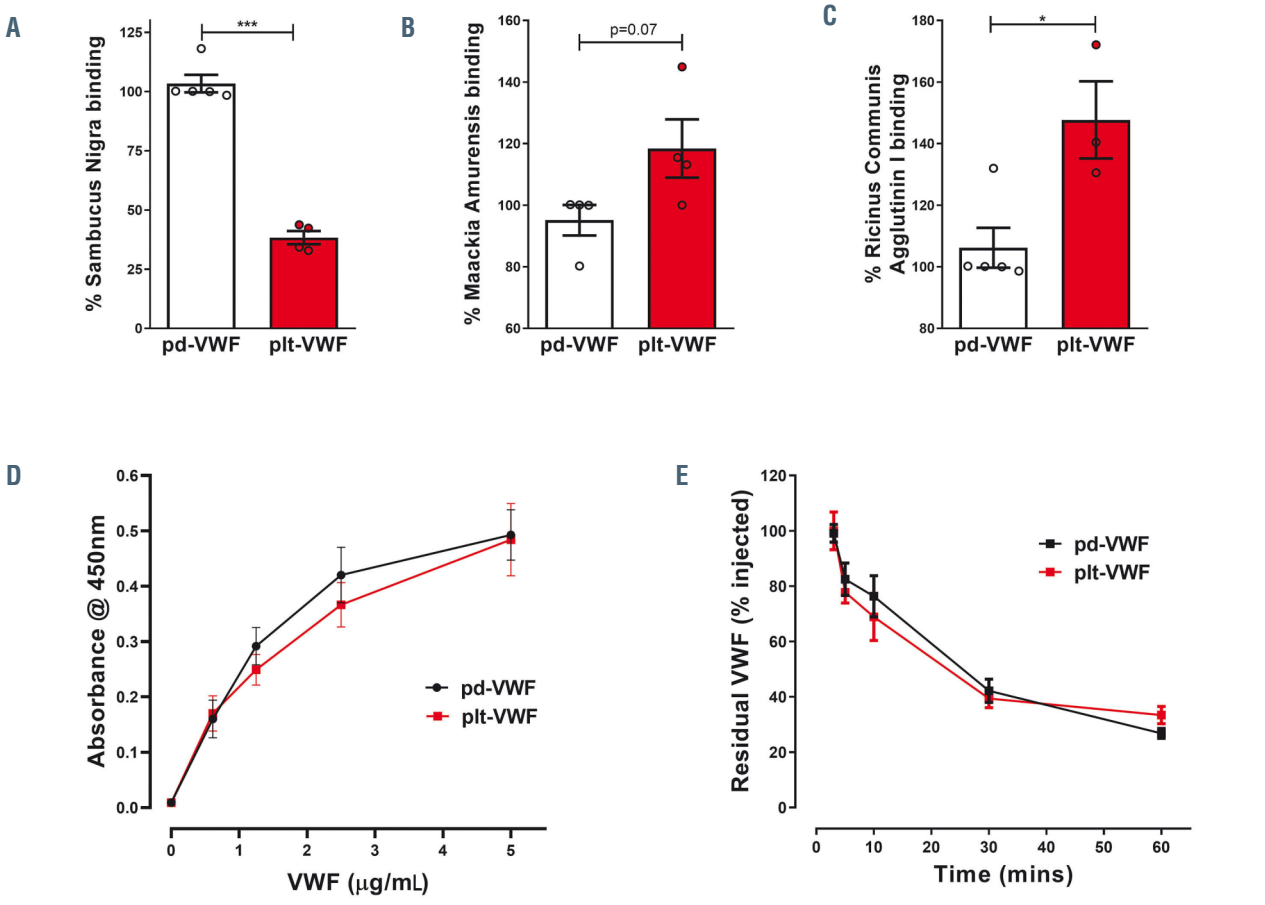


Figure 7. α2-3 linked sialic acid on platelet von Willebrand factor protects from enhanced circulatory clearance. (A-C) Platelet-derived (plt) von Willebrand factor (VWF) sialylation was assessed using lectin binding assays with *Sambucus nigra* (A), *Maackia amuresis* (B) and *Ricinus communis* (C). Plasma-derived (pd) VWF was used as a control. (D) Solid phase binding assay was used to assess the binding of plt-VWF to immobilized human MGL and again compared to human pd-VWF. (E) *In vivo* pharmacokinetic experiments were performed in *VWF*$^{-/-}$ mice to compare the clearance rates or plt-VWF compared to pd-VWF. At each time point, residual circulating VWF:antigen (VWF:Ag) concentration was determined by enzyme-linked immunosorbent assay. All results are plotted as percentage residual VWF:Ag levels relative to the amount injected. Data are represented as mean ± standard error of mean (SEM). In some cases, the SEM cannot be seen because of its small size. *P<0.05, **P<0.01, ns: not significant.

will play a key role in mediating this pathogen-associated enhanced VWF clearance. Interestingly, two previous studies have demonstrated that complete loss of O-linked carbohydrate structures is associated with significantly increased VWF clearance *in vivo*.[28,55] Given that O-glycans are known to influence protein conformation, the observation that complete removal triggers enhanced clearance is likely attributable to conformational changes in VWF.

In addition to MGL, other macrophage receptors that can also interact with VWF include LRP1, SR-A1, Siglec-5, Gal-1 and Gal-3.[3,56,57] Some of these receptors have also been shown to bind with enhanced affinity to hyposialylated VWF (ASGPR, Gal-1 and Gal-3). Additional studies will be necessary to fully elucidate the relative roles of these other macrophage receptors in regulating the physiological and/or pathological clearance of hyposialylated VWF. Although it remains unclear whether these receptors may function synergistically in regulating desialylated VWF clearance, recent studies have demonstrated that LRP1 can form heterologous functional complexes with other macrophage receptors including β2-integrins. Importantly, Deppermann et al recently demonstrated that MGL on hepatic Küpffer cells plays a significant role in the removal of desialylated platelets, and that MGL and ASGPR appear to function collaboratively in physiological platelet clearance.[58]

Disclosures

JSO'D has served on speakers' bureaux for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Leo Pharma, Takeda and Octapharma. He has also served on advisory boards for Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim, Takeda and Pfizer and has received research grant funding awards from Baxter, Bayer, Pfizer, Shire, Takeda and Novo Nordisk. JMO'S has received research grant funding from LEO Pharma and Grifols

Contributions

SEW, ABM, JF and AC performed experiments; SEW, JMO'S, ABM, DS, RG, JS, JF, MM, TAJM, AC, SH and JSO'D designed the research and analyzed the data. All authors were involved in writing and reviewing the paper.

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Data-sharing statement

All original data and protocols can be made available to other investigators upon request.

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