Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Macrophage Galactose Lectin Contributes to the Regulation of FVIII (Factor VIII) Clearance in Mice—Brief Report

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BACKGROUND: Although most plasma FVIII (Factor VIII) circulates in complex with VWF (von Willebrand factor), a minority (3%–5%) circulates as free-FVIII, which is rapidly cleared. Consequently, 20% of total FVIII may be cleared as free-FVIII. Critically, the mechanisms of free-FVIII clearance remain poorly understood. However, recent studies have implicated the MGL (macrophage galactose lectin) in modulating VWF clearance.

METHODS: Since VWF and FVIII share similar glycosylation, we investigated the role of MGL in FVIII clearance. FVIII binding to MGL was assessed in immunosorbent and cell-based assays. In vivo, FVIII clearance was assessed in *MGL1*$^{-/-}$ and *VWF*$^{-/-}$/*FVIII*$^{-/-}$ mice.

RESULTS: In vitro–binding studies identified MGL as a novel macrophage receptor that binds free-FVIII in a glycan-dependent manner. *MGL1*$^{-/-}$ and *MGL1*$^{-/-}$ mice who received an anti-MGL1/2 blocking antibody both showed significantly increased endogenous FVIII activity compared with wild-type mice (P=0.036 and P<0.0001, respectively). MGL inhibition also prolonged the half-life of infused FVIII in *FVIII*$^{-/-}$ mice. To assess whether MGL plays a role in the clearance of free FVIII in a VWF-independent manner, in vivo clearance experiments were repeated in dual *VWF*$^{-/-}$/*FVIII*$^{-/-}$ mice. Importantly, the rapid clearance of free FVIII in *VWF*$^{-/-}$/*FVIII*$^{-/-}$ mice was significantly (P=0.012) prolonged in the presence of anti-MGL1/2 antibodies. Finally, endogenous plasma FVIII levels in *VWF*$^{-/-}$ mice were significantly increased following MGL inhibition (P=0.016).

CONCLUSIONS: Cumulatively, these findings demonstrate that MGL plays an important role in regulating macrophage-mediated clearance of both VWF-bound FVIII and free-FVIII in vivo. We propose that this novel FVIII clearance pathway may be of particular clinical importance in patients with type 2N or type 3 Von Willebrand disease.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: mechanisms ■ pathophysiology ■ physiology ■ vascular biology

In normal plasma, 95% to 97% of FVIII (Factor VIII) circulates as part of a high affinity complex (Kd ≈0.2 nmol/L) with VWF (von Willebrand factor).[1,2] The remaining 3% to 5% circulates as free-FVIII. The VWF-FVIII complex protects FVIII against premature clearance, with the half-life of VWF-bound FVIII being ≈12 hours compared with only ≈2 hours for free-FVIII.[1,3] Although the proportion of free-FVIII in plasma is small, this fraction exists in dynamic reversible equilibrium with the FVIII-VWF complex.[4] Consequently, recent studies have estimated that 20% of total plasma FVIII may be cleared as free-FVIII.[5] Enhanced clearance of free-FVIII is also a hallmark of type 2N and type 3 von Willebrand disease. Critically however, the biological mechanisms underlying the regulation of rapid clearance of free-FVIII remain poorly understood.[1,3,6]

Prior to secretion, FVIII undergoes complex posttranslational modification including significant glycosylation.[7] As a result, mature FVIII contains 18 occupied N-glycan sites.[8]

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For Sources of Funding and Disclosures, see page 546.

Nonstandard Abbreviations and Acronyms

ASF	asialofetuin
ASGPR	asialoglycoprotein receptor
BMDM	bone marrow–derived macrophage
FVIII	Factor VIII
FVIII:C	Factor VIII coagulant activity
MGL	macrophage galactose-type lectin
PNGase F	Peptide:N-glycosidase F
VWD	Von Willebrand disease
VWF	von Willebrand factor

Biantennary complex-type structures constitute the commonest N-glycans on FVIII, with smaller populations of high mannose and hybrid-type chains.[8,9] Terminal sialic acid residues are present on 67% of the complex-type chains.[8] In addition, human FVIII contains 7 O-linked glycans clustered within the B-domain.[10] FVIII glycans are known to modulate interaction with a number of lectin receptors including the ASGPR (asialoglycoprotein receptor),[11] Galectins-1 and -3 (Gal-1, Gal-3),[12] the macrophage mannose receptor (MMR/CD206), Siglec-5[13] and C-type lectin domain family 4 member M (CLEC4M).[5] Functional roles for these glycans has been demonstrated. For example, loss of capping sialic acid residues from FVIII has been shown to trigger rapid clearance in vivo. This finding is biologically relevant since desialylation accompanies glycoprotein ageing in plasma.[14] In addition, some pathogens (eg, *Steptococcus pneumoniae* and *Haemophilus influenzae*) express neuraminidases that desialylate plasma glycoproteins.[15] Previous studies suggested that the enhanced clearance of hyposialylated FVIII occurs via ASGPR, which is predominantly expressed on hepatocytes.[11] However, a number of other lectin receptors also bind to hyposialylated glycoproteins. In particular, roles for the MGL (macrophage galactose lectin) in regulating clearance of hyposialylated platelets and VWF have recently been described.[16–18] In view of the complex glycans expressed on FVIII, we hypothesized that MGL may also directly interact with FVIII and further influence VWF-bound and/or free-FVIII clearance in vivo.

HIGHLIGHTS

- Macrophage galactose lectin (MGL) binds to factor VIII (FVIII) in a dose-dependent manner.
- O-linked glycans in the B domain of FVIII play a critical role in regulating MGL-interaction.
- Hyposialylated FVIII binds to MGL with increased affinity.
- MGL contributes to the regulation of FVIII clearance in mice.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

In Vitro FVIII-Binding Studies

Bone marrow was harvested from the pelvis, femurs and tibias of 8- to 12-week-old C57BL/6J mice and differentiated into bone marrow–derived macrophages (BMDMs) by culturing in RPMI containing Macrophage Colony Stimulating Factor (25 ng/mL) for 6 days. Following differentiation, BMDM cultures were >97% pure as indicated by dual CD11b and F4/80 positivity by flow cytometry (Figure S1). As described in detail in the Supplemental Material, FVIII binding to murine BMDM and THP-1 macrophages was assessed by flow cytometry. Bound FVIII was detected using a FITC-labelled anti-FVIII antibody (Affinity Biologicals). Where indicated, studies were performed in the presence or absence of ASF (asialofetuin; 2 mg/mL). Immunosorbent assays were used to evaluate FVIII binding to MGL.[17] Briefly, recombinant human MGL (Stratech, United Kingdom) was immobilized and FVIII was incubated at 37°C. Bound FVIII was detected using mouse monoclonal anti-FVIII (Green Mountain). To assess the role of FVIII glycans in regulating macrophage and MGL-binding, exoglycosidases were used to modify FVIII glycosylation, including α2-3 neuraminidase (*Streptococcus pneumonia*; Sigma Aldrich, Ireland), α2-3,6,8,9 neuraminidase (*Arthrobacter ureafaciens*; New England Biolabs, United Kingdom) and PNGase F (peptide N glycosidase F; *Flavobacterium meningosepticum*; New England Biolabs, United Kingdom) as previously described.[12] Following each digestion, residual FVIII glycans was analyzed using lectin ELISAs (see Supplemental Material and Figure S2).[12,19] Human embryonic kidney 293 (HEK293T) cells were transfected with human MGL DNA (Invivogen) using TransIT (Mirius Bio) transfection reagent.

In Vivo FVIII Clearance Studies

VWF$^{-/-}$ and *FVIII*$^{-/-}$ mice on C57BL/6J and 129S4/SvJae backgrounds, respectively were obtained from the Jackson Laboratory (Sacremento, CA). These mice were crossbred to generate *VWF*$^{-/-}$*FVIII*$^{-/-}$ double-knockout mice and bred for 10 generations to C57BL/6J background. *MGL1*$^{-/-}$ mice were also obtained from the Jackson Laboratory. Mice were fed ad libitum with standard chow (LabDiet, IPS United Kingdom). In keeping with previous murine studies of VWF-FVIII clearance, studies were performed in both male and female mice,[19–24] and data combined in order to improve power. Consequently, potential sex differences were not investigated in the study. All in vivo studies were performed in accordance with the Health Product Regulatory Authority, Ireland (AE19127/P060) and approved by the Animal Research Ethics Committee of The Royal College of Surgeons in Ireland and Trinity College Dublin (REC1585 and 260219, respectively). Where indicated, clearance studies were repeated in the presence or absence of (1) clodronate-induced macrophage depletion[16,17] or (2) polyclonal goat anti-mouse MGL1/2 antibody (R&D systems, Minneapolis) as previously described.[16,17,20] Plasma FVIII:C levels were measured using a chromogenic assay (HyphenBiomed, France).

Data Presentation and Statistical Analysis

Experimental data were analyzed with GraphPad Prism version 8.0 (GraphPad Software, San Diego). Where samples with n>5

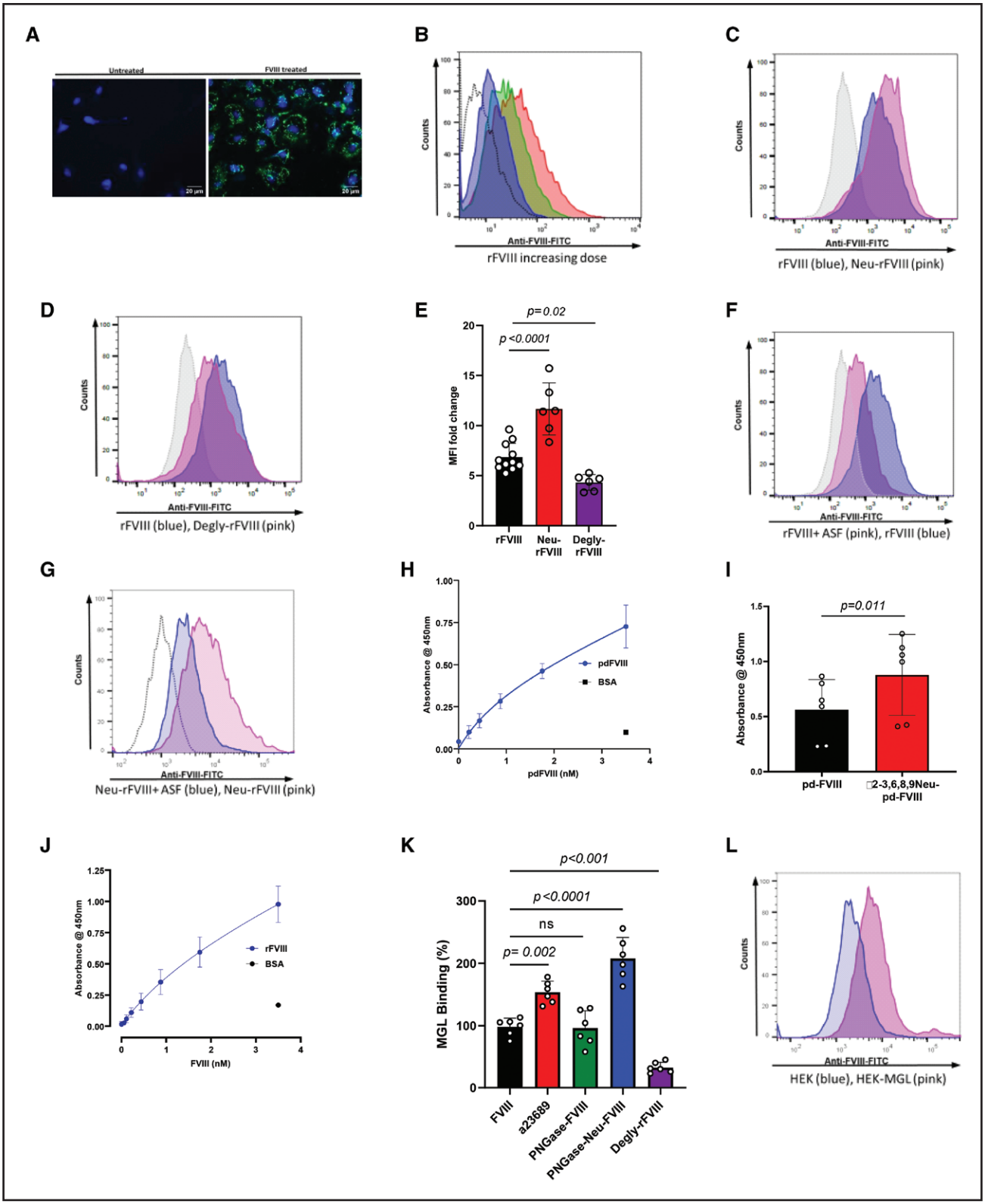

Figure 1. MGL (macrophage galactose lectin) interacts with FVIII (Factor VIII) in a glycan-dependent manner.
A, Binding of FVIII (80 nM) to primary murine bone marrow–derived macrophages (BMDMs) was assessed in vitro using epifluorescent microscopy as detailed in Materials and Methods. The negative control depicts BMDMs incubated with buffer in place of rFVIII. FVIII staining in green; nuclear DAPI staining in blue. **B**, Increasing supra-physiological concentrations of pdFVIII (5 nM blue, 10 nM green, 20 nM red) were incubated with BMDMs for 1 hour on ice and cells were analyzed by flow cytometry. Representative histograms are presented where grey represents control cells not treated with FVIII. **C** through **E**, To investigate the role of FVIII carbohydrate determinants in modulating macrophage interaction, rFVIII was treated with either α2-3,6,8,9 neuraminidase to remove capping sialic acid residues (*Continued*)

were to be compared, data were analyzed for normality using the Shapiro-Wilk test and analyzed for equal variance using Bartlett test. For normal data, either 1-way ANOVA (with Tukey Multiple Comparison test) or Student *t* test for independent samples was conducted. In vivo FVIII clearance experiments and in vitro plate binding curves were analyzed by 2-way ANOVA with Šídák multiple comparisons test. All normally distributed data are shown as mean±SD. Data that were not normally distributed or with n≤5 were analyzed using nonparametric tests. Pairwise analyses were tested using the Mann-Whitney *U* test. Group analyses were tested by Kruskal-Wallis test with multiple comparison testing computed with Corrected Dunn test. All nonparametric data are shown as median±95% CI. *P* values <0.05 were considered significant.

RESULTS AND DISCUSSION

MGL Interacts With FVIII in a Glycan-Dependent Manner

In keeping with previous reports,[25] human FVIII dose-dependently binds to differentiated murine BMDMs (Figures 1A and 1B). To investigate whether glycans influence this binding to macrophages, FVIII glycans were modified using exoglycosidases digestions as previously described (Figure S2C through S2F).[12,19] Treatment with α2-3,6,8,9 neuraminidase (to remove α2-3 and α2-6 linked sialylation) significantly (*P*<0.0001) enhanced FVIII binding to macrophages (Figure 1C through 1E). Removal of both N- and O-glycan structures significantly (*P*=0.02) attenuated FVIII binding to macrophages (Figure 1D and 1E). In contrast, digestion with PNGase F alone had no significant effect on FVIII-binding to macrophages (data not shown), suggesting a specific role for FVIII O-glycans in modulating macrophage interaction. To further investigate the importance of FVIII sialylation, macrophage binding was repeated in the presence or absence of asialo-receptor inhibition. Binding of FVIII and α2-3,6,8,9 Neu-FVIII to macrophages were both inhibited in the presence of ASF (Figure 1F and 1G). Collectively, these findings show that FVIII glycan determinants regulate macrophage interaction and further demonstrate that terminal sialylation protects FVIII against macrophage binding.

We next investigated whether MGL is involved in macrophage binding to FVIII. We first demonstrated that plasma-derived FVIII (pd-FVIII) dose-dependently bound to immobilized human MGL (Figure 1H). Treatment with α2-3,6,8,9 neuraminidase to remove terminal sialylation significantly (*P*=0.011) enhanced pd-FVIII binding to MGL (Figure 1I). Interestingly, although the majority of FVIII sialylation is α2-6 linked, there was no statistically significant difference in binding of α2-3,6,8,9 Neu-FVIII binding to MGL compared with α2-3 Neu-FVIII. Recent studies have demonstrated that rFVIII glycans varies dependent upon the cell line used for expression.[8] However, dose-dependent saturable MGL-binding was also observed for recombinant full-length human FVIII (rFVIII) expressed in Chinese hamster ovary cells (Figure 1J). Desialylation of rFVIII again significantly (*P*=0.002) enhanced MGL binding (Figure 1K). Consistent with our macrophage cellular binding data, PNGase treatment alone had no significant effect but combined N- and O-glycans digestion markedly attenuated (*P*<0.001) rFVIII binding to MGL (Figure 1K; Figure S3), suggesting that O-glycans located within the FVIII B-domain regulate MGL-binding. In contrast, previous studies demonstrated that N-glycans within the B-domain play a key role in modulating FVIII binding to the asialoglycoprotein receptor on hepatocytes.[11]

Transient expression of recombinant human MGL in human embryonic kidney cells was sufficient to facilitate rFVIII binding (Figure 1L). Binding to human embryonic kidney cells-MGL cells was increased for neuraminidase-treated FVIII, and attenuated in the presence of asialofetuin (Figure S4). Cumulatively, these in vitro data identify MGL as a novel macrophage receptor that binds free FVIII in a glycan-dependent manner and demonstrate that sialylation on the O-linked glycans of FVIII specifically protects against MGL-binding.

MGL Regulates Free-FVIII Clearance In Vivo

Mice have two MGL homologs, murine MGL1 (mMGL1) and murine MGL2 (mMGL2).[26] Consistent with previous reports,[27] we observed that mMGL1 is the predominant form expressed on BMDMs (Figure S5). However full-length human FVIII was shown to bind to both mMGL1 and mMGL2 (Figure 2A). In support for a potential role for

Figure 1 Continued. (Neu-rFVIII) or a combination of PNGase F and O-glycosidase to remove both N- and O-glycans (Degly-rFVIII). Binding to BMDM was then studied by flow cytometry for each glycoform (pink) compared with cells incubated with untreated FVIII (blue) or untreated controls (gray). Flow histograms are representative of a minimum of three replicates. Change in MFI were analyzed by 1-way ANOVA with Tukey multiple comparison test. Data are presented as mean MFI fold change±SD (n=6–11). **F** and **G**, To further study the importance of sialic acid in regulating macrophage binding, flow cytometry experiments were repeated to assess BMDM binding of FVIII and desialylated FVIII (Neu-FVIII) in the presence or absence of the asialo-receptor inhibitor ASF (asialofetuin, 2 mg/mL). **H**, In vitro binding of purified pdFVIII (pdFVIII) to recombinant human MGL was assessed using an immunosorbent assay compared with a BSA negative control. **I**, Binding to human MGL was assessed for pdFVIII following treatment with α2-3,6,8,9 neuraminidase (α2-3,6,8,9 Neu-pdFVIII). Absorbance at 450 nm was plotted and differences between groups analyzed by Student *t* test (n=6). Data are displayed as mean±SD. **J**, In vitro binding of clininical grade recombinant full-length FVIII expressed in CHO cells (CHO-rFVIII) to human MGL was assessed using plate binding assay. **K**, Binding to human MGL was assessed for CHO-rFVIII following treatment with α2-3,6,8,9 neuraminidase (α2-3,6,8,9 Neu-rFVIII), PNGase F (PNGase-rFVIII) or combined PNGase F and O-glycosidase (PNGase, Ogly-rFVIII). Data were analyzed by 1-way ANOVA with Tukey multiple comparison test and are presented as mean±SD (n=6). **L**, Finally, human-MGL was transiently expressed in HEK-293T cells (HEK-MGL). Binding of FVIII to HEK and HEK-MGL was assessed using flow cytometry. All plate binding experiments were performed with three technical replicates. For flow cytometry experiments, a minimum of three biological replicates were used.

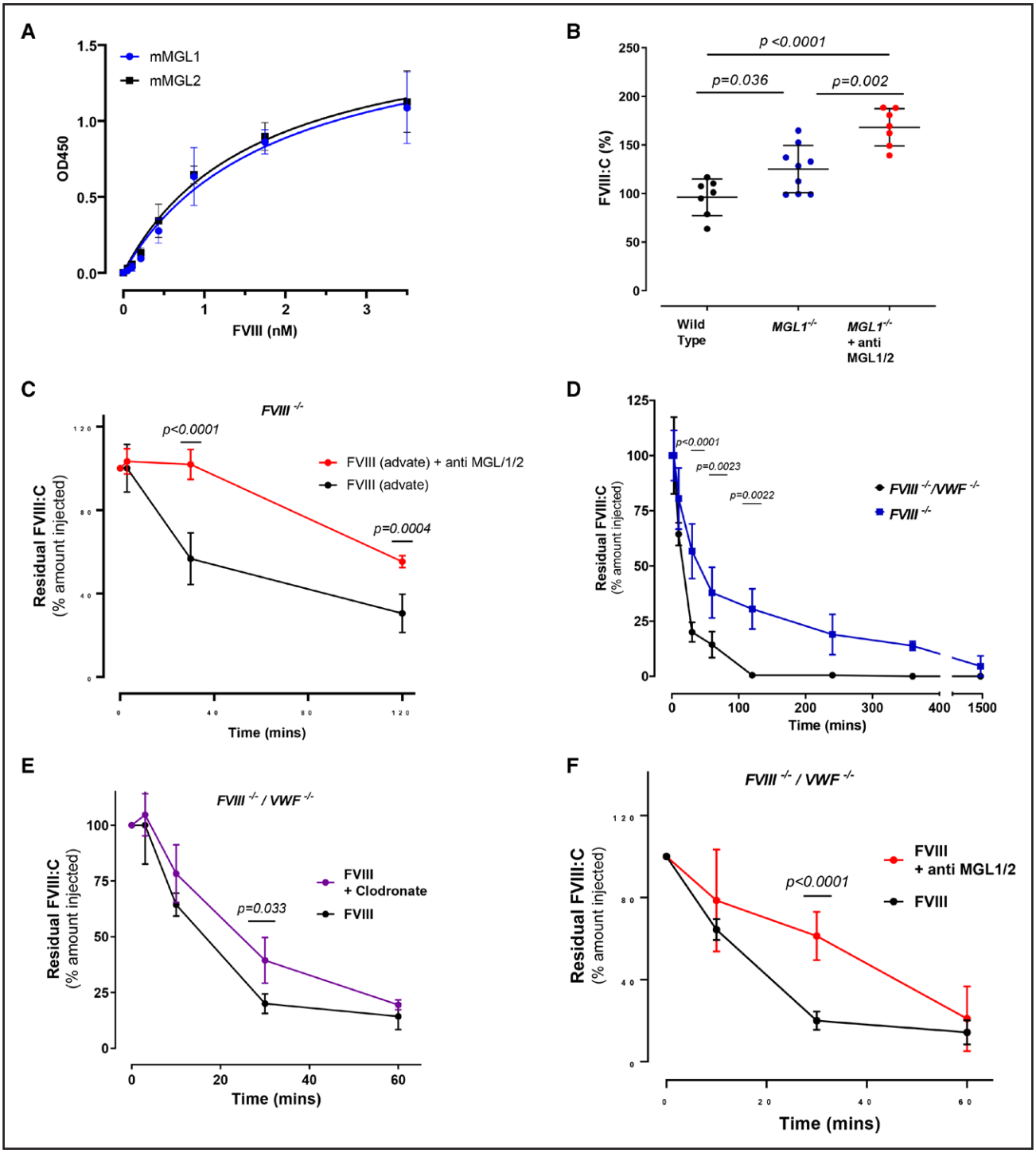

Figure 2. MGL (macrophage galactose-type lectin) regulates free-FVIII (Factor VIII) clearance in vivo.
A, In vitro binding of human pdFVIII to murine MGL1 and MGL2 (mMGL1 and mMGL2) receptors was assessed using plate binding assay as detailed in the Materials and Methods section. **B**, Plasma FVIII levels were measured using chromogenic assay in wild-type mice, *MGL1*$^{-/-}$ mice, and *MGL1*$^{-/-}$ mice 24 hours following infusion of anti-MGL2 antibody. Data were analyzed by 1-way ANOVA and are presented as mean FVIII activity±SD (n=7–9). **C**, In vivo clearance of infused rFVIII was assessed in *FVIII*$^{-/-}$ mice in the presence or absence of combined mMGL1 and mMGL2 antibody inhibition. At each time point, residual circulating rFVIII concentration was determined by chromogenic assay. All results are plotted as percentage residual rFVIII levels relative to the amount injected. Data are represented as mean±SD (n=2–6). Data were analyzed by 2-way ANOVA with Šídák's multiple comparisons test. **D**, The clearance of infused free-rFVIII was assessed in *VWF*$^{-/-}$/*FVIII*$^{-/-}$ dual knockout mice. Data are plotted as mean percentage residual rFVIII levels relative to the amount injected ±SD (n=2–6). Data were analyzed by 2-way ANOVA with Šídák multiple comparisons test. **E**, In order to assess the contribution of macrophages in modulating enhanced clearance of free-rFVIII, clearance experiments were repeated in *VWF*$^{-/-}$/*FVIII*$^{-/-}$ mice 24 hours after clodronate-induced macrophage depletion. Data are plotted as mean residual FVIII activity ±SD (n=3–4). Data were analyzed by 2-way ANOVA with Šídák multiple comparisons test. (*Continued*)

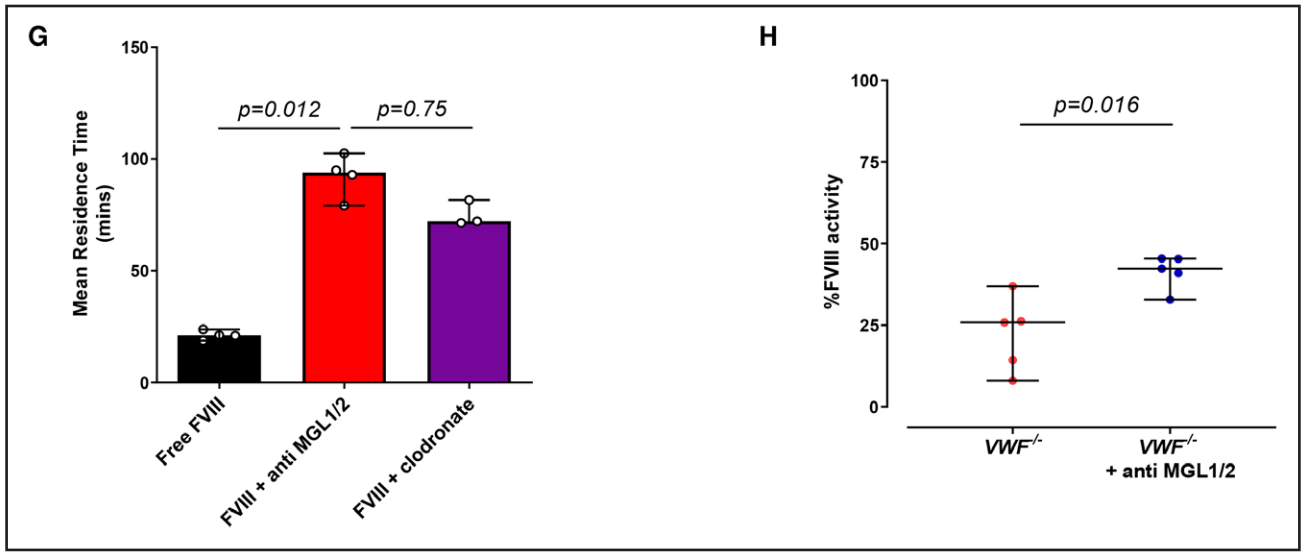

Figure 2 Continued. **F** and **G**, To investigate whether MGL plays a role in macrophage-mediated clearance of free-rFVIII, in vivo clearance studies in *VWF*$^{-/-}$/*FVIII*$^{-/-}$ mice were performed in the presence or absence of anti-MGL1/2 blocking antibodies. Mean residual FVIII activity is shown ±SD (n=3–4). Data were analyzed by 2-way ANOVA with Šídák's multiple comparisons test. **F**, Mean residence time was analyzed by Kruskal-Wallis test with Dunn multiple Comparison test and is presented as median±95% CI (n=3–4). **G**, Endogenous plasma FVIII levels were measured in *VWF*$^{-/-}$ mice 24 hours post-treatment with anti-mMGL1/2 blocking antibodies compared with isotype control IgG. **H**, Endogenous plasma FVIII:C levels were measured in *VWF*$^{-/-}$ mice in the presence or absence of anti-MGL1/2 blocking antibodies. Data were analyzed by Mann-Whitney *U* test and presented as median FVIII activity ± 95% CI (n=5). All experiments were performed with three technical replicates and a minimum of two biological replicates per time point.

MGL in FVIII clearance, endogenous plasma FVIII:C levels were significantly elevated in *MGL1*$^{-/-}$ mice compared with wild-type controls (125±24.32% versus 96.13±18.86%, respectively; *P*=0.036, Figure 2B). Moreover, plasma FVIII:C levels were further significantly (*P*=0.002) increased following infusion of anti-MGL 1/2 antibodies to block both murine MGL receptors (168.1±19.16%; Figure 2B). The in vivo clearance of infused FVIII in *FVIII*$^{-/-}$ mice was also attenuated following MGL inhibition (mean residence time 113.4±16 versus 233.5±67 minutes; Figure 2C). Together, these in vivo findings are consistent with a role for MGL in modulating physiological clearance of the VWF-FVIII complex.[16]

To formally investigate whether macrophages and/or MGL may also play a role in regulating free-FVIII clearance, in vivo clearance experiments were performed in dual *FVIII*$^{-/-}$*VWF*$^{-/-}$ mice. Consistent with previous studies on roles for VWF in FVIII clearance, the mean residence time of infused FVIII was reduced in *VWF*$^{-/-}$*FVIII*$^{-/-}$ compared with *VWF*$^{+/+}$*FVIII*$^{-/-}$ mice (21.2±2.1 versus 158.9±32.4 minutes, respectively; Figure 2D). The in vivo clearance of free-FVIII in *VWF*$^{-/-}$*FVIII*$^{-/-}$ mice was mediated by macrophages,[28] with a reduction following clodronate-induced macrophage depletion (mean residence time 75.06±5.72 versus 21.2±2.1; Figure 2E). To investigate whether MGL plays a role in macrophage-mediated clearance of free-FVIII, infused FVIII clearance studies were repeated in *VWF*$^{-/-}$*FVIII* $^{-/-}$ mice in the presence or absence of combined MGL1 and MGL2 inhibition (Figure 2F). Anti-MGL 1/2 blocking significantly attenuated the enhanced clearance of free-FVIII (mean residence time 92.37±9.71 versus 21.2±2.1 minutes, respectively; *P*=0.012 Figure 2G). Finally, in keeping with the short half-life of free-FVIII, endogenous plasma FVIII:C levels were significantly reduced in *VWF*$^{-/-}$ mice compared with wild-type control mice. Importantly however, we observed that combined MGL1 and MGL2 inhibition could significantly increase endogenous murine plasma FVIII:C levels in *VWF*$^{-/-}$ mice (25.25±11.29% versus 41.34±5.13%; *P*=0.016; Figure 2H). Finally, in keeping with the concept that O-linked glycans regulate FVIII interaction with MGL, minimal binding of B-domain deleted (BDD)-FVIII to human MGL was seen (Figure S6A). Importantly however, free BDD-FVIII was still cleared rapidly in the absence of endogenous murine VWF (Figure S6B).

Collectively, these findings demonstrate that MGL receptor plays an important role in regulating the macrophage-mediated clearance of both VWF-bound FVIII and free-FVIII in vivo. We propose that this novel FVIII-MGL clearance pathway may contribute to enhanced FVIII clearance in patients with type 2N or type 3 Von Willebrand disease. However, current evidence suggests that a number of different cellular clearance pathways (including LRP-, ASGPR-, and MGL-mediated)[11,29] likely contribute to the short half-life of free-FVIII. Further studies will be required to determine the relative biological importance of these pathways, and whether their contribution may vary for different types of FVIII therapies.

ARTICLE INFORMATION

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Disclosures

P.L. Turecek is full-time employee of Baxalta Innovations GmbH, a member of the Takeda group of companies, and shareholder of Takeda Pharmaceutical Company Limited. J.S. O'Donnell has served on the speaker's bureau for Baxter, Bayer, Novo Nordisk, Sobi, Boehringer Ingelheim, Leo Pharma, Takeda and Octapharma. He has also served on the advisory boards of Baxter, Sobi, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim, Takeda, and Pfizer. J.S. O'Donnell has also received research grant funding awards from 3M, Baxter, Bayer, Pfizer, Shire, Takeda, 3M, and Novo Nordisk. The remaining authors declare no conflicts of interest.

Supplemental Material

Expanded Materials and Methods
Figures S1–S6
Major Resources Table

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