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Title

Thrombomodulin enhances complement regulation through strong affinity interactions with factor H and factor H-C3b complex

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Running title: Thrombomodulin molecular interactions with complement.

Highlights

- Thrombomodulin binds with high affinity to Factor H and C3b-Factor H
- Recombinant human thrombomodulin analogs differ in binding affinity with factor H and thrombin
- Soluble Thrombomodulin regulates complement in serum
- TM456 regulates complement in serum

Abstract

Introduction

Coagulation and complement systems are simultaneously activated at sites of tissue injury, leading to thrombin generation and opsonisation with C3b. Thrombomodulin

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(TM) is a cell-bound regulator of thrombin activation, but can also enhance the regulatory activity of complement factor H (FH), thus accelerating the degradation of C3b into inactive iC3b.

Objectives

This study sought to determine the biophysical interaction affinities of two recombinant TM analogs with thrombin, FH and C3b in order to analyze their ability to regulate serum complement activity.

Methods

Surface plasmon resonance (SPR) analysis was used to determine binding affinities of TM analogs with FH and C3b, and compared to thrombin as positive control. The capacity of the two recombinant TM analogs to regulate complement in serum was tested in standard complement hemolytic activity assays.

Results

SPR analysis showed that both TM analogs bind FH with nanomolar and C3b with micromolar affinity; binding affinity for its natural ligand thrombin was several fold higher than for FH. At a physiological relevant concentration, TM inhibits complement hemolytic activity in serum via FH dependent and independent mechanisms.

Conclusions

TM exhibits significant binding affinity for complement protein FH and C3b-FH complex and its soluble form is capable at physiologically relevant concentrations of inhibiting complement activation in serum.

Keywords

Complement, thrombomodulin, hemolytic assay, Surface Plasmon Resonance

Abbreviations

AP, alternative pathway; CH50, the amount of serum (μl) giving 50% hemolysis of sheep erythrocytes; CFD, complement fixation diluents buffer; CP, classical pathway; DIC, disseminated intravascular coagulation; EGF, endothelial growth

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factor domain; FB, Factor B; FH-H50, the amount of functional FH needed to lyse 50% ShEA; Factor H; FI, Factor I; HUS, hemolytic uremic syndrome; IC50, the concentration of an inhibitor where the response is reduced by 50%; LP, lectin pathway; MAC, membrane attack complex; NHS, normal human serum; PPACK, Phenylalanyl-propyl-arginine chloromethyl ketone; SCR, short consensus repeat; ShEA, Amboceptor-sensitized sheep erythrocytes; SPR, surface plasmon resonance; TAFI, thrombin-activatable fibrinolysis inhibitor TM, thrombomodulin;

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1. Introduction

Thrombomodulin (TM; CD141) [1, 2] is an integral membrane protein while heterogeneous soluble fragments of TM (sTM) are present in blood [3] and urine [4] and act as an effective marker of endothelial cell injury. Endotoxin and inflammatory cytokines decrease membrane-bound TM expression on the endothelium and increase soluble TM in blood [5], [6].

TM's role in haemostasis regulation is primarily to down-regulate thrombin generation [7]. The TM-thrombin complex converts protein C to activated protein C (APC), which in turn mediates inactivation of factors Va and VIIIa in the presence of protein S [8]. TM-thrombin complex also activates thrombin-activatable fibrinolysis inhibitor (TAFI) and hence down-regulates fibrinolysis [9]. Interestingly, TAFI inactivates C3a and C5a [10] revealing a central role for this molecule in the regulation and interface between complement and coagulation.

Complement and coagulation are both innate blood defense systems, evolutionary linked with functional similarities and many shared structural motifs. Crosstalk events between the complement and coagulation pathways have been investigated, but are as yet not comprehensively characterised [11, 12].

Complement protects against pathogens – primarily bacterial infection – by rapid activation of serine proteases and generation of active products. Cell killing is achieved directly through lytic pore formation (membrane attack complex; MAC), and indirectly via anaphylatoxins (C3a, C5a) and marking targets for phagocytosis (opsonisation; C3b, iC3b). Complement is activated via three distinct pathways, triggered respectively by antibody (classical, CP), bacterial carbohydrate (mannose binding lectin, LP) or contact activation (alternative, AP). All pathways lead to enzymatic cleavage of complement C3 to the active fragments C3b and C3a. The opsonin C3b binds indiscriminately to pathogens and adjacent host cells. To prevent damage to self, complement is controlled by regulators, limiting complement activation by inactivating C3b, dissociating C3/C5 convertase enzymes or inhibiting MAC formation [13, 14]. The regulator factor H (FH) accelerates C3 convertase dissociation and acts as a co-factor for the factor I (FI)-mediated inactivation of C3b, regulating both in the fluid phase and on cell surfaces [15].

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TM appears to modulate complement activity by enhancing FH co-factor activity. TM co-precipitates with FH and C3b and both of these bind to the surface of TM-expressing HEK293 cells. In fluid phase cleavage assays, TM enhances FH/FI-mediated C3b inactivation in a dose-dependent manner [16, 17], although the molecular basis by which this occurs is incompletely understood.

Complement and coagulation crosstalk in disease, and the potential therapeutic application of TM, is the subject of ongoing investigation. Recombinant human soluble TM (ART-123; Recomodulin™) has been investigated for treatment of disseminated intravascular coagulation (DIC) and sepsis-induced DIC [18, 19], and approved for treatment in Japan. Recently, studies have investigated the role of soluble TM in patients with hemolytic uremic syndrome (HUS) [20, 21] and atypical HUS (aHUS) [22]. aHUS has been associated with TM mutations [16], but is primarily linked to complement dysregulation caused by mutations in complement proteins [23-27] or autoantibodies [28, 29].

In this study, we utilize two human recombinant TM analogs generated in different expression systems, using SPR analysis to determine binding affinity for complement C3b and FH. We further test the capacity of TM to act as a complement regulator in serum.

2. Materials and Methods

2.1 Complement and coagulation components

Complement factor H (FH) and factor B (FB) were affinity-purified from EDTA-plasma of healthy volunteers on anti-FH (mAb 35H9, kind gift from Prof. Santiago Rodríguez de Cordoba, Madrid, Spain) or anti-Bb (mAb JC1, in-house) immobilized on HiTrap columns (GE Healthcare UK Limited, Amersham, UK). HRP-conjugated polyclonal anti-FH antibody was prepared in-house. Factor D (FD) and C3b were purchased from Complement Technology Inc (Tyler, Texas, USA). Polyclonal anti-TM antibody and the two human recombinant soluble TM were from R & D Systems Europe Ltd (Abingdon, UK) and Abcam plc (Cambridge, UK); the first was produced in a mouse myeloma cell line (NS0-derived) with sequence: Ala19-Ser515, with C-terminal 6-His-tag; TM_1, the second was from HEK293 cells, sequence: Ala19-Ala509; TM_2. Phenylalanyl-propyl-arginine chloromethyl ketone (PPACK)-inhibited

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thrombin and active thrombin were from Enzyme Research Laboratories Ltd (Swansea, UK). Recombinant S195A thrombin [30], a catalytically inactive mutant; and TM456, a recombinant fragment of TM comprising EGF 4-6 including the thrombin binding sites EGF5-6 [31], was a kind gift from Prof. J. A. Huntington (Cambridge University, UK). Rabbit thrombomodulin was purchased from Haematologic Technologies Inc (Vermont, USA), Complement fixation diluent (CFD) was from Oxoid Ltd (Basingstoke, UK),sheep erythrocytes from TCS Biosciences Ltd (Buckingham, UK) and Amboceptor from Siemens Healthcare GmbH (Erlangen, Germany). Thrombin generation assay reagents (platelet-poor-plasma reagent, fluorogenic substrate, thrombin calibration standard) were purchased from Thrombinoscope B.V. (Maastricht, Netherlands). Chromogenic substrate Biophen CS-21 for APC was from Cambridge Bioscience Ltd (Cambridge, UK). OPD and Chondroitinase ABC from *Proteus vulgaris* was from Sigma-Aldrich Company Ltd. (Gillingham, UK). Polystyrene plates (MaxiSorp^{TM}) were from Fisher Scientific UK Ltd (Loughborough, UK) Biacore chips and reagents from GE Healthcare UK Limited (Amersham, UK).

2.2 Generation of depleted sera for hemolysis assays

Normal human serum (NHS) isolated from pooled whole blood of three healthy volunteers was sequentially affinity-depleted (ΔBH-NHS) on anti-Bb (mAb JC1, in-house) and anti-FH (mAb 35H9) affinity columns in the presence of CFD buffer. FB, a key activator of the alternative pathway (AP), is depleted alongside FH to prevent consumption of complement due to spontaneous AP activation in the absence of FH; FB is added back immediately prior to assay activation (addition of activated ShEA).

FH was quantified in the depleted serum using an in-house developed ELISA. In ΔBH-NHS (compared to NHS), FH was undetectable at standard serum dilution (1:3200) and even at 1:100 dilution no specific signal was observed above background - residual FH was <1.5ug/ml or <0.5% of average concentration in NHS.

2.3 Mass spectrometry analysis of thrombomodulin analogs

TM variants were subjected to mass spectrometry analysis (Applied Biosystems 4800 MALDI TOF/TOF Analyser, CBS Cardiff) to determine the molecular weights of the TM analogs.

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2.4 Anticoagulant function of thrombomodulin variants

The anticoagulant function of TM in normal, platelet-poor, pooled plasma was assessed using a Fluoroskan Ascent plate reader (Thermo Lab System) by incubating 80 μL of citrated plasma with 20 μL of platelet-poor plasma reagent containing 5pM tissue factor (TF) and 4μM phospholipids in the presence or absence of TM (5nM). Thrombin generation was initiated by automatic dispensation of fluorogenic thrombin substrate (FluCa) and determined using a thrombin calibration standard (T-Cal). Measurements were taken at short intervals for 162 minutes.

Activated protein C (APC) was measured by adding varying concentrations of TM (2.5-10 nM) to a fixed concentration of thrombin (0.2 nM) and protein C (500 nM) in 10 mM Hepes, 150 mM NaCl, 3 mM $CaCl_2$, pH7.4 then incubated at 37°C for 2 hours. Thrombin was inhibited by adding 1 ul (1U) Hirudin for 15 minutes at 37°C. APC-generation was measured by adding a chromogenic substrate (Biophen CS-21), incubated for 10 minutes and colorimetric output read at 405 nm absorbance.

2.5 Ligand binding assay of thrombomodulin to complement factor H

TM (1 μg/ml) or 10ug/ml TM456 was coated onto polystyrene plates in 50 μl carbonate buffer, pH 9.6 for 2 hours at room temperature, blocked with 1% gelatine in phosphate buffered saline containing 0.1% Tween (PBST) and incubated with varying concentrations of FH (1-5 μg/ml). Bound FH was detected using a HRP-conjugated polyclonal anti-FH antibody (10μg/ml), adding OPD substrate, stopped using 10% H_2SO_4 and absorbance read at 492 nm. Background (gelatine-only coated wells) was subtracted from the binding signal as blank.

2.6 Surface plasmon resonance (SPR) binding interaction and affinity analysis

All analyses were performed on a Biacore T100 (GE Healthcare) in Hepes buffered saline (HBS), 0.01% surfactant P20, pH 7.4. Kinetics were analyzed at 20μl/min flow rate at 25°C. Due to its low isoelectric point, TM immobilization to a CM5 Biacore chip via standard amine coupling was not feasible. Therefore an indirect capture method was used, as described previously [32]. Briefly, polyclonal anti-TM antibody was immobilized via amine coupling to a CM5 chip at 2000RU, a density chosen to

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ensure that a sufficient proportion of the captured molecules exposed a binding epitope. TM analogs were captured stably on the antibody surface on adjacent flow cells. Binding stability of the TM-antibody complex was assessed over time prior to analysis and shown to be sufficiently stable for use as a baseline surface for the duration of the analysis (data not shown).

To measure binding kinetics of each interaction, complement FH, C3b or preformed C3b/FH complexes were flowed across captured TM at varying concentrations. PPACK-thrombin and S195A thrombin interaction with captured TM was tested as positive control. We used PPACK-inhibited and S195A thrombin to prevent non-specific proteolysis during the analysis; as the active site is not directly involved in TM binding [33], inhibition should not interfere with affinity analysis. As a direct comparison to the Ca^{2+}-independent C3b-FH interaction, all analyses were performed in the absence of Ca^{2+}; the effect of Ca^{2+} removal on TM-thrombin interaction is documented [32]. The surface was regenerated with 10 mM sodium acetate pH 4, 1 M NaCl, this did not affect TM binding to the capture antibody.

TM binding with C3b was also tested in the reverse orientation by flowing TM at 1 μM over a 6000RU thioester-deposited C3b surface. A C3bBb convertase was formed in Hepes buffer saline pH 7.4, 1mM $MgCl_2$ by flowing FB (200 μg/ml) and FD (0.5 μg/ml) over a 1000RU amine-coupled C3b surface; 200nM TM was flowed to test binding and TM impact on C3bBb decay/dissociation. As a positive control, FH (100 nM) was flowed over and decayed the convertase efficiently. The effect of TM on FH-mediated C3bBb decay was tested by simultaneously flowing 200nM TM and 100nM FH over the C3bBb surface.

2.7 Hemolysis assays

2.7.1 Standard serum titration assay

Hemolytic assays have traditionally been used to assess the functional activity of the complement system based on protocols first described by Mayer [34]. Serial dilutions of NHS were incubated with antibody (Amboceptor)-sensitized sheep erythrocytes (ShEA), resulting in complement-mediated lysis of the cells. To test complement regulation by TM, a fixed concentration of human recombinant TM was added to neat (100%) NHS before serially diluting the NHS in CFD and subsequent addition of

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a 2% ShEA suspension in CFD buffer. The complement regulating ability of both TM analogs TM_1 and TM_2 was tested twice each on different days using NHS pooled from three healthy donors and freshly made ShEA suspension.

Lysis was calculated by measuring hemoglobin release, control incubations were ShEA incubated in buffer only (0% lysis) or in 0.001% Triton X (100% lysis). Percentage hemolysis = 100 × (A410 test sample – A410 0% control)/(A410 100% control – A410 0% control). The resulting sigmoidal titration curves are expressed as % hemolysis over serial % NHS dilutions. Curves were fitted using nonlinear regression. CH50, the amount (μl) NHS giving 50% hemolysis (K) was calculated by plotting a log-log graph of NHS volume vs Y/(1-Y) giving a linear trace [35]. At 50% hemolysis, Y/1-Y=1, hence the intercept on the x-axis from this point is K and K corresponds to 1 CH50 unit in serum. Statistical significance was evaluated using a two-tailed unpaired t test.

2.7.2 Functional add-back assay

The effects of TM on complement activity at varying FH concentrations was tested by adding the proteins to FH-depleted serum (ΔBH-NHS) prepared as described above. TM456 or rbTM was titrated in CFD and added to 1% ΔBH-NHS, a serum dilution selected to give 70-80% lysis. FH and FB were added back together with 2% ShEA to induce lysis.

FH/FB-double depleted serum (ΔBH-NHS) is used to assay the ability of factor H to regulate the amplification system of the alternative pathway in the presence of TM456 or rabbit TM. By adding 1% ΔBH-NHS to serial FH dilutions (0.001-1μM) we stabilize the alternative pathway; then adding 2% ShEA and 2ug/ml FB with or without 2μM TM456 or 50nM rbTM in CFD buffer to induce lysis.

Curves were fitted using nonlinear regression and are expressed as % hemolysis over serial factor H concentrations (nM) giving IC50 (inhibition of hemolysis by 50%) and maximum response (i.e. complement-mediated cell lysis $Max_{\%Lysis}$). A similar unit to CH50 is the FH-H50, which we used to quantitate the activity of FH in ΔBH-NHS in the presence or absence of TM456 or rbTM. A FH-H50 unit is the amount of functional FH needed to regulate lysis of 50% of antibody- sensitized sheep erythrocytes (ShEA) when incubated with 1% ΔBH-NHS.

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3 Results

3.1 Molecular analysis of recombinant thrombomodulin variants

Before commencing with the biochemical analysis, the two commercially available human recombinant soluble TM preparations (termed TM_1 and TM_2), were characterized. Molecular weights were confirmed with mass spectrometry (MS); this demonstrated a molecular weight for TM_1 ~66 kDa and TM_2 ~64kDa, the difference accounted for by the sequence differences (TM_1, Ala19-Ser515 plus 6xHis-tag; TM_2, Ala19-Ala509) and variable glycosylation for TM from different expression systems. Both TM analogs were functional and mediated the activation of protein C (APC) in the presence of thrombin and calcium and also significantly reduced the endogenous thrombin potential (ETP; the area under the curve) in plasma thrombin generation assays (data not shown).

3.2 Thrombomodulin binds C3b with micromolar and FH with nanomolar affinity

To test binding, TM_1 was immobilized on plastic, for affinity analysis TM_1 and TM_2 were captured on SPR chips. In order to confirm that indirect SPR surface capture did not affect TM capacity to bind ligands, we first analyzed thrombin affinity as a positive control. Catalytically inactive thrombin was used to prevent proteolysis during analysis. Both TM_1 and TM_2 bound thrombin with nM affinity (Supplementary Figure 1), similar to results for thrombin interaction with the TM analog Solulin^{TM} in an equivalent experimental setting [32].

There was a several-fold difference in binding affinity for thrombin between TM_1 to TM_2, with TM_1 showing 3-5 fold higher affinity to both thrombin molecules (PPACK-inhibited thrombin: TM_1, KD 15.4+/-4.5 nM and TM_2, KD 72+/-7.07; inactive S195A thrombin: TM_1, KD: 19.1+/-1.4 nM and TM_2, KD: 49.6+/-2.4 nM; Supplementary Figure 1 A and B). There was no statistically significant difference in binding of S195A-thrombin versus PPACK-thrombin to either TM_1 ($p=0.3848$) or TM_2 ($p=0.0515$), confirming published results, where recombinant TM456, bound PPACK-thrombin compared to active thrombin with the same affinity [36].

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The direct interaction of CFH and C3b with TM was shown by coprecipitations and immunoblotting, and this interaction is increased in the presence of FH [16]; however, there is no published knowledge about their respective complement binding affinities compared with TMs' natural ligand thrombin when antibody-captured rather than on thrombin-sepharose. First, we confirmed binding of our TM analogs to human FH in a solid phase ligand binding assay (Figure 1 A). We then tested binding by SPR analysis; this yielded binding affinities for TM_1 and TM_2 with FH in the nanomolar range (TM_1 KD: 276+/-60 nM and TM_2 KD: 114+/-25nM; Fig. 1 B and C).

Both TM_1 and TM_2 also bound fluid-phase C3b with similar affinities (TM_1 KD: 1.13+/-0.09 μM and TM_2 KD: 1.16+/-0.02 μM; Fig. 2 A and B), albeit much more weakly than FH binding to TM. When the pre-formed C3b-FH complex was flowed over immobilized TM it displayed the highest binding affinity (TM_1 KD~ 91 nM and TM_2 KD~76 nM; Fig. 2 C and D); the data suggest that TM binds the complex better (or at least as well) than the individual components. However, from these data we cannot deduct whether this is an additive effect or due to binding to a new epitope present in the C3b-FH complex.

While TM_1 and TM_2 differed in their binding affinities for thrombin and FH, there was no difference between these analogs with C3b binding.

3.3 Thrombomodulin binds immobilized C3b but not C3bBb

C3b was immobilized on an SPR chip surface and, for some studies, the C3bBb convertase was formed in situ on the chip as previously described [37]. TM was flowed over densely immobilized C3b and specific binding was observed (Fig. 3 A). When TM was flowed over a low density C3b or preformed C3bBb complex, no binding was observed (Fig. 3 B); further, flowing TM did not accelerate decay of the C3bBb convertase (Fig. 3 B). Together, these data suggest that TM binding C3b alone is very weak and that TM cannot bind to or accelerate decay of the convertase.

It has been reported that TM enhances FH cofactor activity [16, 17]. FH co-factor activity is mediated by its domains SCR1-4 (short consensus repeat 1, 2, 3 and 4) [38], which also promote FH-mediated C3bBb convertase decay. Therefore, we

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tested whether TM is able to enhance FH-mediated decay of C3bBb. FH caused accelerated decay of C3bBb as expected but there was no significant change in FH-accelerated decay when a pre-formed FH-TM were flowed, indicating that the observed interactions do not impact FH-mediated decay (Fig. 3 C, D).

3.4 Thrombomodulin regulates complement activity in serum

The capacity of TM to bind individual complement proteins has been shown. In order to determine whether TM impacts plasma complement activity, we used a standard hemolytic assay, and added in TM_1 and TM_2.

To be able to add-back a therapeutically relevant concentration, we used available data on the therapeutic soluble recombinant TM (ART-123/Recomodulin™) which is similar to the recombinant protein analogs used in this work. This agent is used at a dose of 380 U/kg;0.06mg/kg (Japanese Ministry of Health, Labour and Welfare) by daily intravenous administration for treatment of disseminated intravascular coagulation [18]. Plasma levels in patients are ~1500 ng/ml [39], while the highest concentration achieved with no bleeding event in the non-clinical toxicology studies was 5400 ng/mL with a therapeutic range of TM 300–5400 ng/mL [40]. We used the concentration of 5400 ng/mL (83~104nM) in our studies.

NHS was spiked with TM to achieve a final concentration of 100 nM and compared to untreated NHS in a hemolysis assay (Fig. 4). TM_1 and TM_2 (each at 100nM in undiluted NHS) significantly reduced complement activity in NHS by 19% and 16% respectively (TM_1 in NHS CH50 ~ 1.53+/-0.04 compared to NHS alone CH50 ~ 1.25 +/- 0.05, P~0.008, Fig. 4A and B and TM_2 in NHS CH50 ~ 1.75 +/- 0.06 compared to NHS CH50 ~ 1.48 +/- 0.02, P~0.011, Fig. 4C and 4D). These relatively small changes in CH50 were replicated in several assays executed on different days with fresh NHS and ShEA (data summarized in Fig. 4E). Absolute CH50 values vary from day-to-day; however, each individual measurement reproduces the significant difference between NHS titration curve with or without TM. The data suggest that TM at doses used for therapy has a small but consistent inhibitory effect on serum complement activity.

3.5 Complement regulation by human recombinant TM456 fragment

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Thrombin binds to the EGF5-6 domains of TM [41], which comprise the minimum functional domain involved in anticoagulant activity via a thrombin-mediated protein C activation mechanism [42]. To see whether thrombin-binding sites of TM were important for complement regulating activity we tested the TM456 fragment, comprising EGF456 only.

In a solid-phase binding assay, we first confirmed the binding interaction of immobilized TM456 to FH (Figure 5 A). SPR confirmation of binding was attempted but capture of the TM456 on the antibody surface was inefficient. We could further show that TM456 regulates complement lysis in a dose-dependent manner when added back to ΔBH-NHS alongside FB and FH. In the absence of TM456, 85% lysis was achieved, whereas in the presence of the maximum dose of 2μM TM456, lysis was reduced to 61% (Figure 5 B).

FH-H50 was 15 +/-4nM in the presence of TM456 and 41 +/-3nM in its absence, showing that in the presence of TM456, less FH is needed to reduce lysis to 50% (Figure 5 C). A clear reduction in the maximum response (i.e. complement-mediated maximum cell lysis; $Max_{\%Lysis}$) was observed when titrating FH in the presence ($Max_{\%Lysis}$ 60.9+/-2.8) compared to absence of TM456 ($Max_{\%Lysis}$, 73.6+/-0.5, p=0.018). TM456 inhibited complement activity even in the absence of FH, implying that TM456-mediated complement regulation is not exclusively via interaction with FH (Figure 5 D).

3.6 Complement regulation by purified rabbit thrombomodulin

In order to test whether native, purified TM expressed complement regulatory activity we used purified rabbit thrombomodulin (rbTM), which has been traditionally used as a human analog with similar function [43] as the isolation of human thrombomodulin from for instance placental tissue was not feasible.

In a solid-phase binding assay, we first confirmed the binding interaction of immobilized rbTM to human FH (Figure 6 A). We further showed that rbTM also regulates complement lysis in a dose-dependent manner when added back to ΔBH-NHS alongside FB and FH (Figure 6 B).

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FH-H50 was 59 +/-2nM in the presence of rbTM and 134 +/-3nM in its absence, showing that in the presence of rbTM, less FH is needed to reduce lysis to 50% (Figure 6 C, $p<0.0001$). A clear reduction in the maximum response ($Max_{\%Lysis}$) was observed when titrating FH in the presence ($Max_{\%Lysis}$ 87.5+/-0.4) compared to absence of rbTM ($Max_{\%Lysis}$, 102+/-2.5, $p=0.0005$). As seen with TM456, rbTM inhibited complement activity even in the absence of FH (Figure 6 D).

4. Discussion

TM expression on endothelial cells (ECs) aids the maintenance of a continuous barrier along the vasculature through coagulation regulation via its interaction with thrombin and activation of protein C [44]. The endothelium is also a target for complement [45] and upon activation and generation of C5a, induction of tissue factor (TF) has been observed [46], linking both complement and coagulation to sites of endothelial dysfunction. TMs' role in complement regulation is bi-fold, it aids the generation of TAFIa [9] which in turn inactivates complement C3a and C5a [10], and it further promotes the inactivation of iC3b by enhancing FH-cofactor activity [16, 17].

Normal vascular endothelium expresses an anti-inflammatory phenotype and does not support complement activation in the absence of vascular damage [47]. Endothelial FH binding via polyanionic markers such as sialic acid and sulphated polysaccharides, such as heparin sulfate plays a crucial role in 'self' (healthy) versus 'non-self' (damaged) recognition [48, 49]. FH concentration in plasma can range as much 116–562 μg/ml (0.75-3.6 μM, 115 kDa) depending on genetic and environmental factors [50, 51] and FH binds C3b [37, 52] with an affinity of ~1μM. Surface-bound FH concentration is unknown, however, a several fold higher affinity of FH for C3b was observed on host cells in the presence of polyanions/polysaccharides on the surface [53], supporting a role for FH regulating complement activation on the surface in the event of opsonisation.

Here we found that TM binds FH with nanomolar affinity, and also binds C3b albeit with relatively weak micromolar affinity; importantly, TM displayed a significantly increased affinity for the C3b-FH complex. Similar to the observations that the presence of polyanions increases C3b-H affinity [53], TM may be supporting the attachment of FH to the endothelium – enhanced in the presence of complement

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activation and C3b opsonisation (C3b-FH). This might aid FH localization to the endothelial surface, providing vascular protection and supporting the discrimination of host endothelium from complement-activating foreign cells.

We further showed that despite TM's ability to bind to C3b, FH and the C3b-FH complex, it neither bound C3b within the C3bBb convertase complex nor decayed the C3bBb complex; this may be due to different binding sites exposed in C3b-FH versus C3bBb complex. Indeed, a steric clash between FH (SCR1-4) and Bb when aligning C3b-FH and C3bBb was shown in a structural study [54]. FH decay activity (i.e. displacing Bb) is likely located within SCR1-2 of its structure [54], and FH cofactor activity is largely lost without SCR1, implying that the interaction of SCR1 and SCR2 with C3b is crucial for FH regulatory activities [38]. TM binding to FH influences its cofactor but not decay activity, one can speculate that it binds directly within a region that is involved in its regulatory activity (i.e. SCR1-4) or indirectly influences these by affecting the overall structure of the C3b-FH complex.

To directly compare the complement-TM interaction with its natural ligand thrombin, we performed affinity analysis with catalytically inactive thrombin molecules. Thrombin affinities for both TM analogs was several-fold higher than to FH and FH-C3b, indicating that in the event of thrombin generation, the TM-thrombin complex formation is prevalent if FH and/or FH-C3b share the same binding sites on TM as suggested by TM456, a thrombin binding site, having complement regulatory activity.

TM analogs differed in their affinity for binding FH and thrombin indicating that the expression system, folding and glycosylation impact ligand binding [55]. As the TM analogs are of similar molecular weight, it is unlikely the degree of glycosylation is causing the differences in affinity; rather, the type of attached glycans likely influences affinity. Interestingly, no difference was shown for C3b, therefore C3b may involve different binding sites to FH and thrombin for TM.

We next tested the effect of TM on systemic complement activity. In standard hemolysis assays, we showed that full length recombinant soluble TM inhibits complement activity at a therapeutically relevant concentration [40]. The effect of TM analogs was small, but robustly reproducible. The amount of complement regulation observed, even though it is little, might potentially be effective in the context of HUS and aHUS. We have previously shown that even a small impact on complement

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activity can have a significant effect over time [56]; so suggest that this effect of TM is functionally and physiologically relevant. TM influences fluid phase regulation of complement and also binds opsonised surfaces, as demonstrated in real time by binding a densely opsonised C3b surface in vitro. As a consequence we suggest that it is important to determine the hemolytic complement activity in individuals before and during treatment with Recomodulin™ in future clinical trials, This is of particular importance in studies investigating the role of soluble TM in patients with hemolytic uremic syndrome (HUS) [20, 57], a condition caused by complement dysregulation. Here, a therapeutic that can efficiently regulate both coagulation and complement may be of particular value.

aHUS-associated mutations in TM impacting FH-mediated inactivation of C3b were identified in the C-type lectin-like domain of TM and increased complement activation was found in the TMLeD/LeD mice [39], which shows that this domain is important for complement binding interaction. Further TM mutations were located within the serine/threonine-rich region, also affecting FH-cofactor activity, likely another site of complement C3b and FH binding. Recombinant TM456 has similar anticoagulant activity to full length TM, although its short half-life limits its therapeutic application [58]. Additionally, it appears that the EGF456 domains in TM are also of importance to complement regulation. In functional assays, we tested a recombinant fragment of TM456, including the thrombin binding sites EGF5-6. We found that TM456 reduces complement activity in a dose-dependent manner in serum and when titrating FH, further supporting the idea that these domains are involved in complement regulation. This supports the speculation that complement, and particularly regulator FH, binds TM via multiple binding sites including EGF456, especially since the concentration of TM456 in the assay required to see an effect was several fold higher than for full-length TM.

Interestingly, we observed TM456 regulation also in the absence of FH, pointing to another mechanism, possibly via the interaction with C4bp, a CP regulator, which has been previously identified to bind TM [16]. Controversially, a recent report stated the AP enhancing activity of TM by increasing C3 convertase mediated cleavage of C3 into C3b, however, at non-physiological TM concentrations [17].

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Therefore, the complement-regulatory activity of TM is mediated through binding interactions of complement C3b and FH with the C-type lectin-like domain, EGF456 and the serine/threonine-rich region, the latter being the site of chondroitin sulfate attachment, whose presence not only enhances the TM interaction with thrombin [59] but also has been shown to bind both FH and C4bp in vitro [60].

To confirm our observations obtained with human recombinant TM with natural purified TM, we utilized TM from rabbit lung tissue to confirm the above findings of complement regulation in normal human serum and when titrating FH, also reproducing the finding that in the absence of FH, complement regulation remains. The complex interaction of TM in the presence and absence of FH with C4bp or any other complement molecule is part of future investigations.

5. Conclusions

Our work shows the contribution of TM in FH mediated host protection through the high affinity interaction with FH and the FH-C3b complex. These results further support the potential of soluble TM to exert complement regulatory activity in serum.

Additionally, since regulation can be observed by TM456 in the absence of FH, it becomes clear that TM inhibits complement hemolytic activity in serum via FH dependent and independent mechanisms. And that the cross-talk between TM and complement is more complex involving yet another complement protein.

In conclusion, TM may display a dual functional role; in addition to its classical role in binding thrombin to limit clot development it also supports FH ‘policing’ of the endothelium, facilitating down-regulation of complement activation in the event of FH-C3b formation.

Our data imply that soluble TM is a potential therapeutic option in conditions where both coagulation and complement are dysregulated. This may be of particular importance in conditions where surface dysregulation of complement in addition to coagulopathy is a disease hallmark, such as in HUS or aHUS.

As TM analogs from different expression systems differ in their interaction with both thrombin and FH, this may influence soluble TM efficacy in regulating coagulation

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and complement in vivo. Therapeutic TM should therefore be characterized for complement regulatory activity.

Addendum

M. Heurich designed and performed research, analyzed and interpreted the data and wrote the paper; R. J.S. Preston contributed analytical tools, analyzed data and editing the manuscript; V. B. O'Donnell, and B.P. Morgan contributed analytical tools, interpretation of data and editing the manuscript. P.W. Collins contributed to research design, interpretation of data, revising the intellectual content and co-wrote the paper. All authors approved the paper.

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Figure legends

Figure 1. Binding affinity of FH to recombinant human TM analogs. (A) A ligand binding assay confirmed the binding of FH (1,2 and 5 ug/ml) to immobilized TM_1. Data points represent mean ± SD of triplicate determinations (error bars depicted for each point) within one binding assay.

(B) FH was flowed over polyclonal antibody-captured recombinant human thrombomodulin TM_1 and (C) TM_2 at concentrations between 1.55 nM and 1.6 μM and equilibrium binding response was measured. Affinity (KD) was determined by steady-state analysis in duplicate for TM_1 KD 276+/-60 nM and TM_2 KD 114+/-25nM (mean +/- SD, n=2).

Figure 2. Binding affinity of C3b and C3b-FH to recombinant human TM analogs. C3b was flowed over (A) TM_1 and (B) TM_2 at concentrations between 4.4 nM and 5 μM and affinity was determined by 1:1 binding model for TM_1 KD 1.13+/-0.09 μM and TM_2 KD 1.16+/-0.02 μM (mean +/- SD, n=2). Preformed equimolar C3b-FH complex was flowed over (C) TM_1 and (D) TM_2 at concentrations between 7.8 nM and 800 nM and affinity was determined by 1:1 binding model for TM_1 KD~91 nM and TM_2 KD~76 nM (n=1).

Figure 3. TM interaction with C3b and C3bBb. (A) 1μM TM was flowed over 6000 RU thioester-deposited C3b. (B) 200 nM TM_1 was flowed over a preformed C3bBb convertase (black line, injection of TM_1 indicated by arrow) on a 1000RU C3b surface. (C) As indicated by arrows, 100 nM FH was flowed over a preformed C3bBb convertase in the absence (black dashed line) or presence (grey line) of 200 nM TM. (D) To illustrate FH-mediated C3bBb decay, FH binding to the C3b surface alone (Inset) was subtracted from the C3bBb sensorgram.

Figure 4. TM regulates complement activity in serum. Activated sheep erythrocytes (ShEA) were incubated with normal human serum (NHS) spiked with 100 nM human recombinant TM (circles) compared to NHS alone (squares) and lysis was developed. Data points represent mean ± SD of three determinations (error bars depicted for each point). Lysis was calculated by measuring hemoglobin release as a percentage of the controls. Nonlinear regression curves (A, C) and CH50 log-log plot

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(B, D) are shown for TM_1 (A,B) and TM_2 (C,D), respectively. (E) Summary of CH50 values (NHS volume in μl at 50% hemolysis) each for TM_1 (n=2) and TM_2 (n=2) in NHS compared to NHS only and corresponding p values analyzed in independent assays. P values were calculated using a two-tailed unpaired t test.

Figure 5. Complement regulation by human recombinant TM456 fragment. (A) TM456 binding assay confirmed the binding of FH (1,2 and 5 μg/ml) to immobilized TM456. Data points represent mean ± SD of triplicate determinations (error bars depicted for each point) within one binding assay. (B) Serial dilution of TM456 (0.125-2μM) added to 1% ΔBH-NHS with constant FB and FH and ShEA to develop lysis in CFD. In the absence of TM456, 85% lysis was achieved by 1% ΔBH-NHS, whereas the maximum dose of 2μM reduced lysis to 61%. (C) Serial dilutions of FH (0.001-1μM) was added to TM456-sufficient (2μM) or deficient 1% ΔBH-NHS and lysis developed in ShEA, with FB in CFD. FH-H50 was 15 +/-4nM in the presence of TM456 (circles) and 41 +/-3nM (squares) in its absence (p=0.018). (D) The maximum response (% Lysis) was reduced for TM456-suffient 1% ΔBH-NHS (circles, $Max_{\%Lysis}$ 60.9+/-2.8) compared to 1% ΔBH-NHS alone (squares, $Max_{\%Lysis}$, 73.6+/-0.5, p=0.018). Data points represent mean ± SD of three determinations (error bars depicted for each point, the assay was repeated twice with identical results).

Figure 6. Complement regulation by purified rabbit TM

(A) A ligand binding assay confirmed the binding of FH (1, 2 and 5 μg/ml) to immobilized rbTM. Data points represent mean ± SD of triplicate determinations (error bars depicted for each point) within one binding assay. (B) Serial dilution of rbTM (3-200 nM) with FB and FH were added to 1% ΔBH-NHS and ShEA to develop lysis in CFD. (C) Serial dilutions of FH (0.0019-2 μM) were added to rbTM-sufficient (50 nM) or deficient 1% ΔBH-NHS and lysis developed in ShEA with FB in CFD. FH-H50 was 59 +/-2nM in the presence of rbTM (circles) and 134 +/-3nM (squares) in its absence (p<0.0001). (D) The maximum response (% Lysis) was reduced for rbTM-sufficient 1% ΔBH-NHS (circles, $Max_{\%Lysis}$ 87.5+/-0.4) compared to 1% ΔBH-NHS alone (squares, $Max_{\%Lysis}$, 102+/-2.5, p=0.0005). Data points represent mean ± SD of

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three determinations (error bars depicted for each point, the assay was repeated twice with identical results).

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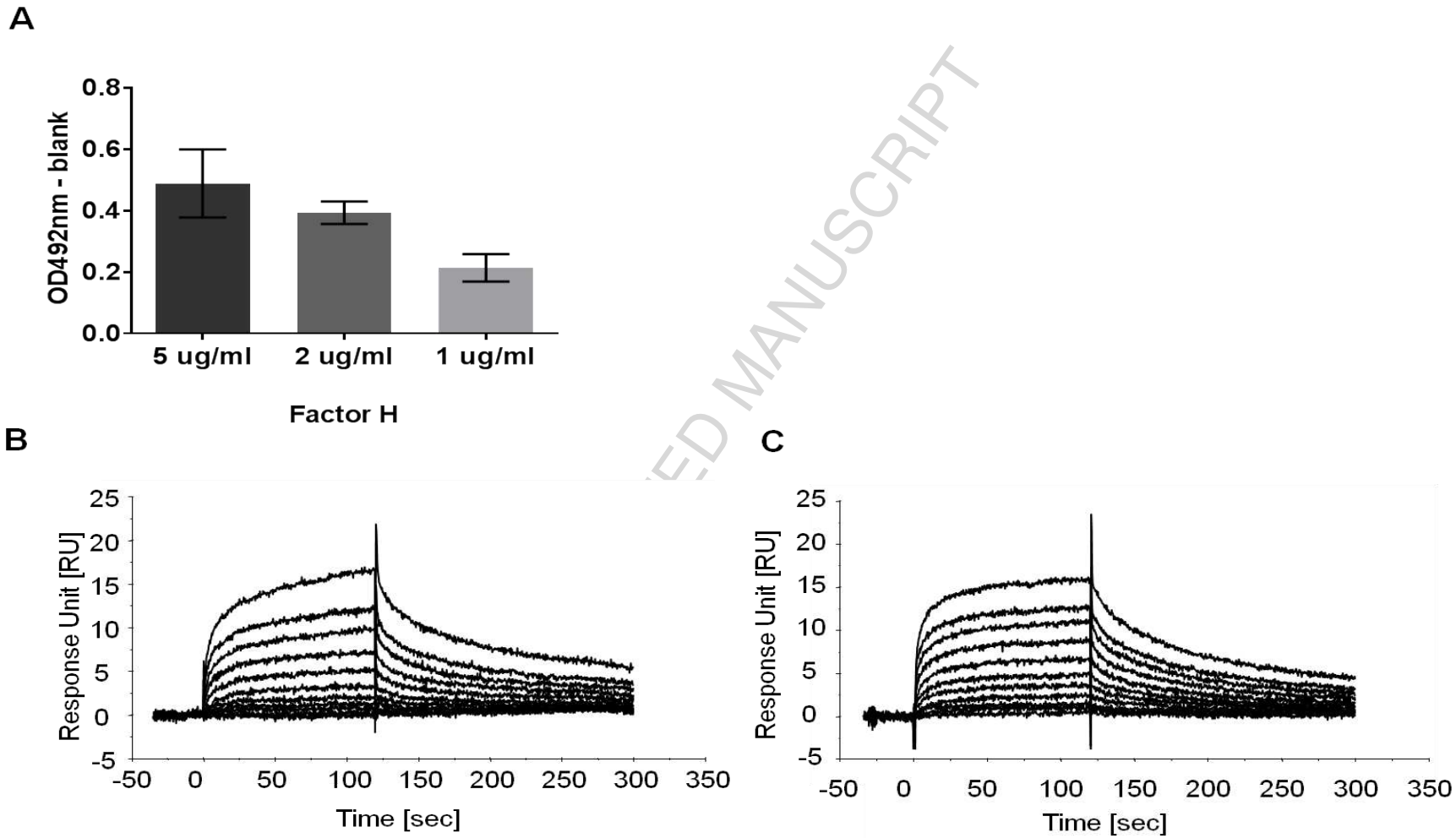

Figure 1

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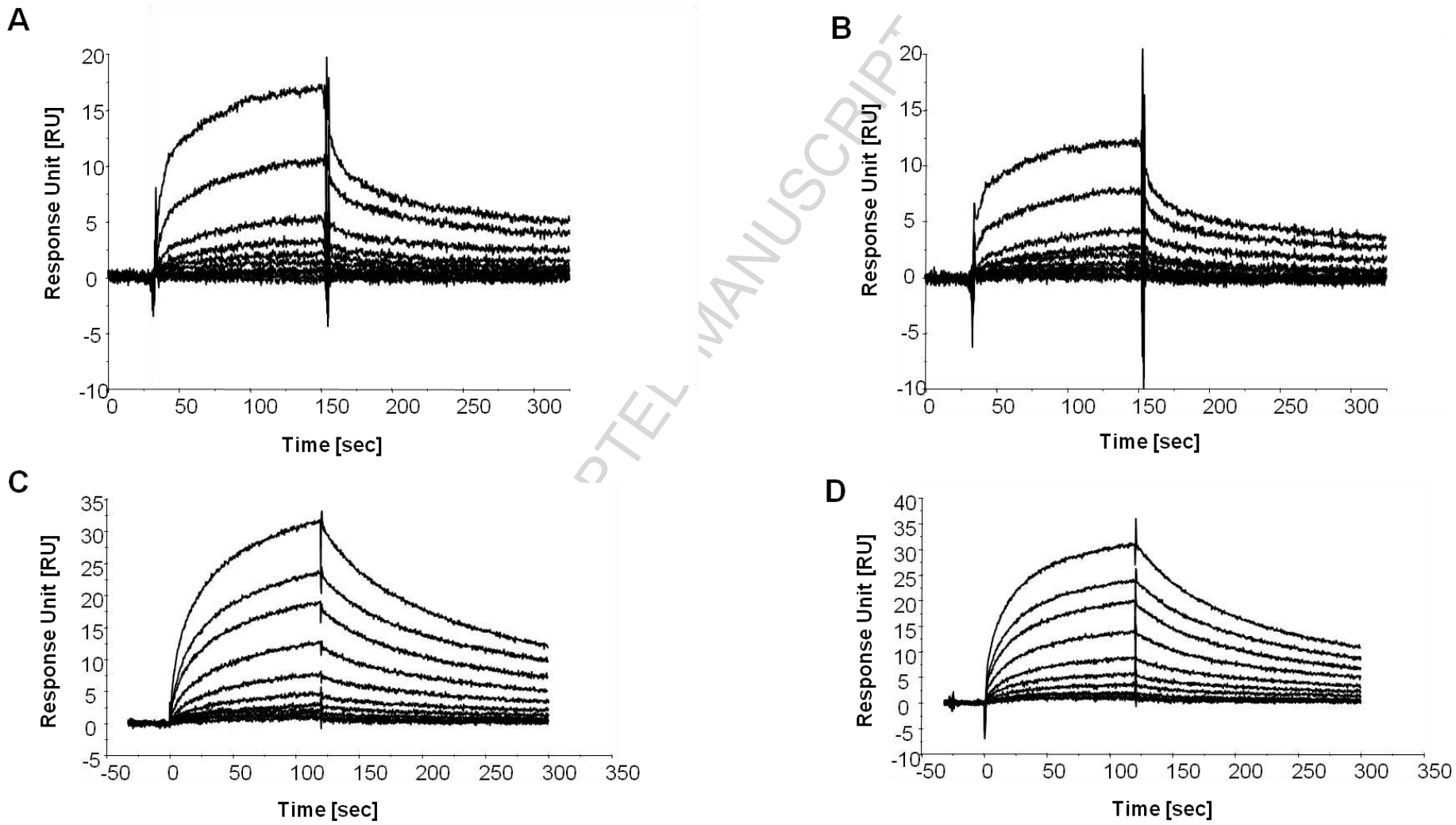

Figure 2

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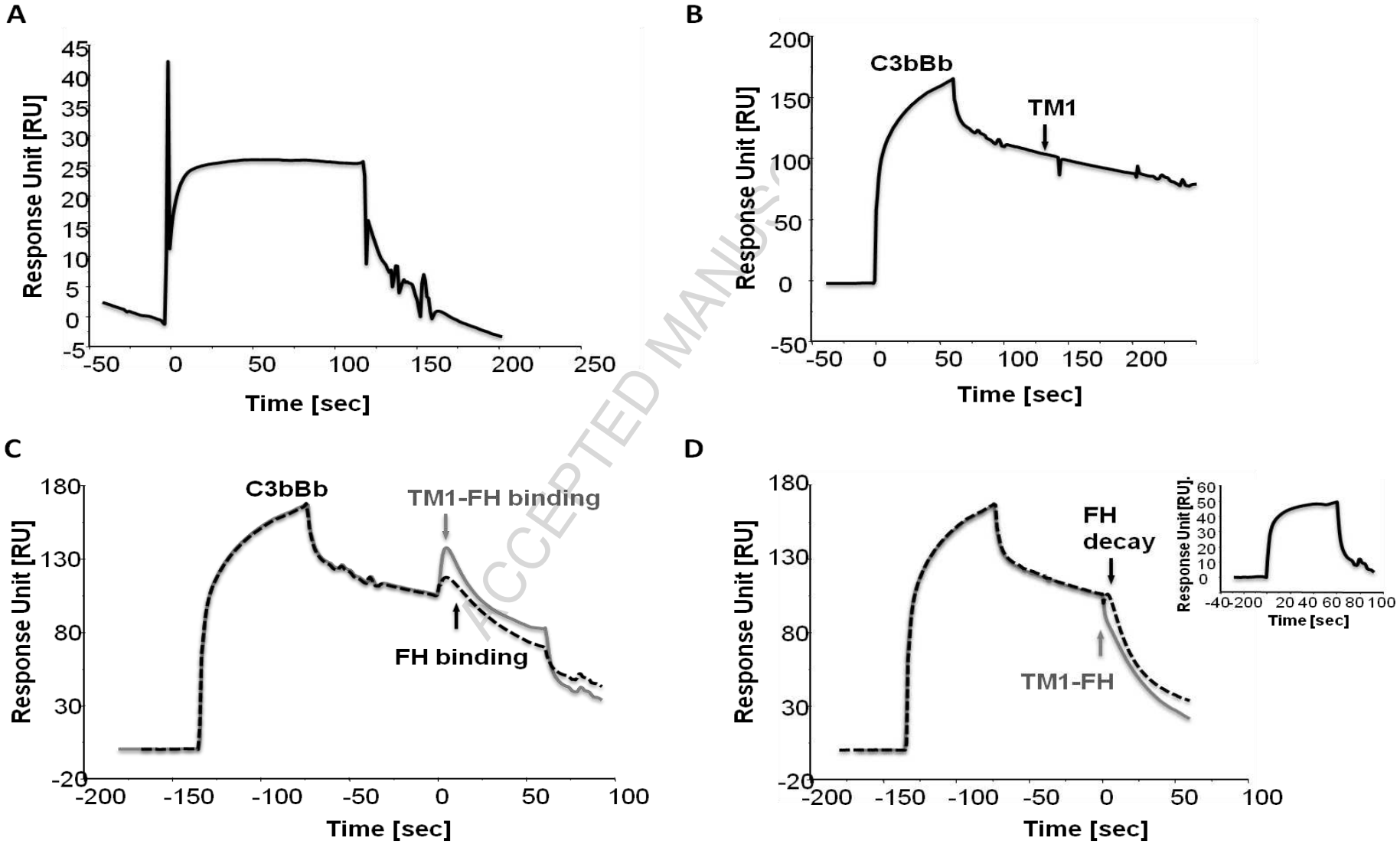
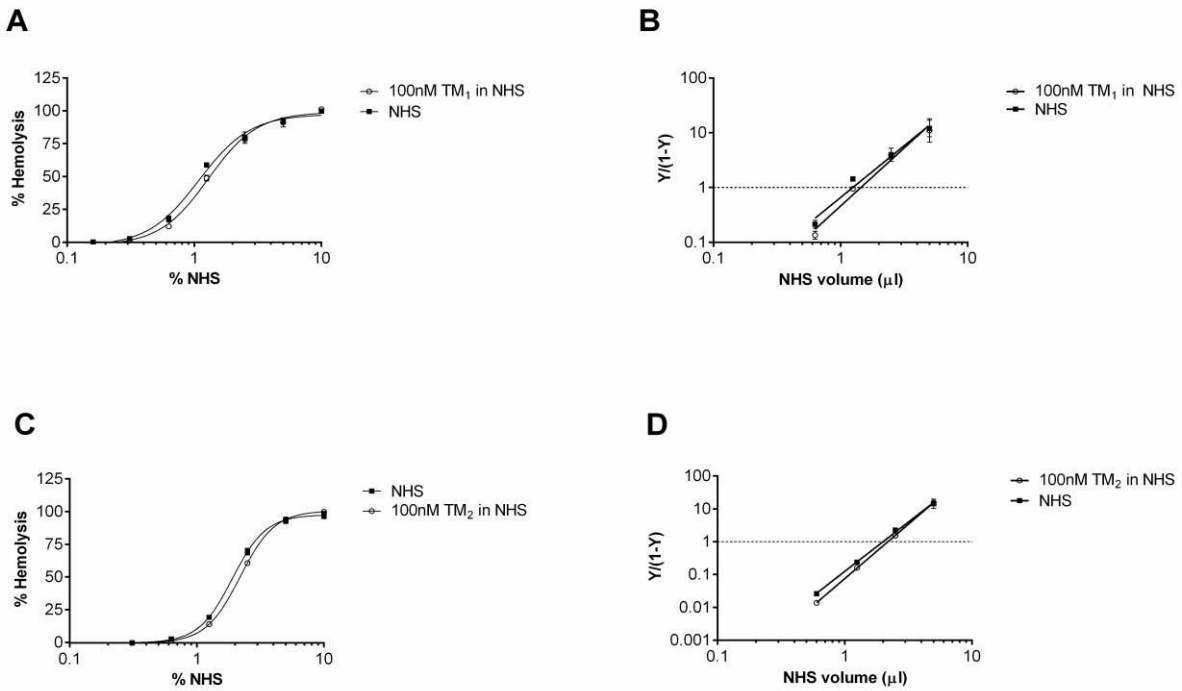


Figure 3



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	CH50 Thrombomodulin in NHS (n = 3)	CH50 NHS (n = 3)	fold difference	P value
TM1 (100nM)	1.53 +/- 0.043	1.25 +/- 0.05	1.22	**0.008**
TM1(2) (100nM)	1.69 +/- 0.05	1.46 +/- 0.086	1.16	**0.033**
TM2 (100nM)	1.75 +/- 0.06	1.48 +/- 0.02	1.18	**0.011**
TM2(2) (100nM)	2.19 +/- 0.03	1.94 +/- 0.04	1.13	**0.002**

Figure 4

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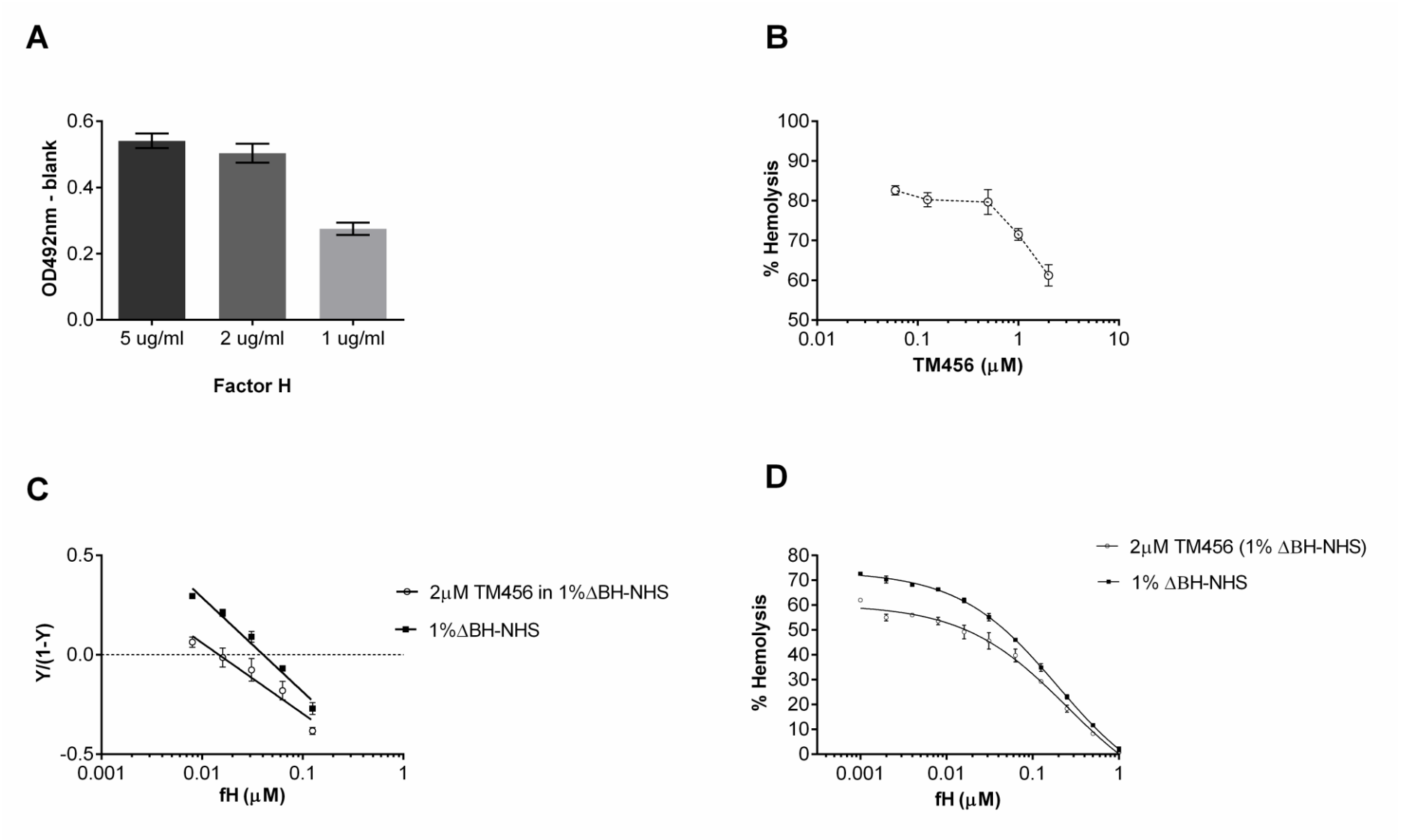

Figure 5

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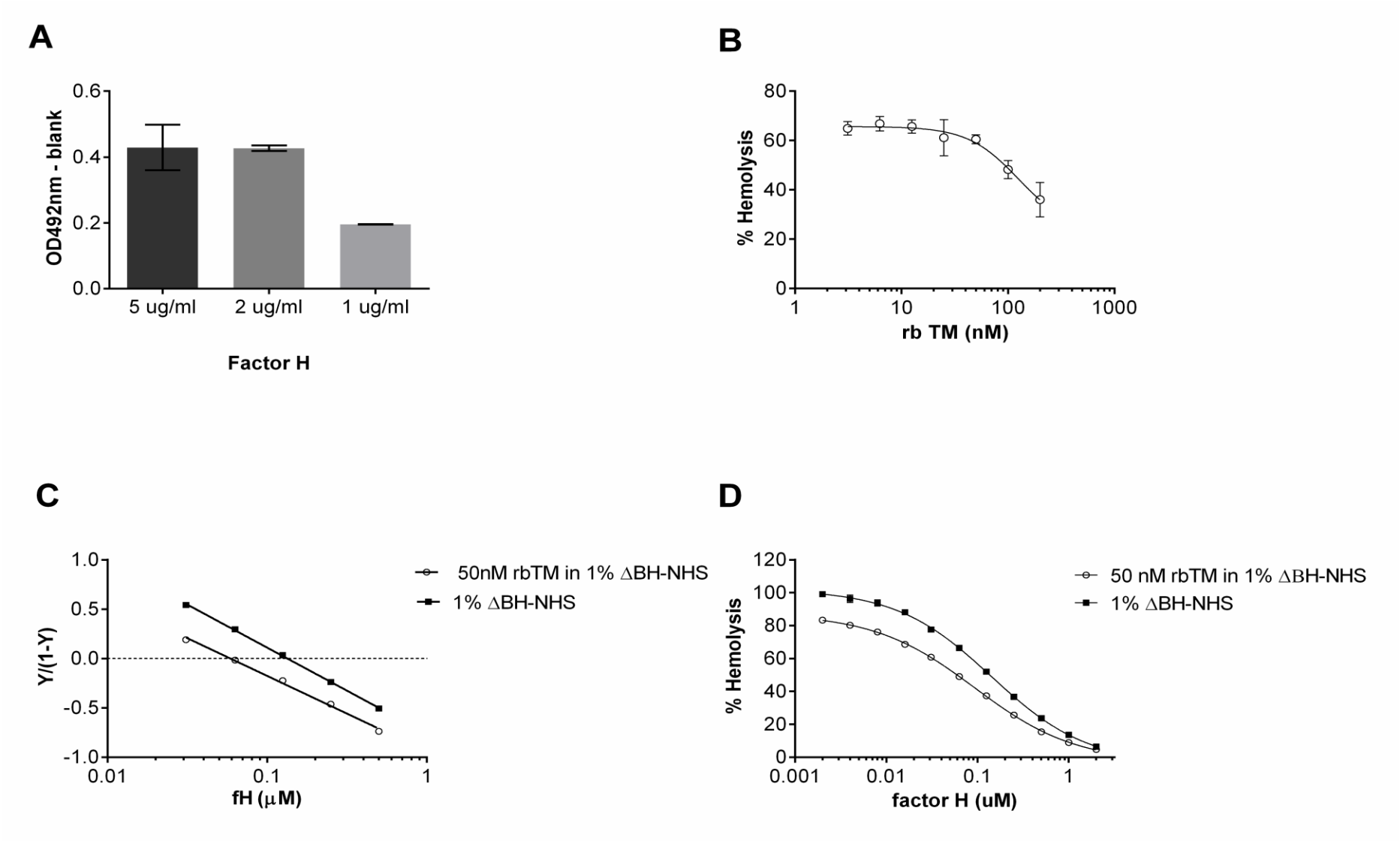

Figure 6