Supplemental Methods

Cell lines

Human umbilical vein endothelial cells (HUVECs) were purchased and maintained in complete EGM (Lonza, Walkersville, MD). Stably transfected Drosophila S2 cells were maintained in Schneider's complete medium (Thermo Fisher Scientific, Philadelphia, PA) with 25 μg/mL blasticidin (Thermo Fisher Scientific, Carlsbad, CA) and transitioned to serum free Insect-Xpress (Lonza, Walkersville, MD) supplemented with Glutamax and 0.8mM CuSO4 (Sigma Aldrich, St. Louis, MO) for recombinant protein expression. Chemically competent One Shot Top10 *E. coli* were used for subcloning as well as for production of scFvs using the pBAD/gIII periplasmic production system (Thermo Fisher Scientific, Carlsbad, CA).

Reagents

Human α-thrombin, human protein C, corn trypsin inhibitor (CTI), and blood collection tubes containing citrate and CTI were all purchased from Haematologic Technologies (Essex Junction, VT). Recombinant human TNF-α was purchased from Corning (Corning, NY). Anti-human CD141 (thrombomodulin) antibody (clone Phx-01) was purchased from BioLegend (San Diego, CA). Calcein AM and flourescent labeling reagents AlexaFluor 647-NHS Ester and AlexaFluor 488-TFP Ester were purchased from Thermo Fisher Scientific (Carlsbad, CA). Anti-human fibrin (clone 59D8) was purified from hybridoma supernatant using protein G and fluorescently labeled with AlexaFluor 568-NHS Ester (Thermo Fisher Scientific). Monoclonal antibodies BRIC256 (anti-GPA), BRAD2 (anti-RhD), BRAD3 (anti-RhD), FOG-1 (anti-RhD), BRIC14 (anti-Band3/Wrb), BIRMA84b (anti-Band3/Wrb), and BRIC200 (anti-Band3) were purchased from the International Blood Group Reference Laboratory (Bristol, England, UK). Antibody BRIC69 (anti-RhCE) was purchased from Thermo Fisher Scientific.

Recombinant protein expression and purification

pMT/hTM-aBand3, pMT/hTM-aRh17, and pMT/shTM were each co-transfected with pCoBLAST in *Drosophila* S2 cells and selected with blasticidin to generate stable cell lines. Expression and purification were performed as described previously[39], using a copper-induced promoter for secreted expression. Proteins harvested from culture supernatants were purified using an anti-FLAG (M2, Sigma, St Louis, MO) affinity resin. Purified proteins were assessed by SDS-PAGE and HPLC (Waters) using a size-exclusion column (Yarra, Phenomenex, Torrance, CA). HPLC was used to removed dimers from purified products when present. scFvs were produced using a pBAD/gIII vector production system (Thermo Fisher Scientific) for periplasmic secretion. Cultures of transformed E. Coli were induced with 0.02% arabinose and grown for at least 6 hours at room temperature. The periplasmic fraction was isolated by osmotic shock and the resulting shock fluid was purified on an L5 anti-FLAG column (Biolegend, San Diego, CA).

Binding assays

Recombinant proteins were radiolabeled with $Na^{125}I$ (Perkin Elmer, Exton, PA) using pre-formulated iodination reagent (Pierce Iodination Reagent, Thermo Fisher Scientific, Carlsbad, CA) per the manufacturer's protocol. Radiochemical purity was verified by instant thin layer chromatography on silica and was typically >95%. Radiolabeled proteins were added to human RBCs at 0.02% hematocrit in PBS with 2% human AB serum. Binding reached equilibrium over 4 hours at 37°C. After binding, cell suspensions were rapidly washed at least four times with cold PBS using multiwell filter plates (Multiscreen, EMD Millipore, Billerica, MA). The resulting cell pellet was counted using a Perkin Elmer Wizard2 gamma counting system. Dissociation kinetics of the fusion proteins was assessed using RBCs saturated with radiolabeled proteins, washing unbound ligands, and placing in dilute suspensions prior to measurement of free and bound ligand at specified time points. Similar binding experiments were performed with fluorescently-labeled recombinant proteins and cells were analyzed by flow cytometry (Accuri C6, BD Biosciences, San Jose, CA). Fluorescently labeled proteins were produced by reaction with amine-reactive derivatives of fluorescent dyes AlexaFlour488 and AlexaFlour647 (typically 10- to 20-fold excess at pH 8) and purified using 10,000 MWCO centrifugal filter devices (EMD Millipore, Billerica, MA).

Activated protein C assay

Generation of APC by TM proteins or TM coupled to RBCs was measured as described previously[39]. In brief, a given concentration of recombinant protein (1-20 nM) or fusion-loaded RBCs was suspended with 300 nM human protein C and 1 nM human alpha thrombin for 1 hour at 37°C. A portion of the reaction supernatant was then added to an excess of hirudin and 500 μM S-2366 chromogenic substrate. The absorbance was read kinetically at 405 nm with the slope of the linear portion of the resulting curve reflecting APC concentration.

Supplemental Tables

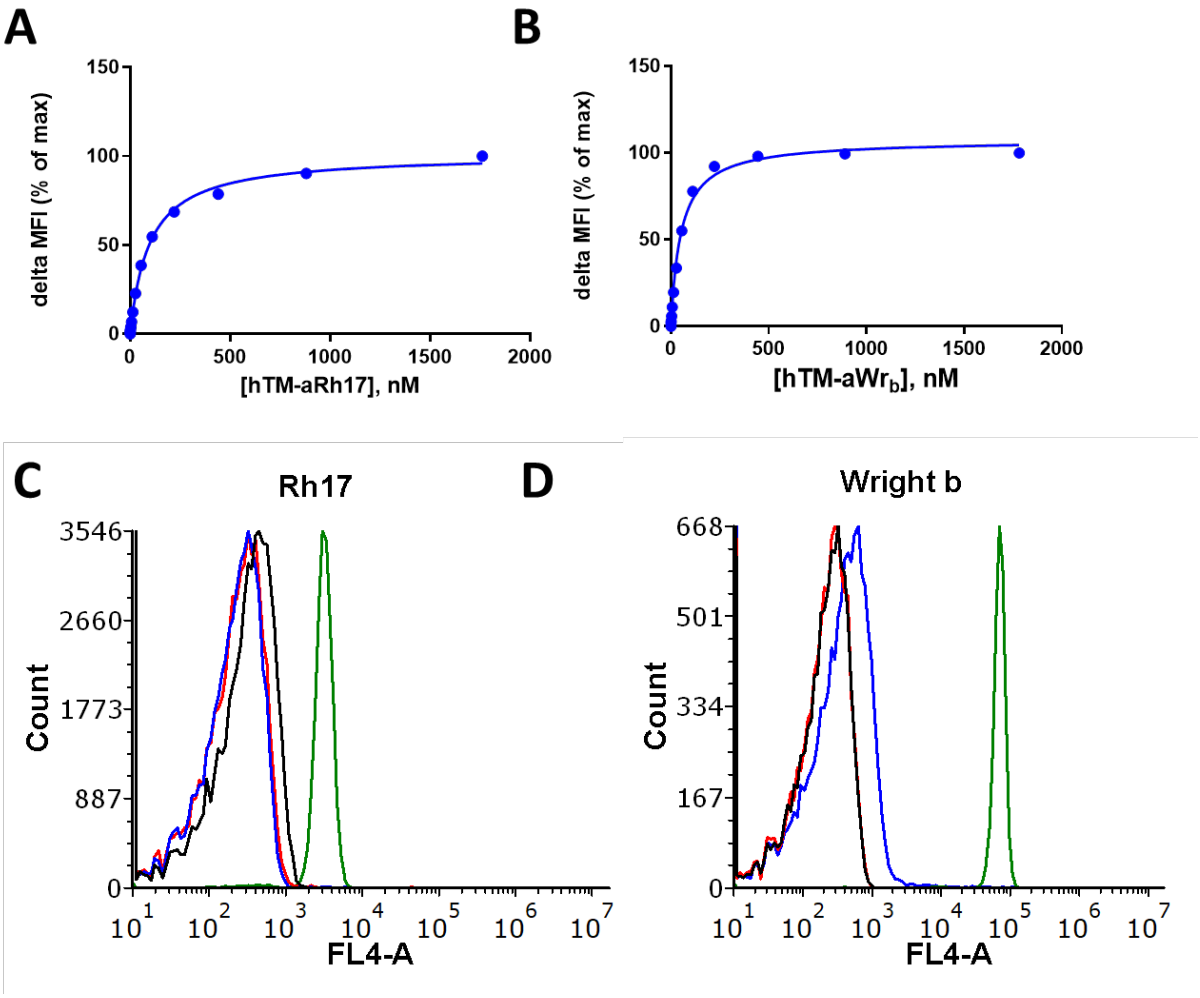
Clone#	Specificity	VH gene family	Vk gene family	VH sequence	VL sequence
KP3-17	**Rh17**	**4**	**1**	EVQLLESGPGLLKPSETLSLTCAVSGAPISNYW WSWIRQSPGKGLEWIGEIDGSIYTTYYNPSLKS RVAISKDTSKNRLSLKLTSVTAADTAVYYCAREG QNPLVPTYGSTGFGLDFWGHGLAVTVSS	AAELTQSPSSLSASVGDRVTITCQASQGISS WLAWYQQKPGKAPKLLIYKASSLQSGVPS RFSGSGSGTDFTLTISSLQSEDFATYYCQQY SSSPRTFGQGTKVEIK
KP2-23	**Wr^b**	**4**	**3**	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYG WNWIRQPPGKGLEWIGSIGGSRDNTNYNPSL KRRVTISKDTSKNQFSLKLKSVTAADTAVYYCA QRGAYGYSYFDYWGQGVLVAVSS	AAELTLTQSPATLSLSPGETATLSCRASQTV GRNLAWYQQRPGQAPNLLVHSAYFRATG IPDRFSGSGSGTDFTLTISSLEPEDAGVYHC QQYNDLLPLTFGGGTKVEIK

Supplemental Table 1 – Anti-Band3 and anti-RHCE antibody clones from phage library. scFv produced as H_2N-VH-$(GGGGS)_3$-VL-FLAGx3-COOH

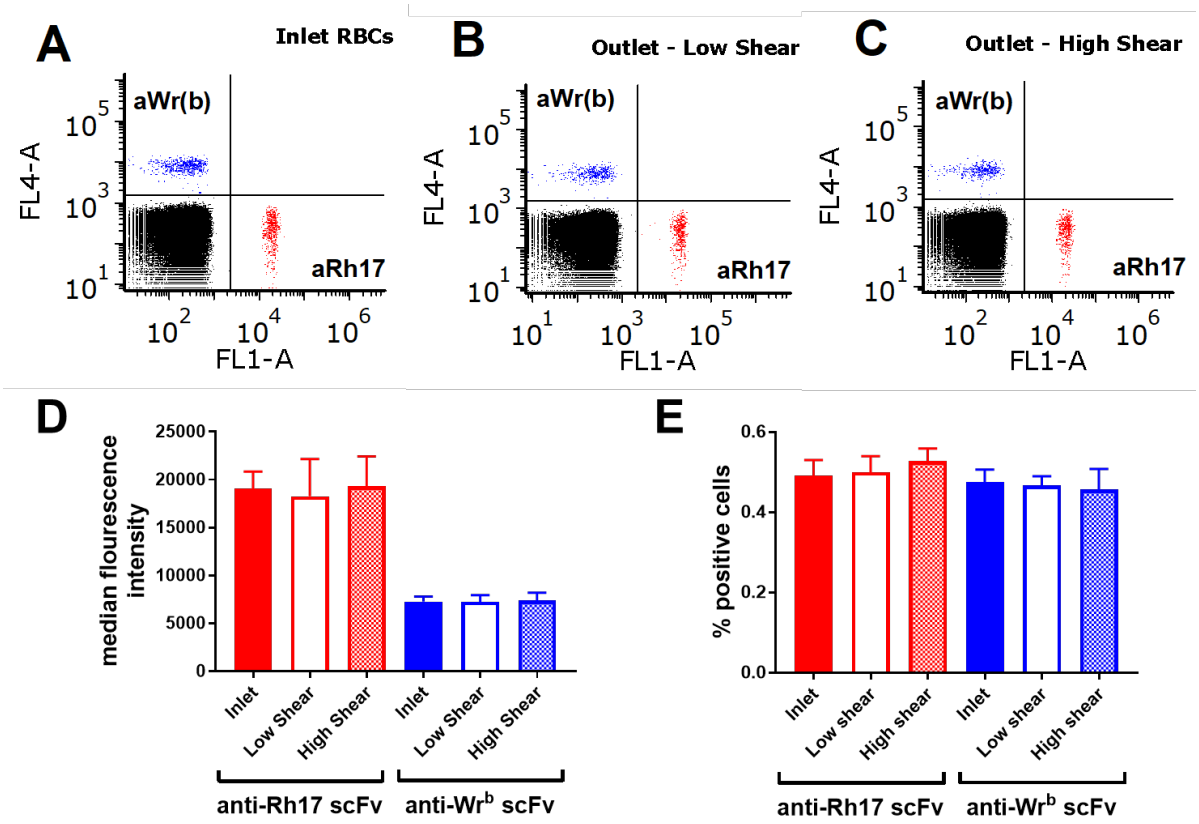
Protein	K_D (95% CI), nM	Bmax (95% CI), copies/RBC x10^3	k_{off} (95% CI), s^{-1}
aRh17 scFv (anti-RhCE)	41.4 (34.1, 50.2)	99 (93,105)	2.0x10^{-5} (1.6, 2.4)
aWr^b scFv (anti-Band3/GPA)	21.3 (17.0, 26.5)	746 (704,790)	2.9x10^{-5} (2.0, 3.8)
hTM-aRh17 (anti-RhCE)	45.6 (34.8, 56.5)	184 (173,195)	4.7x10^{-5} (3.2, 6.5)
hTM-aWr^b (anti-Band3/GPA)	52.6 (40.1, 65.1)	904 (848,961)	4.8x10^{-5} (2.9, 7.0)

S**upplement Table 2** – Binding parameters for radiolabeled anti-RBC ligands. A slight decrease in affinity and increase in k_{off} are seen for fusions in comparison to scFv alone.

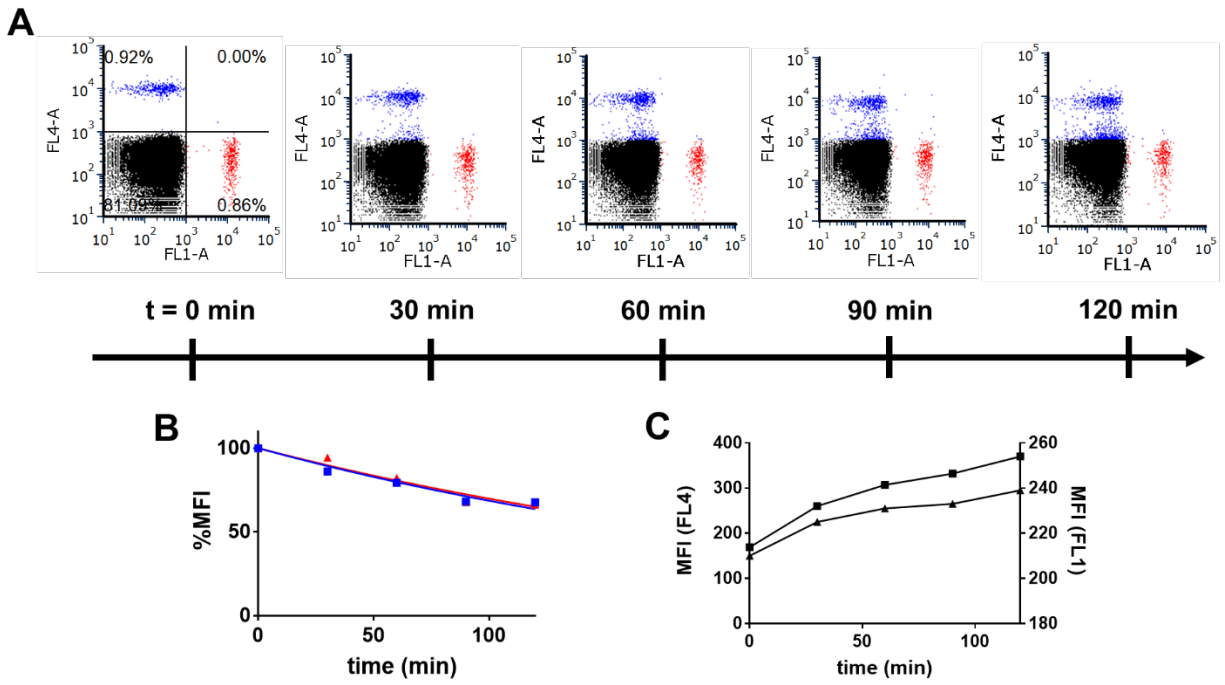
Supplemental Figures



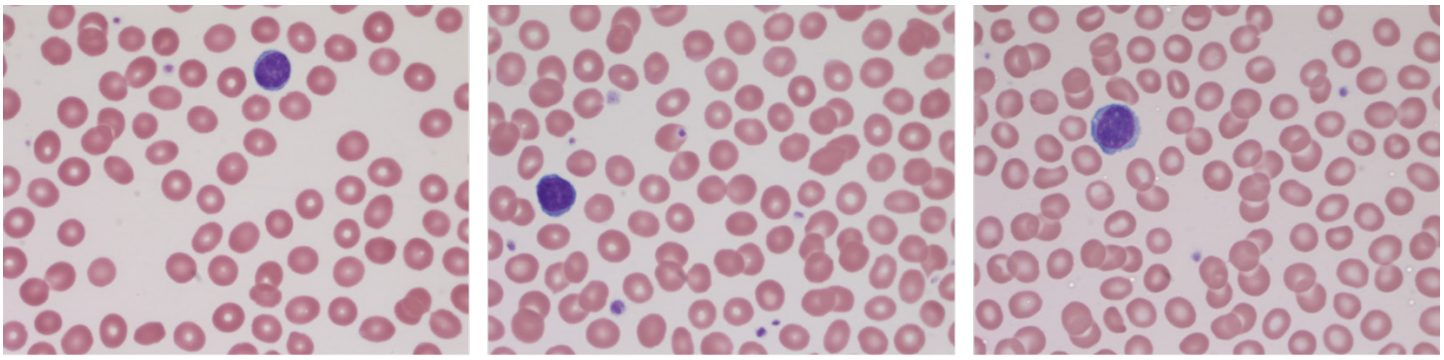
Supplementary Figure S1 – **Binding of fluorescent fusion proteins to RBCs measured by flow cytometry**. Representative binding curves for fluorescently labeled **(A)** hTM-aRh17 and **(B)** hTM-aWr^b fusions demonstrate similar binding parameters as radiolabeled fusions (representative of at least 3 repeated studies). Histograms for mouse(red), pig(blue), rat(black) and human(green) RBCs bound by fluorescently labeled fusion proteins demonstrate that both **(C)** hTM-Rh17 and **(D)** hTM-Wr^b bind to human and not mouse, rat, or pig RBCs.



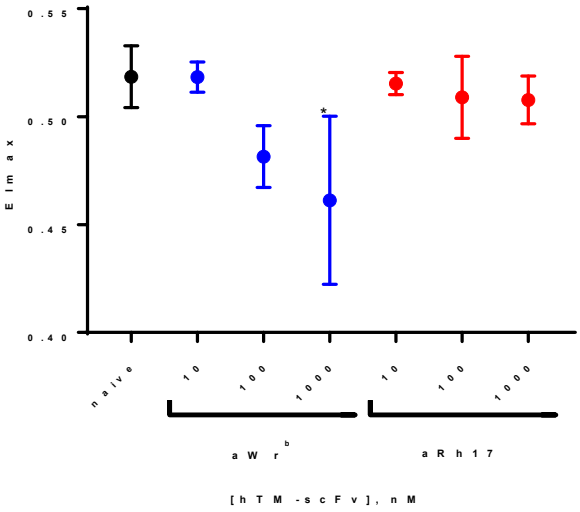
Supplemental Figure S2 – Binding of scFvs to RBCs is maintained after exposure to low (5 dyne/cm^2) and high (200 dyne/cm^2) shear stress flow. A fraction of washed, isolated human RBCs was treated with saturating concentrations of anti-Wr^b or anti-Rh17 scFv labeled with Alexa Flour 647 or Alexa Flour 488, respectively. The labeled RBCs were then added to fresh donor human whole blood (collected in citrate) at 0.5% of the total RBC population. The resulting blood was flowed through the Bioflux microfluidic device at either 5 dyne/cm^2 or 200 dyne/cm^2 and the (A) inlet and (B and C) outlet blood was analyzed by flow cytometry. The results demonstrate that (D) the labeled RBCs maintained the same fluorescence intensity as the inlet populations and (E) were present in equal proportion to the unlabeled RBCs



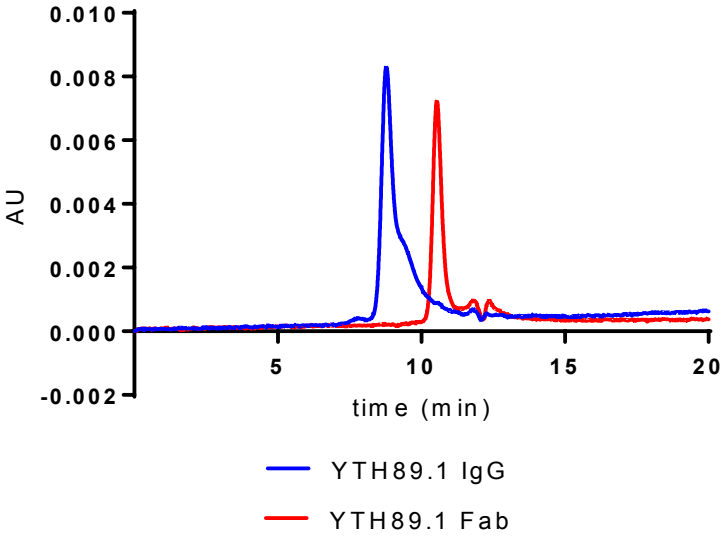
Supplemental Figure S3 – Dissociation and exchange of scFv from pre-treated RBCs onto naïve RBCs under constant mixing at 37°C. A fraction of washed, isolated human RBCs was treated with saturating concentrations of anti-Wr^b or anti-Rh17 scFv labeled with Alexa Flour 647 or Alexa Flour 488, respectively. The labeled RBCs were then added to fresh donor human whole blood (collected in citrate) at 0.5-1% of the total RBC population. This mixture was then incubated at 37°C under constant mixing by inversion. We observed (A) a gradual decrease in fluorescence intensity in the targeted RBCs, with >65% of fluorescence signal retained on the targeted RBCs at two hours. We quantified both the (B) dissociation of the scFvs and their (C) gradual rebinding to the naive population.



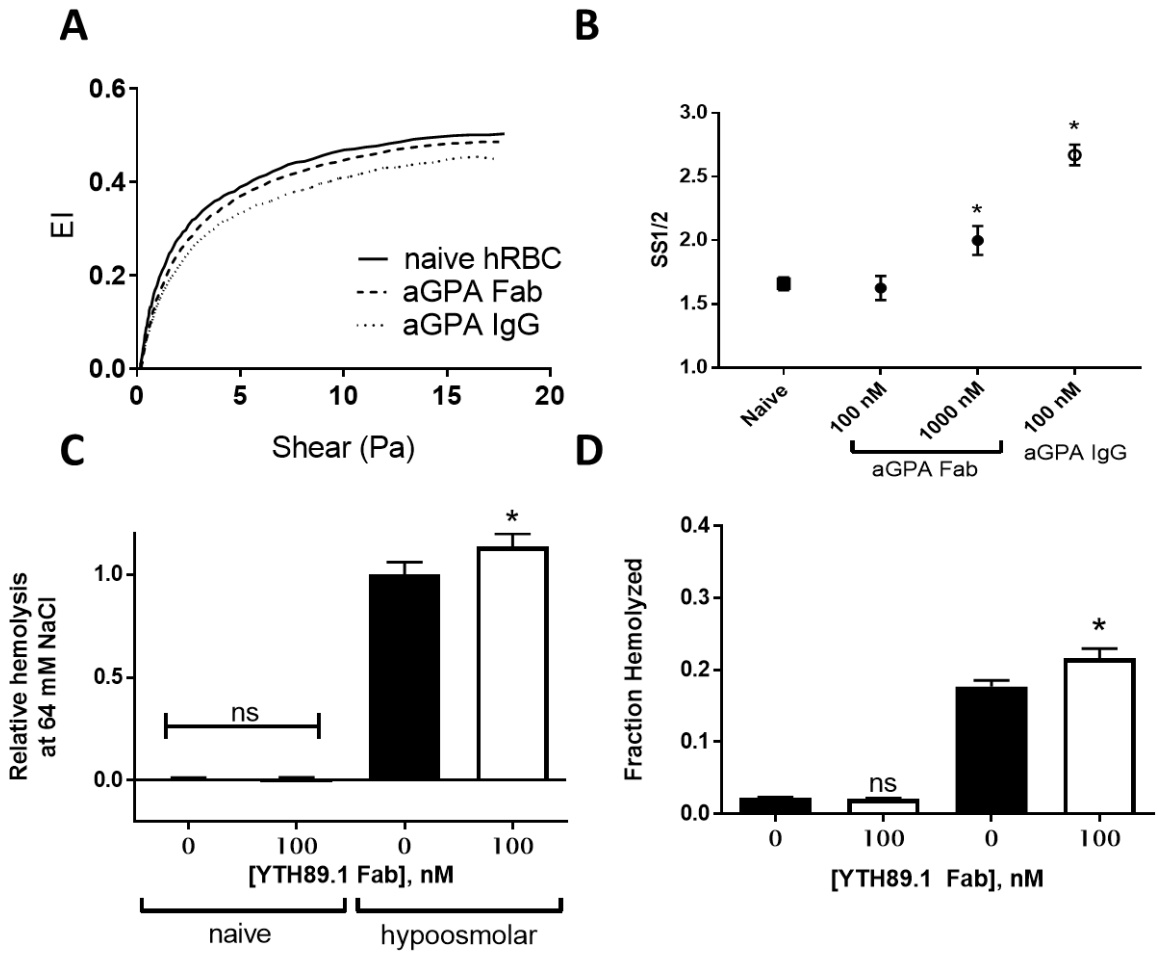
Supplementary Figure S4 – Wright-Giemsa stained blood smears of hTM-scFv treated RBCs. Whole blood was treated with 1 μM hTM-scFv and incubated for 1 hour prior to preparation of smears. At a normal hematocrit, this ratio is ~10^5 fusions/RBC. Slides were dried and stained with a commercial Wright-Giemsa stain (Sigma Aldrich) per package insert.



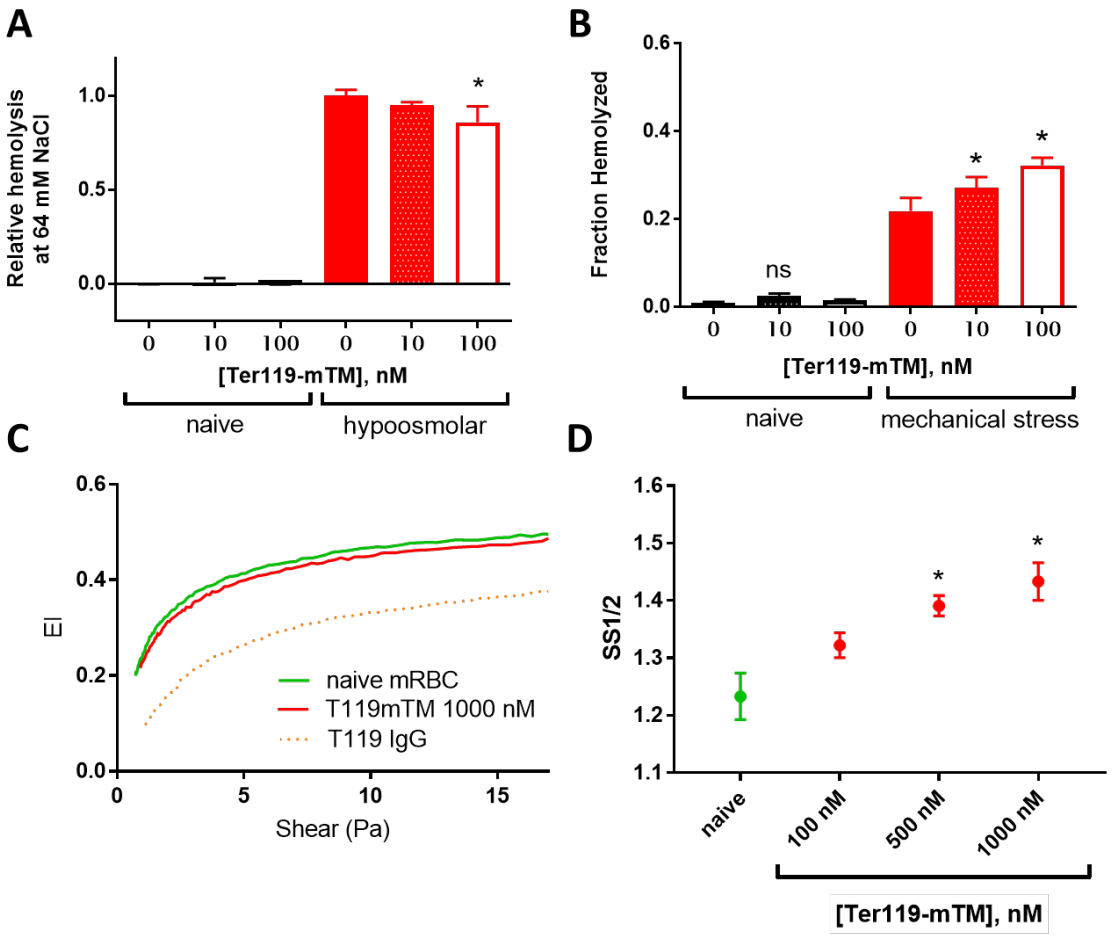
Supplementary Figure S5 – Maximum elongation index (EImax) of human RBCs treated with hTM-aWrb and hTM-aRh17 fusion proteins. Donor RBCs at 5% Hct were treated with the indicated concentration of fusion protein and measured in the ektacytometer. EImax calculated using non-linear regression (see Methods). Mean ± SD is shown (n=3-5 for each condition). (*$p<0.05$ vs naïve RBC, one-way ANOVA with Holm-Sidak correction for multiple comparisons)



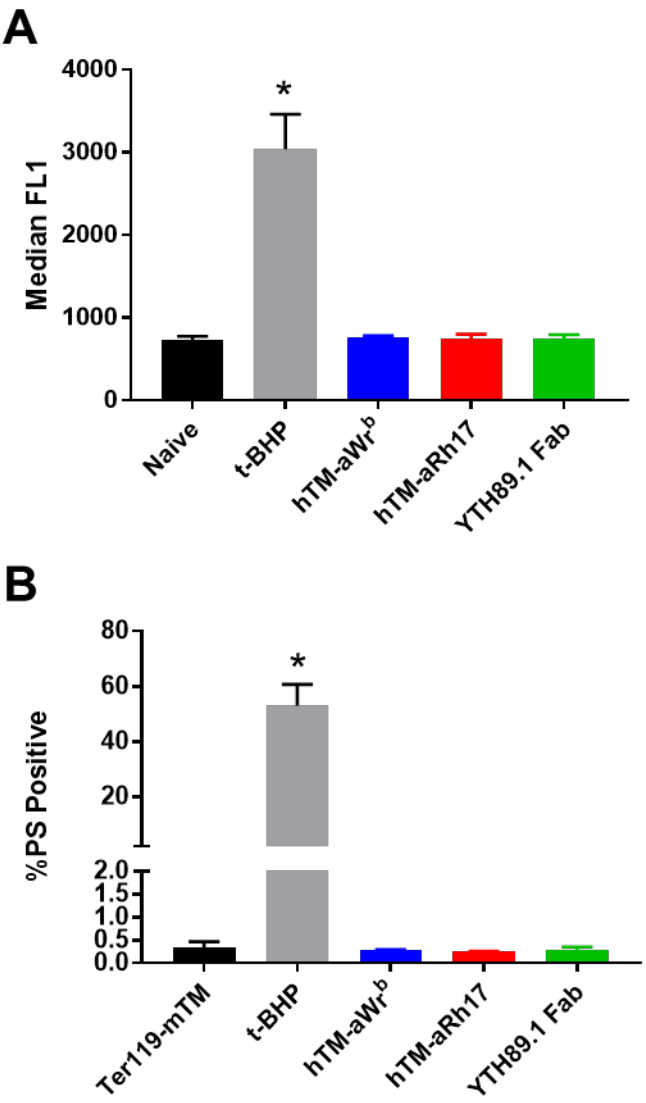
Supplemental Figure S6 – Size-exclusion HPLC of IgG and Fab antibodies against GPA. Antibodies prepared from hybridoma clone YTH89.1 which targets human glycophorin A. Full IgG was prepared from hybridoma supernatant using standard techniques and purified using protein G. Fab was prepared by enzymatic digestion of IgG with papain solution (Immucor) followed by treatment with protein A-sepharose (Thermo Fisher Scientific) for removal of Fc fragments and preparative size-exclusion HPLC for removal of residual papain enzyme. Representative HPLC from two independent antibody production runs.



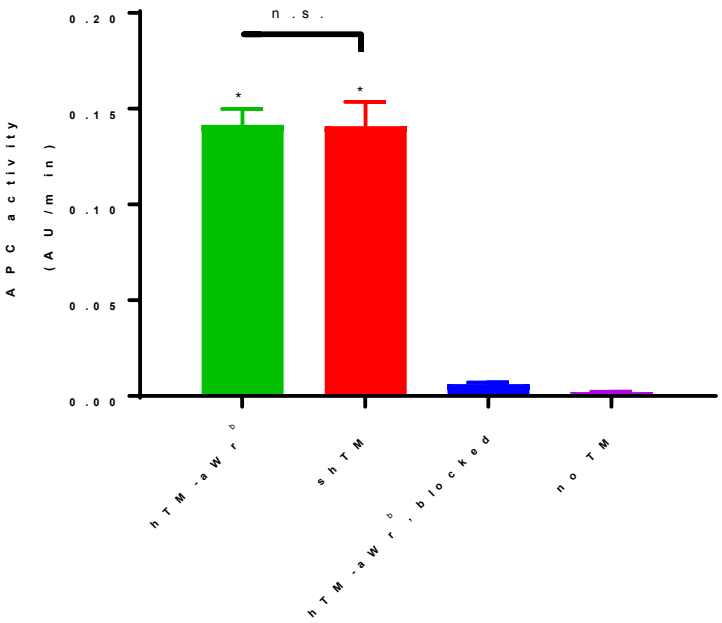
Supplementary Figure S7 – RBCs bound by ligands to human GPA also demonstrate slight increases in rigidity and changes in mechanical and osmotic resistance. (A) Representative ektacytometric curves of at least 3 studies of human RBCs treated with anti-GPA Fab and IgG, derived from antibody clone YTH89.1 demonstrate a rightward shift after antibody treatment (B) At high ligand loading, anti-GPA Fab induced a significant increase in SS1/2 while anti-GPA IgG (100 nM) more potently induced rigidification. Mean ± SD is shown, n=3 for each condition. (*p<0.05, one-way ANOVA with Holm-Sidak correction for multiple comparions) (C) Anti-GPA Fab induced increased hemolysis in response to hypo-osmolar stress and (D) slightly increased hemolysis in response to mechanical stress. Mean ± SD, n=3 is shown, representative of 2 independent experiments. (*p<0.05 vs naïve RBCs, one-way ANOVA with Holm-Sidak correction for multiple comparisons)



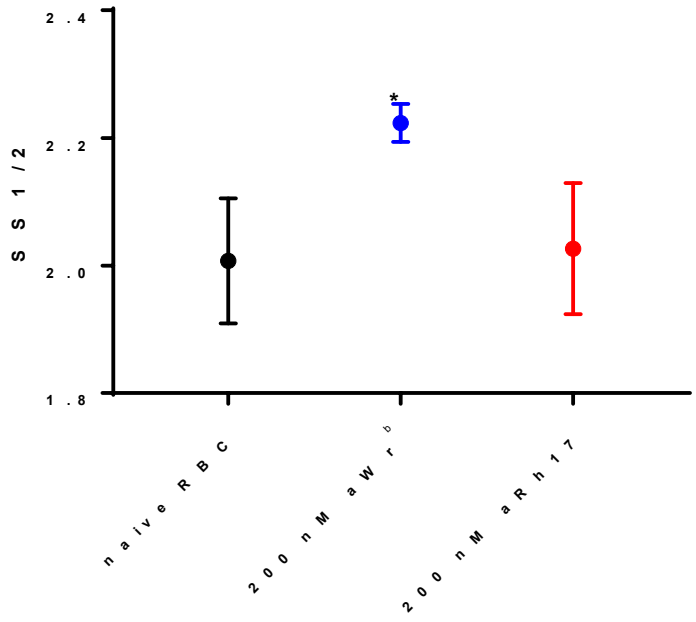
Supplementary Figure S8 – Ter119 ligands induce changes in murine RBCs similar to human RBCs treated with Wr^b ligands. Ter119-TM fusion proteins induce changes to (A) osmotic resistance and (B) mechanical resistance similar to aWr^b fusions in human RBCs. Mean ± SD is sown, n=3 for each condition. (*p<0.05 vs naïve RBCs, one-way ANOVA with Holm-Sidak correction for multiple comparisons) (C) Representative ektacytometric curves of at least 3 independent experiments showing that Ter119-TM (1000induced a slight rightward shift in ektacytometric curves, indicating increased RBC rigidity. The parent Ter119 IgG induced marked ektacytometric changes. (D) SS1/2 derived from ektacytometric curves demonstrates a significant, dose-dependent increase in SS1/2 with Ter119-TM treatment of murine RBCs. Mean ± SD is shown, n= 5-8 for each condition. (*p<0.05 vs naïve RBCs, one-way ANOVA with Holm-Sidak correction for multiple comparisons)



Supplementary Figure S9 – Human RBC ligands do not induce significant ROS generation or PS exposure. (A) No significant ROS generation was observed for cells treated with aWr^b, aRh17, or aGPA ligands. Human RBCs were preincubated with 5 µM dihydrorhodamine 123 (Thermo Fisher Scientific) at 1% hematocrit for 30 min at 37C, washed, then treated with either t-butyl hydrogenperoxide (10 µM) as a positive control or 100 nM of the indicated ligands for 1 hr at 37C. ROS generation was measured as median FL1 fluorescence and the mean ± SD are shown (n=4) **(B)** No significant PS exposure was observed for cells treated with aWr^b, aRh17, or aGPA ligands. Human RBCs were treated with 200 nM of the indicated ligands at 5% hematocrit (~2x10^5 ligands/RBC). Ter119-mTM was used as a non-binding negative control, and 2 mM t-butyl hydrogenperoxide was used as a positive control. Cells were treated at 37°C for 1 hour, washed, and resuspended in annexin V-Alexa Fluor 488 in annexin assay buffer (Thermo Fisher Scientific) per manufacturer protocol. Mean ± SD, n=3 is shown for each condition. (*$p<0.05$ vs non-binding control, one-way ANOVA with Holm-Sidak correction for multiple comparisons)



Supplementary Figure S10 – APC generation by fusion proteins (hTM-scFv). APC generation by fusion proteins in soluble phase (green) is similar to shTM alone (red). shTM or hTM-aBand3 (20 nM) were assayed by chromogenic methods. No significant APC generation was seen in the presence of excess anti-TM blocking antibody (Phx-01, blue) or without TM added (purple). Mean ± SD is shown (n=3). (*p<0.05 vs no TM, one-way ANOVA)



Supplementary Figure S11 – **aWr^{b} scFvs rigidify human RBCs in whole blood at 200 nM**. Whole blood treated with 200 nM aWr^{b} scFv shows significant rigidification (increased SS1/2) while treatment with 200 nM aRh17 scFv shows no change compared to naïve whole blood. Blood was treated at 37C for 1 hour prior to ektacytometry in 5.5% PVP solution. These ratios produce approximately 25,000 ligands per RBC. Mean±SD is shown, n=5-6, three donors tested. (*p<0.05 vs naïve RBC, one-way ANOVA with Holm-Sidak correction for multiple comparisons)

Supplementary Videos

Channels were treated with 10 ng/mL TNF-alpha for 6 hours under flow at 5 dyne/cm^2. The channels were then perfused with whole blood from normal donors that was collected in citrate/corn trypsin inhibitor, recalcified, and stained with anti-fibrin antibodies (red) and calcein AM (green, stains leukocytes and platelets). Fibrin generation and platelet/leukocyte adhesion were quantified as mean fluorescence intensity using image analysis software (ImageJ).

Video 1: duplicate channels treated with TNF, perfused with untreated whole blood

Video 2: duplicate channels treated with TNF, perfused with whole blood treated with 200 nM hTM-aRh17

Video 3: duplicate channels treated with TNF, perfused with whole blood treated with 200 nM hTM-aWr^b

Video 4: duplicate channels treated with TNF, perfused with whole blood treated with 200 nM shTM