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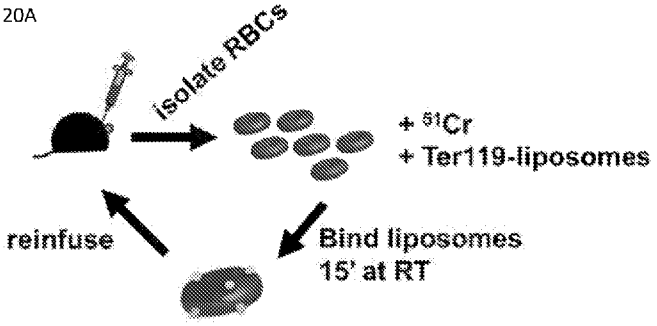
(54) Title: FUSION PROTEINS AND ANTIBODIES TARGETING HUMAN RED BLOOD CELL ANTIGENS

FIG 20A



(57) Abstract: Compositions and methods are provided for loading cargoes onto red blood cells. Provided herein are novel antibodies, fragments, fusion proteins and other conjugates which specifically bind red blood cells via RHCE or Band 3.

WO 2019/113224 A1

FUSION PROTEINS AND ANTIBODIES TARGETING HUMAN RED BLOOD CELL ANTIGENS

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED IN ELECTRONIC FORM

Applicant hereby incorporates by reference the Sequence Listing material filed in electronic form herewith. This file is labeled "17-8117PCT_Seq_Listing_ST25.txt".

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under NIH 5R01HL121134-03, NIH 5P01HL40387, and NIH 5T32HL007775-23 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Drug delivery by red blood cells (RBCs) was envisioned many decades ago[1-3] and the field has recently seen substantial growth [4-6], spurred by advances in drug loading within cells [7, 8] and coupling to the cell surface[9, 10], new technologies for genetic manipulation[11], and clinical successes in cellular therapeutics overall[12]. Furthermore, recent reports that carriage of drugs by RBCs can modulate immunogenicity, even inducing tolerance, expand the potential applications of RBC delivery[13-15]. Delivery by carrier RBCs enhances the pharmacokinetics and, in some cases, the pharmacodynamics of the loaded agents. RBC-encapsulated agents, including dexamethasone and L-asparaginase, have entered clinical trials.

Surface-coupling may offer some advantages with respect to clinical translatability, manufacturing, and bio-compatibility[16]. Animal studies demonstrated highly desirable features of surface-coupled anti-thrombotic and anti-inflammatory agents[10, 17-20]. For example, coupling of thrombomodulin (TM) to murine RBCs improves its efficacy in thrombotic[20], inflammatory, and ischemia-reperfusion injuries[21].

Previous reports have generally used fusion proteins, antibodies, and peptides to couple therapeutics to the surface of murine and porcine, but not human, RBCs. Fusion to murine RBCs is typically accomplished by derivatives of Ter119, an antibody to an epitope associated with glycophorin A (GPA)[22], or with ERY1 peptide, whose putative target is

also GPA[13]. While no overt adverse effects on RBCs have been noted when using these ligands, the effects of their binding to murine RBCs have not been characterized extensively[23].

The translational aspects of RBC delivery are challenging, as the considerable polymorphism of RBC antigenic determinants among species hinders any generalization of the effects of extracellular ligands to human RBCs. While we expect that surface-coupling is comparatively less-damaging than encapsulation methods (for example, hypotonic opening of membrane pores), careful and rigorous examination of affinity-coupling of bio-therapeutics to the surface of human red blood cells, assessment of their perturbation of red cell physiology, and subsequent demonstration of efficacy in humanized models, have not been reported.

It is known that RBC ligands, even monovalent, specifically targeted to GPA and Band 3, have the potential to cause undesirable alterations of RBC, including changes in deformability[24-28], exposure of phosphatidylserine (PS)[29], and generation of reactive oxygen species (ROS)[30] . These effects have been shown to vary even among epitopes within the same target protein. It is critical to examine these effects to identify the optimal RBC target for each therapeutic ligand, which should be erythroid specific, present in sufficient copy number for its therapeutic intent, be widely distributed among human populations, be non-immunogenic, and for most applications, not compromise RBC biocompatibility. Importantly, expression of three blood group systems is largely confined to erythropoiesis, GPA (MNS system), Band 3 (Diego system), and Rhesus family members (RhCE and RhD, Rh system)[34].

Therefore, antibodies and fusion proteins useful for targeting RBCs for drug delivery in subjects are needed.

SUMMARY OF THE INVENTION

The compositions and methods described herein relate to antibodies, fragments, fusion proteins and conjugates which specifically bind red blood cells, specifically via anti-RHCE or anti-Band 3. In one aspect, an antibody or fragment thereof comprising at least a VH or VL sequence as shown in Table 2 or Table 5 is provided, wherein said antibody or fragment thereof specifically binds an erythrocyte. In one embodiment, the antibody or

fragment comprises a VH and a VL sequence as shown in Table 2 or Table 5. In one embodiment, the antibody is an scFv.

In another aspect, compositions are provided in which any pharmacological, therapeutic, prophylactic, imaging or diagnostic agent which is coupled to, bound, fused, associated with or conjugated to an anti-RHCE or anti-Band 3 antibody described herein. In one embodiment, the cargo is a liposome.

In another aspect, a method for delivering an agent using red blood cells is provided. The method includes administering any of the compositions described herein to a subject in need thereof. In another embodiment, a method of prolonging circulation of an agent in the body is provided. The method includes administering any of the compositions described herein to a subject in need thereof. In another aspect, a method for preventing or reducing coagulation is provided. The method includes administering any of the compositions described herein to a subject in need thereof. In yet another aspect, a method of treating or preventing thrombosis, tissue ischemia, acute myocardial infarction (AMI), non-segmented elevated AMI, deep vein thrombosis, ischemic stroke, hyperoxic injury, transient ischemic attack (TIA), cerebrovascular disease, disseminated intravascular coagulation (DIC), pulmonary embolism, ischemic peripheral vascular disease, inflammation, pulmonary edema, sepsis, malaria, SDC, PNH, hemolytic anemia, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), a bleeding disorder such as hemophilia, or aseptic systemic inflammation is provided. The method includes administering any of the compositions described herein to a subject in need thereof.

Other aspects and advantages of the invention will be readily apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A – FIG. 1G provide characterization of aRh17 and aWrb ligands and their binding to human RBCs. Representative size exclusion HPLC analysis of (FIG. 1A) hTM-scFv fusions and (FIG. 1B) scFvs alone directed to Band3/GPA (aWrb, solid blue lines) and RhCE (aRh17, dashed red lines) demonstrates high purity of recombinant proteins and elution times consistent with theoretical molecular weights. Direct binding assays with radiolabeled proteins demonstrates high affinity and Bmax (Table 7) consistent with reported

copy number of the surface targets for both the (FIG. 1C) hTM-scFv fusions and the (FIG. 1D) scFv antibodies. No significant non-specific binding to control murine RBCs was seen. Representative data of 3 independent experiments are shown. Ligand dissociation studies demonstrated slow dissociation kinetics (>50% bound at 3 hours) for both the (FIG. 1E) TM-scFv fusions and (FIG. 1F) scFv antibodies. (FIG. 1G) Binding assay by hemagglutination techniques demonstrated that when anti-hTM IgG antibody (100 nM) was added to RBC pre-bound with the indicated concentration of hTM-scFv fusions, agglutination was observed when ~1000 copies of hTM would be expected on the surface. Representative data of 3 independent experiments are shown. No agglutination was seen with RBCs treated with either scFv or hTM-scFv alone or with mouse, rat, or pig RBCs treated with scFv or hTM-scFv followed by anti-hTM.

FIG. 2A – FIG. 2F show that aRh17 and aWrb antibodies demonstrate differential effects on RBC resistance to osmotic and mechanical stress. Osmotic stress was induced by incubation in buffered (10 mM sodium phosphate) saline at a range of osmolalities (0-308 mOsm). Mechanical stress was induced by rotation in the presence of glass beads at 1% Hct. Antibodies were added at 10 nM and 100 nM to 5% Hct RBC suspension, which produces a ratio of approximately 104 and 105 ligands per RBC and is below saturation for both target antigens. (FIG. 2A) RBCs treated with 500 nM aWrb scFv (blue) showed a left shift in the osmotic lysis curve compared to naïve (black) or aRh17 scFv treated RBCs (red). (FIG. 2B) RBCs treated with aWrb but not aRh17 showed a significant change in the concentration required for 50% hemolysis (128 vs 120 mOsm, n=3, *$p<0.05$ one-way ANOVA with Holm-Sidak correction for multiple comparisons) (FIG. 2C) aWrb scFv treated RBCs (blue) show a dose-dependent decrease in hemolysis in response to osmotic stress at 128 mOsm (EC50 for naïve RBCs) and (FIG. 2D) a dose-dependent increase in hemolysis in response to mechanical stress. aRh17 scFv treated RBCs (red) do not demonstrate any significant change in response to (FIG. 2E) osmotic stress or (FIG. 2F) mechanical stress. In all experiments means ± SD are shown, n = 3 for each condition. (*$p<0.05$ compared to naive, one-way ANOVA with Holm-Sidak correction for multiple comparisons).

FIG. 3A – FIG. 3D show that aRh17 and aWrb hTM-scFv fusion proteins demonstrate similar patterns of changes in RBC resistance to osmotic and mechanical stress as the parent scFv. Fusion proteins were added at 10 nM and 100 nM to 5% Hct RBC

suspension, which produces a ratio of approximately 104 and 105 fusion proteins per RBC and is below saturation for both target antigens. (FIG. 3A) aWrb hTM-scFv shows a dose-dependent decrease in hemolysis in response to osmotic stress and (FIG. 3B) a dose-dependent increase in hemolysis in response to mechanical stress. aRh17 hTM-scFv does not demonstrate any significant change in response to (FIG. 3C) osmotic stress or (FIG. 3D) mechanical stress. In all experiments means ± SD are shown, n = 3 for each condition. (*$p<0.05$ compared to naive, one-way ANOVA with Holm-Sidak correction for multiple comparisons)

FIG. 4A – FIG. 4D show that aWrb scFv and hTM-scFv increase RBC rigidity, while aRh17 scFv and hTM-scFv show no changes compared to naïve RBC. Ektacytometry was performed on 5% Hct RBC suspensions incubated with scFv or hTM-scFv at the indicated concentrations. Elongation index (as calculated automatically by the instrument) was read as a function of shear stress and non-linear regression was used to calculate the shear stress required for half-maximal deformation and the maximum elongation index. Representative curves of at least 3 independent experiments with different donors. (FIG. 4A) hTM-scFv fusions and (FIG. 4C) scFv antibodies targeted to Band3/GPA (aWrb blue dotted lines) demonstrated a rightward shift in the ektacytometry curves compared to naïve (solid line) while aRh17 fusions and scFv (red dashed lines) showed no change from naïve (scFvs and fusion proteins added at 1000 nM) (FIG. 4B and FIG. 4D) The shift in deformability was quantified as the SS1/2, which showed dose-dependent increases in response to Band3/GPA targeted ligands and not RhCE ligands. In (FIG. 4B) and (FIG. 4D), mean ± SD is shown, n=3-5 per condition. (*$p<0.05$ compared to naive, one-way ANOVA with Holm-Sidak correction for multiple comparisons)

FIG. 5A – FIG. 5F show that IgG antibodies against Band3 and GPA rigidify RBCs, while IgGs against RhCE and RhD do not. Representative ektacytometric curves (at least 3 separate donors studied per antibody) of RBCs treated with antibodies targeting (FIG. 5A) RhD or RhCE, (FIG. 5B) Band 3 or Wrb, or (FIG. 5C) GPA. A 5% suspension of RhD+ human RBCs in PBS was treated with 100 nM of the indicated antibody clones (~100,000 IgG/RBC). After incubation for 1 hour at 37°C, the red cell suspensions were read on an ektacytometer in 5.5% PVP. Legends indicate antibody clones. (FIG. 5D) Ektacytometric dose-response of anti-RhCE versus anti-Wrb IgG antibodies. Selected antibody clones

against RhCE (BRIC69, red) and Wrb (BRIC14, blue) were added at 100 nM to varying hematocrit RBC suspensions (2.5,5,10, and 20%, 6 donors tested) to result in ligand ratios of 25,000-100,000 IgG/RBC. aWrb demonstrated a significant increase in SS1/2 at all ligand loading ratios, while no significant difference was seen for aRhCE antibodies. Mean ± SD is shown, n=3-6 for each condition. (FIG. 5E) Flow cytometry on aRhCE (BRIC69, red) and aWrb (BRIC14, blue) IgG treated RBCs stained with AlexaFluor488 labeled anti-mouse secondary antibodies shows no significant difference in bound IgGs (based on median fluorescence) at the indicated loading ratios. (FIG. 5F) Representative histogram demonstrating similar antibody loading for RBCs treated with aRhCE (BRIC69, red) and aWrb (BRIC14, blue) antibodies.

FIG. 6A – FIG. 6D provide characterization of the activity of RBCs bound by hTM-scFv fusions and their therapeutic efficacy in a microfluidic model of inflammatory thrombosis (FIG. 6A) APC generation by RBCs loaded with hTM-scFv demonstrates a dose- and copy-number dependent response in APC generation as measured by chromogenic assay. hTM-aBand3 (circles) showed about 2-fold higher APC generation per RBC compared to hTM-aRhCE (triangles), although copy numbers are expected to 5- to 10-fold higher. Soluble hTM (shTM) treated RBCs are shown as a non-binding control (open squares) (FIG. 6B) Comparison of APC generative capacity of sTM versus hTM-scFv fusions (added at 50 nM) in a high hematocrit (20%) RBC suspension. Mean ± SD is shown, n=3 for each condition. (*$p<0.05$ vs sTM, one-way ANOVA with Holm-Sidak correction for multiple comparisons) A slight reduction in activity was seen for hTM-aBand3 but not hTM-aRhCE. (FIG. 6C) Fibrin generation on TNF-alpha activated, endothelialized microfluidic channels perfused with human whole blood preincubated with either PBS control (open squares), shTM control (crosses), hTM-Wrb (blue circles), or hTM-aRh17 (red triangles). Both fusion proteins (and shTM positive control) significantly reduced fibrin generation. (*$p<0.05$ vs untreated, one-way ANOVA with Holm-Sidak correction for multiple comparisons) as compared to the control channel. An increase in fibrin generation was noted toward the end of the observation period for the hTM-aWrb treated channels. (FIG. 6D) hTM-aRh17 treatment (red triangles) more effectively reduced platelet and leukocyte adhesion (quantified with calcein AM fluorescence) than hTM-aWrb (blue circles) versus untreated control (open squares). hTM-Rh17 treatment was similar to shTM positive control (crosses). For (FIG. 6C) and (FIG. 6D)

mean ± SEM for 2 independent channels is shown. (FIG. 6E) Representative composite images of whole blood (fibrin in red, platelets and leukocytes in green, brightfield image in gray) flowing through endothelialized channels at the end of the observation period (t=20 min). Fibrin is decreased in both fusion treated channels. An increase in platelet adhesion with associated fibrin (yellow, arrowhead) is seen in the hTM-aWrb treated channels compared to hTM-aRh17. Videos of the full time-course are not provided.

FIG. 7A – FIG. 7D show that binding of fluorescent fusion proteins to RBCs measured by flow cytometry. Representative binding curves for fluorescently labeled (FIG. 7A) hTM-aRh17 and (FIG. 7B) hTM-aWrb 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 (FIG. 7C) hTM-Rh17 and (FIG. 7D) hTM-Wrb bind to human and not mouse, rat, or pig RBCs.

FIG. 8A – FIG. 8E shows that binding of scFvs to RBCs is maintained after exposure to low (5 dyne/cm2) and high (200 dyne/cm2) shear stress flow. A fraction of washed, isolated human RBCs was treated with saturating concentrations of anti-Wrb 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/cm2 or 200 dyne/c2 and the (FIG. 8A) inlet and (FIG. 8B and FIG. 8C) outlet blood was analyzed by flow cytometry. The results demonstrate that (FIG. 8D) the labeled RBCs maintained the same fluorescence intensity as the inlet populations and (FIG. 8E) were present in equal proportion to the unlabeled RBCs.

FIG. 9A – FIG. 9C show that 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-Wrb 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 (FIG. 9A) 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 (FIG. 9B) dissociation of the scFvs and their (FIG. 9C) gradual rebinding to the naive population.

FIG. 10 shows 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 ~105 fusions/RBC. Slides were dried and stained with a commercial Wright-Giemsa stain (Sigma Aldrich) per package insert.

FIG. 11 provides 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. 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)

FIG. 12 shows 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.

FIG. 13 shows RBCs bound by ligands to human GPA also demonstrate slight increases in rigidity and changes in mechanical and osmotic resistance. (FIG. 13A) 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 (FIG. 13B) 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) (FIG. 13C) Anti-GPA Fab induced increased hemolysis in response to hypo-osmolar stress and (FIG. 13D) 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)

FIG. 14A – FIG. 14D show Ter119 ligands induce changes in murine RBCs similar to human RBCs treated with Wrb ligands. Ter119-TM fusion proteins induce changes to (FIG. 14A) osmotic resistance and (FIG. 14B) mechanical resistance similar to aWrb 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) (FIG. 14C) 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. (FIG. 14D) 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).

FIG. 15A – FIG. 15B show human RBC ligands do not induce significant ROS generation or PS exposure. (FIG. 15A) No significant ROS generation was observed for cells treated with aWrb, 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). (FIG. 15B) No significant PS exposure was observed for cells treated with aWrb, aRh17, or aGPA ligands. Human RBCs were treated with 200 nM of the indicated ligands at 5% hematocrit (~2x105 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)

FIG. 16 shows 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)

FIG. 17 shows aWrb scFvs rigidify human RBCs in whole blood at 200 nM. Whole blood treated with 200 nM aWrb 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).

FIG. 18 is a schematic of the hTM-aRHCE vector which includes a human thrombomodulin domain (hTM), and Rh17 VH and VL chains.

FIG. 19A – FIG. 19C demonstrate that RBC-targeted liposomes are maintained in circulation significantly longer than conventional 'stealth' liposomes. (FIG. 19A) Whole animal biodistribution of Ter119-liposomes (100-200 scFv:liposome) loaded onto RBCs in vivo by direct injection into the blood stream (blue) or unconjugated PEGylated liposomes (red). For in vivo loading liposomes were injected at a ratio of approximately 50 liposomes per RBC. (FIG. 19B) Blood PK curves demonstrate that the large majority of both in vivo loaded Ter119-liposomes (blue) are maintained in circulation at 3 hours and gradually drop off over 24 hours. Compared to traditional "stealth" liposomes (red), there is approximately a 2-fold increase in area under the curve (p<0.05) (FIG. 19C) Ter-119 liposomes are found mostly (>80%) in the RBC pellet of collected blood and gradually clear this compartment while free liposomes are largely in the plasma fraction.

FIG. 20A – FIG. 20B demonstrate circulation of ex vivo liposome loaded RBCs is dependent on the number of loaded nanocarriers. (FIG. 20A) Schema for ex vivo loading of RBCs with liposomes, 15 min of incubation typically resulted in >65% of liposomes bound (FIG. 20B) 30 min biodistribution of 51-Cr labeled RBCs loaded with either 200 or 20,000 liposomes per RBC demonstrates that while high loading leads to rapid clearance, low loading maintains near normal circulation of RBCs. For liposomes loaded ex vivo at a 200:1 ratio, PK data (up to 3 hours) were nearly identical to in vivo loading approaches.

FIG. 21A – FIG. 21B demonstrate effects of liposome binding on RBC agglutination and RBC membrane deformability. (FIG. 21A) High ratios of liposome loading on RBCs

leads to agglutination in vitro, while lesser ratios (<200:RBC) do not induce macroscopically detectable agglutination, as measured in a round-bottom well agglutination assay. Human RBCs shown as negative control. (FIG. 21B) Ektacytometry demonstrates that RBCs maintain normal membrane deformability at ratios up to 200 liposomes per RBC, above which dose-dependent rigidification of the membrane was observed

FIG. 22 demonstrates that Ter119IgG-liposomes are less stably retained on circulating RBCs and produce greater RBC rigidification than Ter119scFv-liposomes. (left panel) Mice were injected with radiolabeled liposomes conjugates with similar numbers (100-200/lipo) of either Ter119 IgG or Ter119 scFv. A higher percentage of Ter119-scFv liposomes remained in the RBC pellet, while Ter119-IgG was more rapidly cleared. (Middle and right panels) Ter119-IgG liposomes produced a higher degree of RBC membrane rigidification, as measured by ektacytometry (curves in middle panel, quantification in right panel, *$p<0.05$)

FIG. 23 shows that rigidification of RBC membranes by loaded liposomes is target dependent. Ektacytometry on human RBCs loaded with liposomes targeted to Wright(b) antigen (red) or RHCE (blue), compared to human RBCs mixed with untargeted liposomes (green). Liposomes loaded onto Wright(b) demonstrate significant rigidification while RHCE targeted liposomes preserve normal RBC deformability. Liposomes were targeted with IgGs to human RHCE (BRIC69) or Wright(b) (BRIC14).

FIG. 24A – FIG. 24B show that whole blood treated with aWrb scFv shows increased platelet adhesion in response to flow over TNF-α activated endothelium compared to blood treated with aRh17. (FIG. 24A) Representative image of endothelialized channels subjected to flow with either (top) aWrb scFv treated whole blood or (bottom) aRh17 scFv treated whole blood. Blood was collected in citrate with corn trypsin inhibitor, incubated with scFv (500 nM) for 15 minutes, recalcified, and flowed over channels for 15 min after which images were captured across the channels. Prior to flow, platelets and leukocytes were stained by addition of calcein AM dye. (FIG. 24B) Quantification of the experiments in panel A (mean fluorescence intensity) demonstrate a significant increase in calcein AM signal in the aWrb scFv treated blood but not aRh17 scFv ($n = 4$, *$p<0.05$, one-way ANOVA).

FIG. 25 is a Western blot that demonstrates that anti-Rh17 recognizes a linear epitope in human RhCE. A Western blot was performed to assess the binding of KP3-17 (anti-Rh17) to proteins extracted from mouse and human erythrocyte ghosts. Because proteins were

denatured in reducing SDS-PAGE buffer prior to gel electrophoresis, the presence of binding is due to interaction with linear, and not conformational, epitopes. This is in contrast to anti-RhCE mAbs described by other groups, which recognize conformational epitopes.

FIG. 26 is a bar graph which demonstrates that KP3-17 (anti-Rh17) recognizes an epitope present in the 6th extracellular loop of human RhCE. Flow cytometry was used to assess the binding of anti-Rh17 to human erythrocytes in the presence and absence of linear peptides corresponding to the amino acid sequence 6th extracellular loop of human RhD (negative control) and human RhCE. A decrease in binding signal only in the presence of the RhCE-derived peptide demonstrates that the 6th extracellular loop of RhCE is involved in the binding of Rh17 to human erythrocytes. (* denotes $p<0.05$ by 1-way ANOVA with Tukey's post-hoc test).

FIG. 27 is a 3D model of human RHCE (looking top down onto a membrane), with 6^{th} extracellular loop boxed.

DETAILED DESCRIPTION OF THE INVENTION

Carriage of drugs by red blood cells (RBCs) enhances pharmacokinetics and pharmacodynamics, modulates immune responses, and is approaching clinical translation. The effects of attaching therapeutics to human RBCs have not been well defined and optimal RBC surface determinants have not been identified. As described herein, non-human-primate single chain antibodies (scFv) directed to human RBCs were engineered and fused with human thrombomodulin (hTM) as a representative therapeutic cargo (hTM-scFv). Binding these fusions to RBC determinants Band3 (Wrb) and RHCE (Rh17) endowed RBC with hTM activity, but differed in their effect on RBC physiology and specific activity. scFv and hTM-scFv targeted to Band3 increased membrane rigidity, sensitized RBCs to hemolysis induced by mechanical stress, and decreased hypo-osmotic hemolysis. Similar trends were seen for monovalent ligands bound to glycophorin A (GPA) on human and murine RBCs. In contrast, binding of scFv and hTM-scFv to RHCE did not alter RBC deformability or sensitivity to mechanical and osmotic stress at similar copy numbers per RBC. Although RBC-bound hTM-scFv fusions all generated APC in the presence of thrombin, RHCE-bound TM demonstrated superior specific activity. Both fusion proteins were efficacious in endothelialized microfluidic models of inflammatory thrombosis in human whole blood

wherein they significantly decreased fibrin deposition in response to TNF-alpha activation, but RHCE-bound hTM-scFv more effectively reduced platelet and leukocyte adhesion.

As used herein, the term "subject" means a mammalian animal, including a human, a veterinary or farm animal, a domestic animal or pet, and animals normally used for clinical research. In one embodiment, the subject of these methods and compositions is a human. Still other suitable subjects include, without limitation, murine, rat, canine, feline, porcine, bovine, ovine, non-human primate and others. As used herein, the term "subject" is used interchangeably with "patient".

The term “immunoglobulin” or “antibody” is used herein to include antibodies, including functional fragments thereof. As used herein, the term antibody includes scFvs. As used herein, the term antibody also includes FABs, single domain antibodies, heavy chain antibodies (camelids), DARTs, F(ab’)2, BITEs, and immunoadhesins. These antibody fragments or artificial constructs may include a single chain antibody, a Fab fragment, a univalent antibody, a bivalent of multivalent antibody, or an immunoadhesin. An scFv is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids. The antibody may also be a monoclonal antibody, a “humanized” antibody, a multivalent antibody, or another suitable construct. An “immunoglobulin molecule” is a protein containing the immunologically-active portions of an immunoglobulin heavy chain and immunoglobulin light chain covalently coupled together and capable of specifically combining with an antigen. Immunoglobulin molecules are of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass. The terms “antibody” and “immunoglobulin” may be used interchangeably herein. An “immunoglobulin heavy chain” is a polypeptide that contains at least a portion of the antigen binding domain of an immunoglobulin and at least a portion of a variable region of an immunoglobulin heavy chain. Thus, the immunoglobulin derived heavy chain has significant regions of amino acid sequence homology with a member of the immunoglobulin gene superfamily. For example, the heavy chain in a Fab fragment is an immunoglobulin-derived heavy chain. An “immunoglobulin light chain” is a polypeptide that contains at least a portion of the antigen binding domain of an immunoglobulin and at least a portion of the variable region. Thus, the immunoglobulin-derived light chain has significant regions of

amino acid homology with a member of the immunoglobulin gene superfamily. An "immunoadhesin" is a chimeric, antibody-like molecule that combines the functional domain of a binding protein, usually a receptor, ligand, cell-adhesion molecule, or 1-2 immunoglobulin variable domains with immunoglobulin constant domains, usually including the hinge or GS linker and Fc regions. A "fragment antigen-binding" (Fab) fragment" is a region on an antibody that binds to antigens. It is composed of one constant and one variable domain of each of the heavy and the light chain. With respect to immunoglobulins or antibodies as described herein, each fragment of an immunoglobulin coding sequence may be derived from one or more sources, or synthesized. Suitable fragments may include the coding region for one or more of, e.g., a heavy chain, a light chain, and/or fragments thereof such as the constant or variable region of a heavy chain (CH1, CH2 and/or CH3) and/or or the constant or variable region of a light chain. Alternatively, variable regions of a heavy chain or light chain may be utilized. Where appropriate, these sequences may be modified from the "native" sequences from which they are derived, as described herein.

Antibodies may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, camelid heavy chain only (V_HH) antibodies, intracellular antibodies ("intrabodies"), recombinant antibodies, multispecific antibody, antibody fragments, such as, Fv, Fab, $F(ab)_2$, $F(ab)_3$, Fab', Fab'-SH, $F(ab')_2$, single chain variable fragment antibodies (scFv), tandem/bis-scFv, Fc, pFc', scFvFc (or scFv-Fc), disulfide Fv (dsfv), bispecific antibodies (bc-scFv) such as BiTE antibodies; humanized camelid antibodies, resurfaced antibodies, humanized antibodies, shark antibodies, fully human antibodies, single-domain antibody (sdAb, also known as NANOBODY®), chimeric antibodies, chimeric antibodies comprising at least one human constant region, and the like. "Antibody fragment" refers to at least a portion of the variable region of the immunoglobulin that binds to its target, *e.g.*, the RHCE protein. In one embodiment, the antibody referred to herein is an scFv.

The term "heterologous" when used with reference to a protein or a nucleic acid indicates that the protein or the nucleic acid comprises two or more sequences or subsequences which are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid. For example, in one embodiment, the nucleic

acid has a promoter from one gene arranged to direct the expression of a coding sequence from a different gene. Thus, with reference to the coding sequence, the promoter is heterologous. With regard to the antibodies described herein, in one embodiment the constant regions of the heavy and/or light chain are from a different source (e.g., different clone) than the variable regions of the heavy and/or light chain. Thus, with reference to each other, said constant and variable regions are heterologous, or said heavy and light chains are heterologous. The different sources may be from the same species or different species.

As used herein, a "vector" or "plasmid" refers to a nucleic acid molecule which comprises an immunoglobulin coding sequence (*e.g.,* an immunoglobulin VH or VL or another fragment of an immunoglobulin construct, or combinations thereof), promoter, and may include other regulatory sequences therefor, which plasmid or vector may be delivered to a host cell, wherein said coding sequence is expressed recombinantly.

In one embodiment, the "linker" refers to any moiety used to attach or associate the antibody to the cargo. Thus, in one embodiment, the linker is a covalent bond. In another embodiment, the linker is a non-covalent bond. In another embodiment the linker is composed of at least one to about 25 atoms. Thus, in various embodiments, the linker is formed of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 atoms. In still another embodiment, the linker is at least one to about 60 nucleic acids. Thus in various embodiments, the linker is formed of a sequence of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, up to 60 nucleic acids. In yet another embodiment, the linker refers to at least one to about 30 amino acids. Thus in various embodiments, the linker is formed of a sequence of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, up to about 30 amino acids. In still other embodiments, the linker can be a larger compound or two or more compounds that associate covalently or non-covalently. In still other embodiment, the linker can be a combination of the linkers defined herein. The linkers used in the constructs of the compositions and methods are in one embodiment cleavable. The linkers used in the constructs of the compositions and methods are in one embodiment non-cleavable. Without limitation, in one embodiment, the linker is a disulfide bond. In the examples below, the exemplified linker comprises a complex of biotin bound to the construct

oligonucleotide sequence by a disulfide bond, with streptavidin fused to the ligand. In another embodiment, the biotin is bound to the ligand and the streptavidin is fused to the construct oligonucleotide sequence.

Antibodies

As described herein, antibodies and antibody fragments which specifically bind erythrocytes are provided. Antibodies and single chain antibody fragments (scFv) against epitopes on Band 3 protein (Wr^b) and RHCE protein ($Rh17/Hr_0$) on human erythrocytes are described herein. These antibodies and fragments were generated using phage display libraries prepared from immunized cynamolgous macaques (*Macaca fascicularis*). Both antigens are present on RBCs from nearly 100% of the human population and are considered relatively erythroid specific[31, 32].

RHCE

The Rh blood group system is the second most clinically significant of the blood groups, second only to ABO. It is also the most polymorphic of the blood groups, with variations due to deletions, gene conversions, and missense mutations. The Rh blood group includes this gene which encodes both the RhC and RhE antigens on a single polypeptide (RHCE) and a second gene which encodes the RhD protein. The classification of Rh-positive and Rh-negative individuals is determined by the presence or absence of the highly immunogenic RhD protein on the surface of erythrocytes. A mutation in this gene results in amorph-type Rh-null disease. Alternative splicing of this gene results in multiple transcript variants encoding several different isoforms.

As used herein, "RHCE" refers to the above-described polypeptide, including all isoforms thereof (UniProtKB- P18577). The "canonical" sequence can be found under Uniprot Identifier: P18577-1, also called isoform 1 or RHI, and is shown below and as SEQ ID NO: 366.

```
        10         20         30         40         50
MSSKYPRSVR RCLPLWALTL EAALILLFYF FTHYDASLED QKGLVASYQV
        60         70         80         90        100
GQDLTVMAAL GLGFLTSNFR RHSWSSVAFN LFMLALGVQW AILLDGFLSQ
       110        120        130        140        150
FPPGKVVITL FSIRLATMSA MSVLISAGAV LGKVNLAQLV VMVLVEVTAL
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       160        170        180        190        200
GTLRMVISNI FNTDYHMNLR HFYVFAAYFG LTVAWCLPKP LPKGTEDNDQ
       210        220        230        240        250
RATIPSLSAM LGALFLWMFW PSVNSPLLRS PIQRKNAMFN TYYALAVSVV
       260        270        280        290        300
TAISGSSLAH PQRKISMTYV HSAVLAGGVA VGTSCHLIPS PWLAMVLGLV
       310        320        330        340        350
AGLISIGGAK CLPVCCNRVL GIHHISVMHS IFSLLGLLGE ITYIVLLVLH
       360        370        380        390        400
TVWNGNGMIG FQVLLSIGEL SLAIVIALTS GLLTGLLLNL KIWKAPHVAK
       410
YFDDQVFWKF PHLAVGF
```

Provided herein are antibodies which bind to one or more antigens on the RHCE polypeptide. Specifically, the antibodies are reactive against Rh17 (Hro). RH17 is an antigen present on all red blood cells having the common Rh phenotypes, except D-- and Rh null RBCs. Because RBC lacking the rh17 antigen are extremely rare, antibodies against rh17 specifically bind to virtually all erythrocytes. Anti-rh17 antibodies are believed to bind to extracellular loops present in RHCE but not RHD. As is shown herein, the antibody termed KP3-17 (anti-Rh17) recognizes a linear epitope on human, but not mouse RBC (FIG. 25). A model showing the 6th extracellular loop can be seen in FIG. 27. It is believed the anti-rh17 antibodies described herein bind to all or a portion comprising at least 5 consecutive amino acids of SEQ ID NO: 361. In one embodiment, the epitope is HTVWN (SEQ ID NO: 365). In one embodiment, at least 100,000 copies of the epitope to which the subject antibody binds, are present on the erythrocyte.

In another embodiment, an antibody is provided which competes for the binding site of the anti-rh17 antibody.

In one embodiment, the antibodies described herein comprise one or more anti-rh17 antibody CDR sequence. Suitable CDR sequences are shown below in Table 1. In one embodiment, the CDRs from a single clone are used to produce an antibody or antibody fragment, e.g., CDR1, CDR2 and CDR3 from KP3-11, KP3-14 or KP3-17. In another embodiment, the CDRs from one or more clone are used to produce an antibody. As a non-limiting illustrative example, CDR1 from clone KP3-11 and CDR2 and 3 from clone KP3-14 are used in conjunction to produce an antibody. In another embodiment, the VH CDRs from one clone are use with the VL CDRs from another clone. In another embodiment, the CDRs described herein are utilized with heterologous antibody sequences to produce a chimeric antibody. In one embodiment, the antibody comprises 1 CDR sequence selected from SEQ

ID Nos 1-18. In another embodiment, the antibody comprises two CDR sequences selected from SEQ ID Nos 1-18. In another embodiment, the antibody comprises three CDR sequences selected from SEQ ID Nos 1-18. In another embodiment, the antibody comprises four CDR sequences selected from SEQ ID Nos 1-18. In another embodiment, the antibody comprises five CDR sequences selected from SEQ ID Nos 1-18. In another embodiment, the antibody comprises six CDR sequences selected from SEQ ID Nos 1-18.

In one embodiment, the antibodies described herein comprise one or more anti-rh17 antibody light (VL) or heavy (VH) variable chain sequence. Suitable VH and VL sequences are shown below in Table 2. In one embodiment, the VH and VL from a single clone are used to produce an antibody or antibody fragment, e.g., VH and VL from KP3-11, KP3-14 or KP3-17. In another embodiment, the VH from one clone is used in conjunction with a VL from another clone. In one embodiment, only a VH sequence is used. In another embodiment, only a VL sequence is used. In another embodiment, the variable chain sequences described herein are utilized with heterologous antibody sequences to produce a chimeric antibody. In one embodiment, the antibody comprises a VH sequence selected from SEQ ID NO: 19, 21, and 23. In another embodiment, the antibody comprises a VL sequence selected from SEQ ID NO: 20, 22 and 24. In one embodiment, the antibody comprises SEQ DI Nos: 19 and 20. In one embodiment, the antibody comprises SEQ ID Nos: 21 and 22. In another embodiment, the antibody comprises SEQ ID Nos: 23 and 24.

Also provided are nucleic acid sequence encoding the antibodies described herein. Such sequences include those shown in Table 3, SEQ ID Nos: 25-30. Also contemplated are nucleic acid sequences encoding the described antibodies. Such sequences include those which share at least about 60% identity with any of the sequence of SEQ ID Nos: 25-30. In another embodiment, the coding sequences share at least about 65% identity with any of the sequence of SEQ ID Nos: 25-30. In another embodiment, the coding sequences share at least about 70% identity with any of the sequence of SEQ ID Nos: 25-30. In another embodiment, the coding sequences share at least about 75% identity with any of the sequence of SEQ ID Nos: 25-30. In another embodiment, the coding sequences share at least about 80% identity with any of the sequence of SEQ ID Nos: 25-30. In another embodiment, the coding sequences share at least about 85% identity with any of the sequence of SEQ ID Nos: 25-30. In another embodiment, the coding sequences share at least about 90% identity with any of

the sequence of SEQ ID Nos: 25-30. In another embodiment, the coding sequences share at least about 95% identity with any of the sequence of SEQ ID Nos: 25-30.

It is also contemplated that one or more of the antibody sequences useful herein encompasses variants of the antibody sequences described herein where modifications and/or substitutions have been made. In one embodiment, the antibody comprises one or more sequences sharing at least 80% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 85% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 90% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 91% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 92% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 93% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 94% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 95% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 96% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 97% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 98% identity with any of SEQ ID NOS: 1-24. In another embodiment, the antibody comprises one or more sequences sharing at least 99% identity with any of SEQ ID NOS: 1-24.

In one embodiment, the antibody described herein does not significantly adversely alter the membrane deformability of the erythrocyte to which it is bound. As used herein, the term “does not significantly adversely alter the membrane deformability” means less than a 10% change in membrane rigidity as compared to a naïve erythrocyte. Membrane deformability can be measured by the person of skill in the art using known techniques and those described herein, e.g., in Example 4. For example, ektacytometry can be used to test whether alterations in membrane deformability are observed. In this technique, a decrease in the maximal elongation index (EImax) or an increase in the shear stress to reach half-

maximal deformation (SS1/2) reflects an increase in RBC rigidity. See, e.g., Bessis M., Mohandas N., and Feo C., "Automated ektacytometry: A new method of measuring red cell deformability and red cell indices," Blood Cells 6(3), 315–327 (1979) and Chien S., "Principles and techniques for assessing erythrocyte deformability," in Red Cell Rheology, edited by Bessis M., Shohet S., and Mohandas N. (Springer; Berlin Heidelberg, 1978), pp. 71–99, which are incorporated herein by reference.

In another embodiment, the antibody described herein does not significantly alter the resistance to stress of the erythrocyte to which it is bound. As used herein, the term "does not significantly alter resistance to stress" means less than a 10% change to physical, chemical, mechanical and/or other stresses, or combinations of thereof. In one embodiment, the term does not significantly alter resistance to stress" means less than a 10% change in osmotic hemolysis or hemolysis induced by mechanical stress as compared to a naïve erythrocyte. Stress to the erythrocyte can be measured by the person of skill in the art using known techniques and those described herein, e.g., in Example 4. For example, osmotic stress can be measured using an osmotic fragility test. See, Godal et al, The normal range of osmotic fragility of red blood cells, Scand J Haematol. 1980 Aug;25(2):107-12, which is incorporated herein by reference. Mechanical stress can be measured using, e.g., the mechanical stress assay (Pan D, Vargas-Morales O, Zern B, et al. The Effect of Polymeric Nanoparticles on Biocompatibility of Carrier Red Blood Cells. PLoS One. 2016;11(3):e0152074, which is incorporated herein by reference) does not directly represent a pathophysiologic scenario, it is intended to reflect overall integrity of the RBC membrane architecture.

Band 3

Band 3, the human RBC anion exchange protein (AE1), is the most abundant integral membrane protein found in erythrocytes and a well-characterized transporter and is encoded by the SLC4a1 gene. There are two blood group antigens, the low-incidence Wr(a) and the high-incidence Wr(b), that are considered to be antithetical and are produced as allelic forms of the same structural gene defined in the Band 3 protein. The Wr(b) antigen requires glycophorin A for surface presentation. See, Huang et al, Blood, Vol 87, No 9 (May I), 1996: pp 3942-3947, which is incorporated herein by reference.

As used herein, "Band 3" refers to the above-described polypeptide, including all isoforms thereof (UniProtKB- P02730). The "canonical" sequence can be found under Uniprot Identifier: P02730-1, also called isoform 1 or eAE1, and is shown below and in SEQ ID NO: 367. The molecular basis of the Wr(a) /Wr(b) blood group antigens is a single variation in position 658; Lys-658 corresponds to Wr(a) and Glu-658 to Wr(b).

```
         10          20          30          40          50
MEELQDDYED MMEENLEQEE YEDPDIPESQ MEEPAAHDTE ATATDYHTTS
         60          70          80          90         100
HPGTHKVYVE LQELVMDEKN QELRWMEAAR WVQLEENLGE NGAWGRPHLS
        110         120         130         140         150
HLTFWSLLEL RRVFTKGTVL LDLQETSLAG VANQLLDRFI FEDQIRPQDR
        160         170         180         190         200
EELLRALLLK HSHAGELEAL GGVKPAVLTR SGDPSQPLLP QHSSLETQLF
        210         220         230         240         250
CEQGDGGTEG HSPSGILEKI PPDSEATLVL VGRADFLEQP VLGFVRLQEA
        260         270         280         290         300
AELEAVELPV PIRFLFVLLG PEAPHIDYTQ LGRAAATLMS ERVFRIDAYM
        310         320         330         340         350
AQSRGELLHS LEGFLDCSLV LPPTDAPSEQ ALLSLVPVQR ELLRRRYQSS
        360         370         380         390         400
PAKPDSSFYK GLDLNGGPDD PLQQTGQLFG GLVRDIRRRY PYYLSDITDA
        410         420         430         440         450
FSPQVLAAVI FIYFAALSPA ITFGGLLGEK TRNQMGVSEL LISTAVQGIL
        460         470         480         490         500
FALLGAQPLL VVGFSGPLLV FEEAFFSFCE TNGLEYIVGR VWIGFWLILL
        510         520         530         540         550
VVLVVAFEGS FLVRFISRYT QEIFSFLISL IFIYETFSKL IKIFQDHPLQ
        560         570         580         590         600
KTYNYNVLMV PKPQGPLPNT ALLSLVLMAG TFFFAMMLRK FKNSSYFPGK
        610         620         630         640         650
LRRVIGDFGV PISILIMVLV DFFIQDTYTQ KLSVPDGFKV SNSSARGWVI
        660         670         680         690         700
HPLGLRSEFP IWMMFASALP ALLVFILIFL ESQITTLIVS KPERKMVKGS
        710         720         730         740         750
GFHLDLLLVV GMGGVAALFG MPWLSATTVR SVTHANALTV MGKASTPGAA
        760         770         780         790         800
AQIQEVKEQR ISGLLVAVLV GLSILMEPIL SRIPLAVLFG IFLYMGVTSL
        810         820         830         840         850
SGIQLFDRIL LLFKPPKYHP DVPYVKRVKT WRMHLFTGIQ IICLAVLWVV
        860         870         880         890         900
KSTPASLALP FVLILTVPLR RVLLPLIFRN VELQCLDADD AKATFDEEEG
        910
RDEYDEVAMP V
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Provided herein are antibodies which bind to one or more antigens on the Band 3 polypeptide. Specifically, the antibodies are reactive against Wr(b) ("Wrb" also called DI4). See, Pool J., The Diego blood group system- an update, Immunohematology, 15(4), 1999, which is incorporated herein by reference.

In one embodiment, the antibodies described herein comprise one or more anti-Wrb antibody CDR sequence. Suitable CDR sequences are shown below in Table 4. In one embodiment, the CDRs from a single clone are used to produce an antibody or antibody fragment, e.g., CDR1, CDR2 and CDR3 from KP2-01, KP2-02 or KP2-04, KP2-06, KP2-07, KP2-08, KP2-09, KP2-11, KP2-13, KP2-14, KP2-15, KP2-17, KP2-18, KP2-19, KP2-20, KP2-22, KP2-23, KP2-24, KP3-01, KP3-02, KP3-03, KP3-05, KP3-06, KP3-07, KP3-08, KP3-09, KP3-12, KP3-13, KP3-15, KP3-16, KP3-18, KP3-19, or KP3-20. In another embodiment, the CDRs from one or more clone are used to produce an antibody. As a non-limiting illustrative example, CDR1 from clone KP2-01 and CDR2 and 3 from clone KP2-02 are used in conjunction to produce an antibody. In another embodiment, the VH CRDs from one clone are use with the VL CDRs from another clone. In another embodiment, the CDRs described herein are utilized with heterologous antibody sequences to produce a chimeric antibody. In one embodiment, the antibody comprises 1 CDR sequence selected from SEQ ID Nos 31-228. In another embodiment, the antibody comprises two CDR sequences selected from SEQ ID Nos 31-228. In another embodiment, the antibody comprises three CDR sequences selected from SEQ ID Nos 31-228. In another embodiment, the antibody comprises four CDR sequences selected from SEQ ID Nos 31-228. In another embodiment, the antibody comprises five CDR sequences selected from SEQ ID Nos 31-228. In another embodiment, the antibody comprises six CDR sequences selected from SEQ ID Nos 31-228.

In one embodiment, the antibodies described herein comprise one or more anti-Wrb antibody light (VL) or heavy (VH) variable chain sequence. Suitable VH and VL sequences are shown below in Table 5. In one embodiment, the VH and VL from a single clone are used to produce an antibody or antibody fragment, e.g., VH and VL from from KP2-01, KP2-02 or KP2-04, KP2-06, KP2-07, KP2-08, KP2-09, KP2-11, KP2-13, KP2-14, KP2-15, KP2-17, KP2-18, KP2-19, KP2-20, KP2-22, KP2-23, KP2-24, KP3-01, KP3-02, KP3-03, KP3-05, KP3-06, KP3-07, KP3-08, KP3-09, KP3-12, KP3-13, KP3-15, KP3-16, KP3-18, KP3-19, or KP3-20. In another embodiment, the VH from one clone is used in conjunction with a VL

from another clone. In one embodiment, only a VH sequence is used. In another embodiment, only a VL sequence is used. In another embodiment, the variable chain sequences described herein are utilized with heterologous antibody sequences to produce a chimeric antibody. In one embodiment, the antibody comprises a VH sequence selected from SEQ ID NO: 229-261. In another embodiment, the antibody comprises a VL sequence selected from SEQ ID NO: 262-294. In one embodiment, the antibody comprises SEQ ID Nos. 229 and 262. In another embodiment, the antibody comprises SEQ ID Nos: 230 and 263. In another embodiment, the antibody comprises SEQ ID Nos: 231 and 264. In another embodiment, the antibody comprises SEQ ID Nos: 232 and 265. In another embodiment, the antibody comprises SEQ ID Nos: 233 and 266. In another embodiment, the antibody comprises SEQ ID Nos: 234 and 267. In another embodiment, the antibody comprises SEQ ID Nos: 235 and 268. In another embodiment, the antibody comprises SEQ ID Nos: 236 and 269. In another embodiment, the antibody comprises SEQ ID Nos: 237 and 270. In another embodiment, the antibody comprises SEQ ID Nos: 238 and 271. In another embodiment, the antibody comprises SEQ ID Nos: 239 and 272. In another embodiment, the antibody comprises SEQ ID Nos: 240 and 273. In another embodiment, the antibody comprises SEQ ID Nos: 241 and 274. In another embodiment, the antibody comprises SEQ ID Nos: 242 and 275. In another embodiment, the antibody comprises SEQ ID Nos: 243 and 276. In another embodiment, the antibody comprises SEQ ID Nos: 244 and 277. In another embodiment, the antibody comprises SEQ ID Nos: 245 and 278. In another embodiment, the antibody comprises SEQ ID Nos: 246 and 279. In another embodiment, the antibody comprises SEQ ID Nos: 247 and 280. In another embodiment, the antibody comprises SEQ ID Nos: 248 and 281. In another embodiment, the antibody comprises SEQ ID Nos: 249 and 282. In another embodiment, the antibody comprises SEQ ID Nos: 250 and 283. In another embodiment, the antibody comprises SEQ ID Nos: 251 and 284. In another embodiment, the antibody comprises SEQ ID Nos: 252 and 285. In another embodiment, the antibody comprises SEQ ID Nos: 253 and 286. In another embodiment, the antibody comprises SEQ ID Nos: 254 and 287. In another embodiment, the antibody comprises SEQ ID Nos: 255 and 288. In another embodiment, the antibody comprises SEQ ID Nos: 256 and 289. In another embodiment, the antibody comprises SEQ ID Nos: 257 and 290. In another embodiment, the antibody comprises SEQ ID Nos: 258 and 291. In another embodiment, the antibody comprises SEQ ID Nos: 259 and

292. In another embodiment, the antibody comprises SEQ ID Nos: 260 and 293. In another embodiment, the antibody comprises SEQ ID Nos: 261 and 294.

Also provided are nucleic acid sequence encoding the antibodies described herein. Such sequences include those shown in Table 6, SEQ ID Nos: 295-360. Also contemplated are nucleic acid sequences encoding the described antibodies. Such sequences include those which share at least about 60% identity with any of the sequence of SEQ ID Nos: 295-360. In another embodiment, the coding sequences share at least about 65% identity with any of the sequence of SEQ ID Nos: 295-360. In another embodiment, the coding sequences share at least about 70% identity with any of the sequence of SEQ ID Nos: 295-360. In another embodiment, the coding sequences share at least about 75% identity with any of the sequence of SEQ ID Nos: 295-360. In another embodiment, the coding sequences share at least about 80% identity with any of the sequence of SEQ ID Nos: 295-360. In another embodiment, the coding sequences share at least about 85% identity with any of the sequence of SEQ ID Nos: 295-360. In another embodiment, the coding sequences share at least about 90% identity with any of the sequence of SEQ ID Nos: 295-360. In another embodiment, the coding sequences share at least about 95% identity with any of the sequence of SEQ ID Nos: 295-360.

It is also contemplated that one or more of the antibody sequences useful herein encompasses variants of the antibody sequences described herein where modifications and/or substitutions have been made. In one embodiment, the antibody comprises one or more sequences sharing at least 80% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 85% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 90% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 91% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 92% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 93% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 94% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 95% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or

more sequences sharing at least 96% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 97% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 98% identity with any of SEQ ID NOS: 229-294. In another embodiment, the antibody comprises one or more sequences sharing at least 99% identity with any of SEQ ID NOS: 229-294.

The antibody sequences herein were produced by immunizing a non-human primate with human erythrocytes. Thus, it may be desirable to make certain changes to the described sequences to make the antibodies more effective in human subjects. For example, in one embodiment, changes are made to one or more of the described sequences to make the antibody more human like. See, Gao SH, Huang K, Tu H, Adler AS. Monoclonal antibody humanness score and its applications. BMC Biotechnol. 2013;13:55, which is incorporated herein by reference.

Such modifications and/or substitutions can be made at the nucleic acid or amino acid level. In one embodiment, the coding sequence of one or more immunoglobulin chain or region is codon optimized.

Once the target and immunoglobulin are selected, the coding sequences for the selected immunoglobulin (*e.g.*, heavy and/or light chain(s)) may be obtained and/or synthesized. Methods for sequencing a protein, peptide, or polypeptide (*e.g.*, as an immunoglobulin) are known to those of skill in the art. Once the sequence of a protein is known, there are web-based and commercially available computer programs, as well as service-based companies which back translate the amino acids sequences to nucleic acid coding sequences. See, *e.g.*, backtranseq by EMBOSS, http://www.ebi.ac.uk/Tools/st/ ; Gene Infinity (http://www.geneinfinity.org/sms/sms_backtranslation.html); ExPasy (http://www.expasy.org/tools/). In one embodiment, the RNA and/or cDNA coding sequences are designed for optimal expression in human cells.

Codon-optimized coding regions can be designed by various methods. This optimization may be performed using methods which are available on-line (*e.g.*, GeneArt), published methods, or a company which provides codon optimizing services, *e.g.*, DNA2.0 (Menlo Park, CA). One codon optimizing method is described, *e.g.*, in US International Patent Publication No. WO 2015/012924, which is incorporated by reference herein in its

entirety. *See also, e.g.,* US Patent Publication No. 2014/0032186 and US Patent Publication No. 2006/0136184. Suitably, the entire length of the open reading frame (ORF) for the product is modified. However, in some embodiments, only a fragment of the ORF may be altered (e.g., heavy constant, light constant, heavy variable, light variable chains). By using one of these methods, one can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide.

A number of options are available for performing the actual changes to the codons or for synthesizing the codon-optimized coding regions designed as described herein. Such modifications or synthesis can be performed using standard and routine molecular biological manipulations well known to those of ordinary skill in the art. In one approach, a series of complementary oligonucleotide pairs of 80-90 nucleotides each in length and spanning the length of the desired sequence are synthesized by standard methods. These oligonucleotide pairs are synthesized such that upon annealing, they form double stranded fragments of 80-90 base pairs, containing cohesive ends, *e.g.,* each oligonucleotide in the pair is synthesized to extend 3, 4, 5, 6, 7, 8, 9, 10, or more bases beyond the region that is complementary to the other oligonucleotide in the pair. The single-stranded ends of each pair of oligonucleotides are designed to anneal with the single-stranded end of another pair of oligonucleotides. The oligonucleotide pairs are allowed to anneal, and approximately five to six of these double-stranded fragments are then allowed to anneal together via the cohesive single stranded ends, and then they ligated together and cloned into a standard bacterial cloning vector, for example, a TOPO® vector available from Invitrogen Corporation, Carlsbad, Calif. The construct is then sequenced by standard methods. Several of these constructs consisting of 5 to 6 fragments of 80 to 90 base pair fragments ligated together, *i.e.*, fragments of about 500 base pairs, are prepared, such that the entire desired sequence is represented in a series of plasmid constructs. The inserts of these plasmids are then cut with appropriate restriction enzymes and ligated together to form the final construct. The final construct is then cloned into a standard bacterial cloning vector, and sequenced. Additional methods would be immediately apparent to the skilled artisan. In addition, gene synthesis is readily available commercially.

In one embodiment, such variants include sequences in which amino acid substitutions have been made to the known anti-RHCE or anti-Band3 variable chain sequences or heterologous backbone sequences described herein. Substitutions may also be written as (amino acid identified by single letter code)-position #- (amino acid identified by single letter code) whereby the first amino acid is the substituted amino acid and the second amino acid is the substituting amino acid at the specified position. The terms "substitution" and "substitution of an amino acid" and "amino acid substitution" as used herein refer to a replacement of an amino acid in an amino acid sequence with another one, wherein the latter is different from the replaced amino acid. Methods for replacing an amino acid are well known to the person skilled in the art and include, but are not limited to, mutations of the nucleotide sequence encoding the amino acid sequence. Methods of making amino acid substitutions in IgG are described, *e.g.*, for WO 2013/046704, which is incorporated by reference for its discussion of amino acid modification techniques.

The term "amino acid substitution" and its synonyms described above are intended to encompass modification of an amino acid sequence by replacement of an amino acid with another, substituting amino acid. The substitution may be a conservative or non-conservative substitution. The term conservative, in referring to two amino acids, is intended to mean that the amino acids share a common property recognized by one of skill in the art. The term non-conservative, in referring to two amino acids, is intended to mean that the amino acids which have differences in at least one property recognized by one of skill in the art. For example, such properties may include amino acids having hydrophobic nonacidic side chains, amino acids having hydrophobic side chains (which may be further differentiated as acidic or nonacidic), amino acids having aliphatic hydrophobic side chains, amino acids having aromatic hydrophobic side chains, amino acids with polar neutral side chains, amino acids with electrically charged side chains, amino acids with electrically charged acidic side chains, and amino acids with electrically charged basic side chains. Both naturally occurring and non-naturally occurring amino acids are known in the art and may be used as substituting amino acids in embodiments. Thus, a conservative amino acid substitution may involve changing a first amino acid having a hydrophobic side chain with a different amino acid having a hydrophobic side chain; whereas a non-conservative amino acid substitution may involve changing a first amino acid with an acidic hydrophobic side chain with a different

amino acid having a different side chain, *e.g.,* a basic hydrophobic side chain or a hydrophilic side chain. Still other conservative or non-conservative changes change be determined by one of skill in the art.

In still other embodiments, the substitution at a given position will be to an amino acid, or one of a group of amino acids, that will be apparent to one of skill in the art in order to accomplish an objective identified herein.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 70% identity, preferably 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region (*e.g.*, any one of the modified ORFs provided herein when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (see, *e.g.*, NCBI web site or the like). As another example, polynucleotide sequences can be compared using Fasta, a program in GCG Version 6.1. Fasta provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference. Generally, these programs are used at default settings, although one skilled in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program that provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. This definition also refers to, or can be applied to, the compliment of a sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25, 50, 75, 100, 150, 200 amino acids or nucleotides in length, and oftentimes over a region that is 225, 250, 300, 350, 400, 450, 500 amino acids or nucleotides in length or over the full-length of an amino acid or nucleic acid sequences.

Typically, when an alignment is prepared based upon an amino acid sequence, the alignment contains insertions and deletions which are so identified with respect to a reference AAV sequence and the numbering of the amino acid residues is based upon a reference scale provided for the alignment. However, any given AAV sequence may have fewer amino acid residues than the reference scale. In the present invention, when discussing the parental sequence, the term "the same position" or the "corresponding position" refers to the amino acid located at the same residue number in each of the sequences, with respect to the reference scale for the aligned sequences. However, when taken out of the alignment, each of the proteins may have these amino acids located at different residue numbers. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Sequence alignment programs are available for amino acid sequences, *e.g.*, the "Clustal X", "MAP", "PIMA", "MSA", "BLOCKMAKER", "MEME", and "Match-Box" programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. *See, e.g.*, J. D. Thomson *et al*, *Nucl. Acids. Res.*, "A comprehensive comparison of multiple sequence alignments", 27(13):2682-2690 (1999).

Cargoes

For several decades, researchers have used erythrocytes for drug delivery of a wide variety of therapeutics to improve their pharmacokinetics, biodistribution, controlled release and pharmacodynamics. Provided herein are compositions in which both therapeutic and non-therapeutic cargoes are coupled to the surface of the red blood cell using the antibodies described herein.

As used herein, the term "cargo" or "agent" refers to any pharmacological, therapeutic, prophylactic, imaging or diagnostic agent which is coupled to, bound, fused, associated with or conjugated to an anti-RHCE or anti-Band 3 antibody described herein. In one embodiment, the term cargo or agent refers to more than one cargo or agent described herein, e.g., liposomes loaded with other drugs. Drugs whose delivery may be improved by coupling to RBCs include antigens and cytokines to stimulate the immune response,

antibodies for vascular targeting of RBC-loaded cargoes, antibodies and other ligands to capture circulating pathological mediators such toxins and pathogens themselves, therapeutic enzymes and other biomolecules whose targets are localized within the bloodstream, and complement inhibitors to protect RBCs against pathological hemolysis. See, Villa et al, Delivery of drugs bound to erythrocytes: new avenues for an old intravascular carrier, Therapeutic Delivery, 6(7), 2015, which is incorporated herein by reference.

In one embodiment, the cargo is a liposome. Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural non-toxic phospholipids. See, Akbarzakeh et al, Nanoscale Res Lett. 2013; 8(1): 102, which is incorporated herein by reference. Liposomes consist of an aqueous core surrounded by a lipid bilayer, much like a membrane, separating the inner aqueous core from the bulk outside. Liposomes have been used to improve the therapeutic index of new or established drugs by modifying drug absorption, reducing metabolism, prolonging biological half-life or reducing toxicity. Drug distribution is then controlled primarily by properties of the carrier and no longer by physico-chemical characteristics of the drug substance only.

Lipids forming liposomes may be natural or synthetic, and liposome constituents are not exclusive of lipids, new generation liposomes can also be formed from polymers (sometimes referred to as polymersomes). Whether composed of natural or synthetic lipids or polymers, liposomes are biocompatible and biodegradable which make them suitable for biomedical research. The unique feature of liposomes is their ability to compartmentalize and solubilize both hydrophilic and hydrophobic materials by nature. Hydrophobic drugs place themselves inside the bilayer of the liposome and hydrophilic drugs are entrapped within the aqueous core or at the bilayer interface. Liposomal formulations enhance the therapeutic efficiency of drugs in preclinical models and in humans compared to conventional formulations due to the alteration of biodistribution. Liposome binding drugs, into or onto their membranes, are expected to be transported without rapid degradation and minimum side effects to the recipient because generally liposomes are composed of biodegradable, biologically inert and non-immunogenic lipids. Moreover, they produce no pyrogenic or antigenic reactions and possess limited toxicity. Consequently, all these properties as well as the ease of surface modification to bear the targetable properties make liposomes attractive candidates for use as drug-delivery. Additional cargoes may be loaded into the liposomes and

coupled to the described antibodies. Such additional cargoes are selected from any useful agent, including those described herein.

In one embodiment, the cargo may be any anti-thrombotic agent (molecule), anti-inflammatory agent, or pro-drug thereof for which targeting to a red blood cell is desired for purposes of systemic delivery, or alternatively, for delivery to the site of a pathological condition including conditions characterized by the production or presence of an enzyme that can cleave the anti-thrombotic agent, anti-inflammatory agent, or the pro-drug, from the fusion protein.

As used herein, the term "pro-drug" or "prodrug" encompasses any polypeptide encoding an anti-thrombotic or anti-inflammatory agent and a cleavage site for activation of the agent. The pro-drug is inactive (or significantly less active) upon administration, and is metabolized *in vivo* into an active form. In further embodiments, the pro-drug is a pro-drug of an anti-thrombotic or anti-inflammatory agent.

In one embodiment, the anti-thrombotic agent is one that is capable of producing its therapeutic effect when attached to the RBC, i.e., an active anti-thrombotic agent. In another embodiment, the anti-thrombotic agent is a pro-drug which contains a native or synthetic cleavage site and which produces an active anti-thrombotic effect only upon cleavage from its pro-drug state.

Among such anti-thrombotic agents include without limitation, plasminogen activators. In still a further embodiment, the plasminogen activator is tPA, urokinase, tenectase, retavase, streptokinase, staphylokinase, or a plasminogen activator from venoms and saliva of bats, insects, and other animals. In another embodiment, the plasminogen activator is anistreplase, pro-urokinase (pUK), or a hybrid plasminogen activator (*e.g.*, as described in US Pat. No. 4,916,071). In one embodiment, the cargo is thrombomodulin, as shown in the examples herein.

In a further embodiment the anti-thrombotic agent is the low molecular weight single chain urokinase-like plasminogen activator described in the examples below (also termed uPA (as the exemplary plasminogen activator), lUK, lmwUK, and lmw scuPA within the examples). Also included are mutants or variants thereof, which retain plasminogen activator activity, such as variants which have been chemically modified or in which one or more amino acids have been added, deleted or substituted or in which one or more functional

domains have been added, deleted or altered such as by combining the active site of one plasminogen activator or fibrin binding domain of another plasminogen activator or fibrin binding molecule. In a further embodiment, the anti-thrombotic agent contains a moiety presented by a protease domain of a plasminogen activator. Naturally-occurring pro-drugs of these agents may be employed. Synthetically designed prodrugs based on these agents may also be employed. Prodrugs containing modified cleavage sites may also be employed.

In one embodiment, the cargo is a therapeutic protein or pro-drug of an anti-inflammatory agent. In one embodiment, the anti-inflammatory agent is an antibody against a cytokine or other pro-inflammatory mediator. In a further embodiment, the anti-inflammatory agent may comprise a moiety presented by thrombomodulin or a domain thereof. Among other anti-inflammatory agents for use in the fusion proteins described herein are, without limitation, somatostatin, adiponectin, cortistatin, corticotrophin releasing factor, sauvagine, nocifensins, as well as the anti-inflammatory cytokines, IL-1 receptor antagonist (IL-1ra), IL-4, IL-6, IL-10, and IL-13 and the soluble receptors sTNFRI, sTNFRp55, sTNFRII, sTNFRp75, sIL-1RII, mIL-1RII, and IL-18BP, among others. Anti-inflammatory proteins may be native or mutated proteins. Similarly, native, mutated or synthetic anti-inflammatory peptides, including without limitation, peptides described in US Patent Nos. 5480869; 7816449 and 5229367, among other known peptides may also form part of the fusion proteins described herein. One of skill in the art may select or design an appropriate anti-inflammatory agent or prodrug depending on the pathological condition being treated.

In still another embodiment, the therapeutic molecule is a molecule which binds a pro-inflammatory mediator. In one embodiment, the pro-inflammatory mediator is the HMGB1 cytokine. In one embodiment, signaling by HMGB1 is disrupted by binding of the lectin-like domain of thrombomodulin (abbreviated herewith as TM). In other embodiments, the pro-inflammatory cytokine is IL-1-α, IL-1-β, IL-6, TNF-α, TGF-β, LIF, IFN-γ, OSM, CNTF, GM-CSF, IL-8, IL-11, IL-12, IL-17, and IL-18.

In one embodiment, a fusion protein may contain a therapeutically-active site, domain or moiety of any of the anti-thrombotic agents, anti-inflammatory agents, or pro-drugs listed herein or known to the art to be suitable for direct targeted administration to the site of a thrombus. Other useful pro-drugs known to one of skill in the art may be used herein.

In still other embodiments, mutations in protein sequence of the anti-thrombotic agent or anti-inflammatory agent, therapeutically-active site, domain, or moiety thereof allows its conversion into a pro-drug activated and/or released locally at a desired pathological site (*e.g.*, pathological nascent intravascular thrombi) using specific activity of pathological factors that exist only in these pathological sites, such as protease thrombin. Such mutations in the amino acid sequences or nucleotide sequences encoding the therapeutic protein can be employed to insert a desired cleavage, enzymatic or activation site into the therapeutic molecule, or into or adjacent the linker between the antibody and the cargo. Alternatively, such mutations can change a native cleavage site to another desired cleavage site, or to insert a cleavage site where none naturally existed into or adjacent to a cargo.

In one embodiment, the therapeutic pro-drug molecule is activated or the mature drug molecule released from the fusion protein by an enzyme, which level is locally elevated under pathological conditions. In a further embodiment, the enzyme is a protease. In still further embodiments, the protease is a leukocyte protease (*e.g.*, cathepsin), an activated protease in the coagulation cascade (*e.g.*, activated Factor Xa), or an activated protease in the complement cascade. In other embodiments, the protease's activity is elevated locally in tissue. In still other embodiments, the protease is a metalloproteinase, elastase, or collagenase.

In still other embodiments of fusion proteins containing therapeutic pro-drug molecules, the enzyme is a pathological mediator. In further embodiments, the pathological mediator is involved in coagulation or fibrinolysis. In another embodiment, the pathological mediator is thrombin or plasmin. In a further embodiment, the pathological mediator is thrombin. Thus, for example, in one embodiment, the therapeutic pro-drug molecule is the thrombin activatable low molecular weight single chain urokinase-like plasmin activator, described in the examples below. In another embodiment, the therapeutic pro-drug molecule is thrombin-activatable thrombomodulin, or thrombin-activatable tPA (or its mouse analog, mRNK-T).

Other cargoes useful include blood factors including those involved in blood clotting. Such blood factors include factor VIII and factor IX. Further cargoes include small molecule drugs. Other cargoes useful herein include anti-malarial drugs, such as chloroquine, quinine sulfate, hydroxychloroquine, mefloquine, atovaquone and proguanil. Other useful cargoes

include anti-hemolytic agents. In one embodiment, such drugs are loaded into liposomes, polymeric particles, lipid nanoparticles, natural or artificial biomolecules or assemblies. See, e.g., Giri et al, Anticancer Agents Med Chem. 2016;16(7):816-31; WO 2017/023358; Jo et al, Colloids Surf B Biointerfaces. 2014 Nov 1;123:345-63. doi: 10.1016/j.colsurfb.2014.09.029. Epub 2014 Sep 22, each of which is incorporated herein by reference.

Fusion Proteins and Conjugates

The cargoes and antibodies described herein are coupled in one of various appropriate methods. Such methods include fusion proteins, chemical conjugation, chemical crosslinking, use of a linker, click chemistry and the like. Such methods are known in the art. As used herein, terms such as and including "coupled to", "bound", "fused, "associated with" or "conjugated to" are used interchangeably. Where one embodiment is provided utilizing the antibody and cargo as e.g., a fusion protein, another embodiment is contemplated in which the antibody and cargo are coupled via another method, e.g., using click chemistry or the like.

In one embodiment, the antibody and the cargo are expressed as a fusion protein. Fusion proteins are created through the joining of two or more genes that originally coded for separate proteins. In one embodiment, the fusion protein comprises an scFv and a heterologous expression product. Such expression products include certain of the cargoes described herein. In one embodiment, the fusion proteins contain a targeting single chain antigen-binding domain (scFv) that binds to a determinant expressed on the surface of a red blood cell, e.g., RHCE (rh17) or Band3 (Wrb). Use of an scFv (monovalent) avoids cross-linking of binding sites or determinants, thereby avoiding potentially harmful cell membrane modification and cell aggregation.

ScFvs may be generated conventionally, e.g., by the method of Spitzer, et al. (Mol. Immunol. 2003, 40:911-919), or by the methods described herein. Total RNA of a hybridoma cell line is isolated (e.g., by RNeasy, Qiagen, Velencia, CA), followed by reverse transcription, e.g., using the SMARTTM technology (Clontech, Palo Alto, CA) employing known primers (e.g., those of Dübel, et al. (J. Immunol. Methods 1994, 175:89-95)). The resulting heavy (VH) and light (VL) chain variable cDNA fragments are then subcloned into a suitable plasmid, e.g., pCR®2.1-TOPO® (Invitrogen, Carlsbad, CA). The materials utilized are not a limitation of these embodiments. The VH and VL chains generated are combined

with a suitable linker, resulting in the desired scFv (see, e.g., Example 1). In one embodiment, the scFV comprises anti-RHCD sequences. In one embodiment, the scFv comprises SEQ ID Nos. 19 and 20. In another embodiment, the scFv comprises SEQ ID Nos: 21 and 22. In another embodiment, the scFv comprises SEQ ID Nos: 23 and 24. In one embodiment, the scFV comprises anti-Band3 sequences. In one embodiment, the scFv comprises SEQ ID Nos. 229 and 262. In another embodiment, the scFv comprises SEQ ID Nos: 230 and 263. In another embodiment, the scFv comprises SEQ ID Nos: 231 and 264. In another embodiment, the scFv comprises SEQ ID Nos: 232 and 265. In another embodiment, the scFv comprises SEQ ID Nos: 233 and 266. In another embodiment, the scFv comprises SEQ ID Nos: 234 and 267. In another embodiment, the scFv comprises SEQ ID Nos: 235 and 268. In another embodiment, the scFv comprises SEQ ID Nos: 236 and 269. In another embodiment, the scFv comprises SEQ ID Nos: 237 and 270. In another embodiment, the scFv comprises SEQ ID Nos: 238 and 271. In another embodiment, the scFv comprises SEQ ID Nos: 239 and 272. In another embodiment, the scFv comprises SEQ ID Nos: 240 and 273. In another embodiment, the scFv comprises SEQ ID Nos: 241 and 274. In another embodiment, the scFv comprises SEQ ID Nos: 242 and 275. In another embodiment, the scFv comprises SEQ ID Nos: 243 and 276. In another embodiment, the scFv comprises SEQ ID Nos: 244 and 277. In another embodiment, the scFv comprises SEQ ID Nos: 245 and 278. In another embodiment, the scFv comprises SEQ ID Nos: 246 and 279. In another embodiment, the scFv comprises SEQ ID Nos: 247 and 280. In another embodiment, the scFv comprises SEQ ID Nos: 248 and 281. In another embodiment, the scFv comprises SEQ ID Nos: 248 and 281. In another embodiment, the scFv comprises SEQ ID Nos: 249 and 282. In another embodiment, the scFv comprises SEQ ID Nos: 250 and 283. In another embodiment, the scFv comprises SEQ ID Nos: 251 and 284. In another embodiment, the scFv comprises SEQ ID Nos: 252 and 285. In another embodiment, the scFv comprises SEQ ID Nos: 253 and 286. In another embodiment, the scFv comprises SEQ ID Nos: 254 and 287. In another embodiment, the scFv comprises SEQ ID Nos: 255 and 288. In another embodiment, the scFv comprises SEQ ID Nos: 256 and 289. In another embodiment, the scFv comprises SEQ ID Nos: 257 and 290. In another embodiment, the scFv comprises SEQ ID Nos: 258 and 291. In another embodiment, the scFv comprises SEQ ID Nos: 259 and 292.

In another embodiment, the scFv comprises SEQ ID Nos: 260 and 293. In another embodiment, the scFv comprises SEQ ID Nos: 261 and 294.

In one aspect, nucleic acid sequences are provided which encode the scFv. In one embodiment, the coding sequences include one of the sequences of Table 3 or Table 6. A cartoon of an exemplary RHCE scFv- human thrombomodulin fusion protein plasmid is provided in FIG. 18.

In another embodiment, the antibodies are chemically conjugated to their cargoes using molecular cross-linkers, spacers, and bridges. By cross-linkers, spacer and bridges are meant any moiety used to attach or associate the antibody to the cargo. In one embodiment, the cross-linker is a covalent bond. In another embodiment, the linker is a non-covalent bond. In still other embodiments, the linker can be a larger compound or two or more compounds that associate covalently or non-covalently. In still other embodiment, the linker can be a combination of the linkers, e.g., chemical compounds, nucleotides, amino acids, proteins, etc. In one embodiment, the cross-linker is biotin-streptavidin. In this embodiment, interconnecting molecule(s) such as streptavidin can be coupled to RBC either directly via chemical modification, or via biotin derivatives conjugated to the functional groups on RBC, inserted into RBC phospholipids or coupled to other appropriate RBC components such as sugars, with or without additional spacers between the active group anchoring biotin derivative to RBC. In turn, cargo molecules are coupled to streptavidin either via chemical conjugation or via using biotin derivatives as described above. In one embodiment a spacer is positioned between biotin and a reactive group, such as succinimide ester group. Various methods of bioconjugation are known in the art. See, e.g., Kalia and Raines, Curr Org Chem. 2010 Jan; 14(2): 138–147, which is incorporated herein by reference.

In one embodiment, a fusion protein as described herein is prepared by linking (fusing) the above-described scFv to a described cargo, (e.g., a above-described anti-thrombotic agent, anti-inflammatory agent, or pro-drug molecule). Moreover, genetic engineering allows the design and synthesis of targeted pro-drugs which can be cleaved by pathophysiologically relevant enzymes that are generated at the size of disease that cannot be attained using chemical conjugation.

Linkers may also be utilized to join variable heavy and variable light chain fragments. A linker as used herein refers to a chain of as short as about 1 amino acid to as long as about

100 amino acids, or longer. In a further embodiment, the linker is 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids in length. In one embodiment, the linker is 13 amino acids in length.

Further, a cleavage sequence, such as the thrombin-sensitive cleavage sequence or other enzyme cleavage sequence, can be inserted in the linker to provide for release of the drug when the RBC to which it is targeted encounters the appropriate cleaving enzyme at the site of the pathological condition, e.g., upon active thrombosis. This cleavage sequence may be located within a linker or at a terminus thereof. In one embodiment, a thrombin cleavage site -Met-Tyr-Pro-Arg-Gly-Asn- may be inserted in, or appended to, the linker between the scFv and the therapeutic molecule or pro-drug. In another embodiment, the thrombin cleavage site is Pro-Arg. In still a further embodiment, lack of the native Phe-Lys plasmin cleavage site prevents single chain (sc) uPA activation (into fully active two-chain plasminogen activator (tcuPA)) via plasmin.

In another embodiment, antibody-derived scFv with a thrombin releasing site can be cloned by an upstream primer, which anneals to the carboxy terminus and introduces the sequence including a short peptide linker with the thrombin cleavage site. In still another embodiment, the cleavage site is internal to the pro-drug itself.

In one embodiment, the antibody and cargo are conjugated using click chemistry. In one embodiment, the conjugation is done using copper-independent click chemistry. Briefly, the antibody (e.g., scFV) is chemically modified to site-specifically incorporate a strained alkyne for 'click' coupling. The cargo (e.g., liposome) is functionalized with a complementary group, such as DBCO and azide. Other examples of click chemistry reactions, include, without limitation: cycloaddition reactions, such as the 1,3-dipolar family, and hetero Diels-Alder reactions; nucleophilic ring-opening reactions (e.g., epoxides, aziridines, cyclic sulfates, and so forth); carbonyl chemistry, such as the formation of oxime ethers, hydrazones, and aromatic heterocycles; in addition to carbon-carbon multiple bonds, such as epoxidation and dihydroxylation and azide-phosphine coupling (Staudinger ligation). See, Nwe and Brechbiel, Cancer Biother Radiopharm. 2009 Jun; 24(3): 289–302, which is incorporated herein by reference.

Methods of Preparation

The sequences, antibodies, fragments, fusion proteins and conjugates described herein may be produced by any suitable means, including recombinant production, chemical synthesis, or other synthetic means. Suitable production techniques are well known to those of skill in the art. See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (Cold Spring Harbor, NY). Alternatively, peptides can also be synthesized by the well-known solid phase peptide synthesis methods (Merrifield, J. Am. Chem. Soc., 85:2149 (1962); Stewart and Young, Solid Phase Peptide Synthesis (Freeman, San Francisco, 1969) pp. 27-62). Polymerase chain reaction (PCR) and related techniques are described in Derbyshire, et al. (Immunochemistry 1: A practical approach. M. Turner, A. Johnston eds., Oxford University Press 1997, e.g., at pp. 239-273). Plasmids useful herein have been described in Derbyshire, et al. (cited above), as well as Gottstein, et al. (Biotechniques 30: 190-200, 2001). Cloning techniques are also described in these and other suitable production methods are within the knowledge of those of skill in the art and are not a limitation of the compositions and methods described herein. Generation of recombinant proteins provides flexibility in design, rapid production, large-scale production and uniform composition.

In one aspect, a construct is provided which encodes the fusion proteins or antibodies described herein. Such a construct is, in on aspect, delivered to a subject in need thereof via an appropriate viral vector or the like. Suitable viral vectors include, without limitation, retrovirus, adenovirus, adeno-associated virus (AAV), herpes simplex virus, lentivirus, and chimeric viral vectors. These vectors may be designed and employed by the person of skill in the art using the sequences and teachings herein.

As an example, reference is made to the use of an AAV as a viral vector for gene therapy. However, similar vectors can be constructed using other types of viral vectors. Typically, an expression cassette for an AAV vector comprises an AAV 5' inverted terminal repeat (ITR), the immunoglobulin/antibody coding sequences and any regulatory sequences, and an AAV 3' ITR. However, other configurations of these elements may be suitable. In one embodiment, the expression cassette encodes a fusion protein, e.g., the scFv coding sequences in combination with the coding sequence for a cargo. Such a construct is shown in FIG. 18.

The expression cassette may contain at least one internal ribosome binding site, *i.e.*, an IRES, located between the coding regions of the heavy and light chains, or located between the coding regions of the scFv and the cargo (e.g., thrombomodulin as in FIG. 18). Alternatively, the heavy and light chain or scFv and the cargo coding sequences may be separated by a furin-2a self-cleaving peptide linker (*see, e.g.,* Radcliffe and Mitrophanous, Gene Therapy (2004), 11, 1673-1674, which is incorporated herein by reference). The use of AAV for delivering antibody sequences is known. See, e.g., WO 2017/106326, which is incorporated by reference herein.

In one embodiment, the antibody genes described herein are engineered into a genetic element (*e.g.,* a plasmid) useful for generating viral vectors which transfer the immunoglobulin construct sequences carried thereon. The selected vector may be delivered to a packaging cell by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. Stable packaging cells can also be made. The methods used to make such constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. *See, e.g.,* Molecular Cloning: A Laboratory Manual, ed. Green and Sambrook, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012).

Pharmaceutical Compositions and Methods of treatment

Pharmaceutical compositions containing antibodies, fragments, fusion proteins and/or conjugates described herein and a pharmaceutically acceptable carrier or vehicle as described herein are useful for the treatment of a variety of diseases and disorders, depending upon the selection and identity of the cargo. In one embodiment, a composition comprises a pharmaceutically acceptable vehicle for intravenous administration. In another embodiment, a composition comprises a pharmaceutically acceptable vehicle for administration via other vascular routes, including but not limited to, intra-arterial and intra-ventricular administration, as well as routes providing slower delivery of drugs to the bloodstream such as intramuscular administration to an animal in need thereof. As used herein, the terms "subject" and "patient" include any mammal. In a further embodiment, the terms "subject" and "patient" refer to a human.

Pharmaceutically acceptable vehicles/carriers include any of those conventionally used in the art, e.g., saline, phosphate buffered saline (PBS), or other liquid sterile vehicles accepted for intravenous injections in clinical practice. Pharmaceutical compositions may also include buffers, pH adjusting agents, and other additives conventionally used in medicine. Other exemplary carriers include sterile saline, lactose, sucrose, maltose, and water. Optionally, the compositions of the invention may contain excipient, diluent and/or adjuvant, other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. In one embodiment, compositions described herein are administered systemically as a bolus intravenous injection of a single therapeutic dose of the fusion protein. In a further embodiment, the dose is 0.1-5.0 mg/kg. In another embodiment, the dose is 0.01-0.5 mg/kg.

In one embodiment, methods of treatment are provided comprising delivering antibodies, fragments, fusion proteins and/or conjugates described herein, or a pharmaceutical composition described herein, to a mammalian subject, particularly a human. In other embodiments, methods of treatment are provided comprising delivering antibodies, fragments, fusion proteins and/or conjugates described herein, or a pharmaceutical composition described herein, to a blood vessel. In one embodiment, antibodies, fragments, fusion proteins and/or conjugates described herein are administered via a systemic intravascular route, e.g., a vascular catheter. In some embodiments, rapid targeting of an organ or system may be accomplished by delivery via coronary artery (e.g., for prophylaxis of acute myocardial infarction (AMI)) or the cerebral artery (e.g., for prophylaxis of stroke and other cerebrovascular thrombotic events). Further, the antibodies, fragments, fusion proteins and/or conjugates described herein may be administered prophylactically, i.e., in patients predisposed to thrombosis. In a further embodiment, the antibodies, fragments, fusion proteins and/or conjugates described herein may be administered to an organ donor, utilized with an isolated organ transplant (e.g., via perfusion), or used with vascular stents.

Thus, in one embodiment, methods of treating or preventing a cardiovascular disorder, such as thrombosis, tissue ischemia, AMI, ischemic stroke, pulmonary embolism, sepsis, acute lung injury (ALI) or other type of vascular inflammation, or ischemic peripheral vascular disease, involves administering antibodies, fragments, fusion proteins and/or conjugates described herein, or a pharmaceutical composition as described herein, to a blood vessel in a mammal in need thereof. In such disorders, the anti-thrombotic or anti-

inflammatory agent and its dosage in delivery (i.e., the amount fused to an individual RBC may be selected and adjusted by an attending physician with regard to the nature of the disorder, the physical condition of the patient, and other such factors). The selection of the cleavage site, where included, may also be selected to match the disorder, e.g., a thrombin cleavage site suitable for most cardiovascular disorders. Loading red blood cells (RBC) in vivo with anti-thrombotic agents (ATAs) constitutes a new approach to thromboprophylaxis that holds promise for improving the management of patients at high risk of thrombosis for a defined period of time in whom anticoagulation poses an unacceptable risk. Delivery of plasminogen activators (PAs) and thrombomodulin (TM) via RBCs markedly prolongs intravascular lifespan and restricts vascular and tissue damage.

In one embodiment, the compositions described herein are effective in the treatment or prevention of cerebrovascular thrombi. In a further embodiment, the compositions described herein are effective in the treatment or prevention of cerebrovascular disease, such as transient ischemic attack and stroke. In yet another embodiment, the antibodies, fragments, fusion proteins and/or conjugates described herein, or a pharmaceutical composition as described herein are effective in prolonging the circulation of a cargo in a subject in need thereof.

Similarly, in another embodiment, methods of treating or preventing disseminated intravascular coagulation (DIC), sepsis, acute lung injury (ALI/ARDS), aseptic systemic inflammation, and other inflammatory conditions are provided by administering the appropriately designed fusion proteins and/or conjugate described herein, according to the teachings of this specification.

Also provided is the use of antibodies, fragments, fusion proteins and/or conjugates described herein or a pharmaceutical composition as described herein as a medicament. The use of antibodies, fragments, fusion proteins and/or conjugates described herein or a pharmaceutical composition as described herein is provided to treat any of the above conditions.

Provided herein is a method of treating or preventing thrombosis, tissue ischemia, acute myocardial infarction (AMI), non-segmented elevated AMI, deep vein thrombosis, ischemic stroke, hyperoxic injury, transient ischemic attack (TIA), cerebrovascular disease, disseminated intravascular coagulation (DIC), pulmonary embolism, ischemic peripheral

vascular disease, inflammation, pulmonary edema, sepsis, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), aseptic systemic inflammation, malaria, SCD, hemolytic anemia, or a bleeding disorder such as hemophilia. The method includes administering an antibody-cargo conjugate composition as described herein to a subject in need thereof.

The dosages, administrations and regimens may be determined by the attending physician given the teachings of this specification. In one embodiment, the composition is administered in a single dosage. In another embodiment, the composition is administered as a split dosage. Split administration may imply a time gap of administration from intervals of minutes, hours, days, weeks or months. In another embodiment, a second administration of a composition as described herein is performed at a later time point. Such time point may be weeks, months or years following the first administration. In one embodiment, the second administration is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 years or more after the first administration.

In still other embodiments, the compositions described herein may be delivered in a single composition or multiple compositions. Optionally, two or more different antibody conjugates, fusion proteins, or AAV may be delivered, or multiple viruses [see, e.g., WO 2011/126808 and WO 2013/049493]. In another embodiment, multiple viruses may contain different replication-defective viruses (e.g., AAV and adenovirus or lentivirus).

The compositions described herein have been shown to have little effect on RBC cell physiology. Previously used constructs, directed to Ter119, have been shown to induce rigidity in RBC. While targeting Wr^b might be expected to induce rigidity[28], ligands to RHCE determinants were not previously characterized on human RBCs with respect to effects on cell physiology. These antibodies were then fused to the extracellular domain of human thrombomodulin (hTM-scFv) to produce an exemplary multi-faceted thromboprophylactic agent[20]. The binding of the scFv and hTM-scFv was characterized and examination of how both affected several clinically relevant aspects of human RBC physiology including osmotic resistance, mechanical strength, deformability under flow, and exposure of phosphatidylserine was performed. The efficacy of these human RBC-coupled TMs was compared using a whole-blood, microfluidic model of inflammatory microthrombosis recently described [33], as shown in the examples below.

In one aspect, a method of loading red blood cells is provided. In one embodiment, the red blood cells are loaded ex vivo. In said method, red blood cells are collected from a subject. The RBCs are isolated and contacted with an antibody-cargo construct of the invention. The loaded RBCs are then infused into a subject. In one embodiment, the subject is the same subject from which the RBCs were harvested. In another embodiment, the subject or a different subject from which the RBCs were harvested.

As described above, the term "about" when used to modify a numerical value means a variation of ±10%, unless otherwise specified. As used throughout this specification and the claims, the terms "comprise" and "contain" and its variants including, "comprises", "comprising", "contains" and "containing", among other variants, is inclusive of other components, elements, integers, steps and the like. The term "consists of" or "consisting of" are exclusive of other components, elements, integers, steps and the like.

The following examples are illustrative only and are not a limitation on the invention described herein. It is demonstrated herein, that a human antibody was murinized and administered to a mouse to effectively lower cholesterol levels in a model of familial hypercholesterolemia.

Example 1 - Materials and 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 AlexaFlour 647-NHS Ester and AlexaFlour 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 (Carlsbad, CA).

Red blood cells

Human whole blood was obtained from healthy volunteer donors. All studies involving human subjects were approved by the Institutional Review Board of the University of Pennsylvania. Written informed consent was obtained and phlebotomy was performed via the antecubital veins using a 21-gauge butterfly needle. Specimens were drawn into 3.2% sodium citrate vacuum tubes (BD, Franklin Lakes, NJ). To obtain red blood cells, whole blood was spun at 1000xg for 10 min and the plasma and buffy coat were discarded. A portion of the packed red cells was then suspended in phosphate buffered saline (PBS) with 2% normal human AB serum (Sigma Aldrich, St. Louis, MO) at the indicated hematocrit for each subsequent assay. To measure osmotic resistance and mechanical resistance, human RBCs were isolated from the retained segments of non-expired O positive, leukoreduced, irradiated RBCs from our hospital blood bank and prepared similarly. Similar results were seen using either fresh RBCs or donor units.

Derivation and production of antibodies and fusion proteins

An IgG Fab/phage display library was prepared from the peripheral blood lymphocytes of a hyperimmunized macaque using homologous human V-region oligonucleotides (Siegel DL, R.M., Lee H, Blancher A., Production of large repertories of macaque mAbs to human RBCs using phage display. Transfusion, 1999. 39(S10): p. 92S, which is incorporated herein by reference). Fab/phage specific for human RBCs were isolated by panning on intact human RBCs. Monoclonal Fab/phage were grown to produce antibodies

for immunoassays and their corresponding DNA was extracted for sequencing. To identify target epitopes, antibodies were screened against RBCs of known serologic phenotypes, including rare cells lacking highly conserved antigens, using standard immunohematologic techniques (Roback, J.D., Technical Manual. 2014: American Association of Blood Banks (AABB), which is incorporated herein by reference.)

After identification of the target epitopes, clones reactive against Wrb and Rh17 present at the highest titers were chosen to produce scFv derivatives of the encoded antibodies. Sequences of the antibody clones examined herein are available in the supporting information. For each VH and VL region, restriction enzyme sites were introduced for cloning into expression vectors and fusion to the extracellular domain of human TM (Glu22-Ser515). VH and VL were also ligated into a pBAD/gIII expression system (Thermo Fisher Scientific, Carlsbad, CA) to produce scFv alone in *E. coli*. Sequences were modified by custom synthesis of double-stranded gene fragments (gBlock, IDT, Coralville, IA).

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 (Ding, B.S., et al., Anchoring fusion thrombomodulin to the endothelial lumen protects against injury-induced lung thrombosis and inflammation. Am J Respir Crit Care Med, 2009. 180(3): p. 247-56, which is incorporated herein by reference), 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, Carlsbad, CA) 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 was allowed to reach equilibrium over 4 hours at 37 degrees C. After binding, cell suspensions were rapidly washed at least four times with cold PBS. The resulting cell pellet was counted using a Perkin Elmer Wizard2 gamma counting system. Dissociation of the fusion proteins was assessed using RBCs saturated with radiolabeled proteins, washing unbound ligands, and placing in dilute suspensions prior to measurement of 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 Milipore, Billerica, MA).

Activated protein C assay

Generation of activated protein C by TM proteins or TMs coupled to RBCs was measured as described previously (Carnemolla, R., et al., Quantitative analysis of thrombomodulin-mediated conversion of protein C to APC: translation from in vitro to in vivo. J Immunol Methods, 2012. 384(1-2): p. 21-4). 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 degrees 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.

Microfluidic assay

Microfluidic experiments were performed on a Bioflux 1000 (Fluxion Biosciences, San Francisco, CA) multi-well microfluidic system. Microchannels were endothelialized with HUVECs as described previously (Colin F. Greineder, I.H.J., Carlos H. Villa, Douglas B. Cines, Mortimer Poncz, and Vladimir R. Muzykantov, Microfluidic Modeling of Human

Disseminated Intravascular Coagulation Reveals Efficacy and Mechanism of Targeted Thrombomodulin. Submitted, 2017) which typically resulted in complete coverage of the micro-channels. Channels were treated with TNF-alpha (10 ng/mL) under flow (at shear stress of 5 dyne/cm2) for 6 hours to flow condition and induce activation prior to exposure to whole blood. Whole blood was obtained from healthy volunteer donors and collected into citrate collection tubes containing corn trypsin inhibitor (CTI, Essex Junction, CT). The indicated concentrations of recombinant proteins were added to the whole blood for hour at prior to perfusion through the microchannels. Flourescently labeled anti-fibrin antibodies and calcein AM were also added to blood 15 minutes before microfluidic assay to image fibrin deposition and leukocyte and platelet adhesion, respectively. Blood was flowed through the channels under conditions mimicking post capillary venules (5 dyne/cm2) for 20 minutes while images were continuously acquired. Controls and experimental conditions were compared on simultaneously run channels using a motorized stage for real-time acquisition. Images were analyzed using ImageJ for quantification of mean fluorescence intensity.

Osmotic and mechanical resistance assays

Osmotic and mechanical resistance was measured as previously described (Pan, D., et al., The Effect of Polymeric Nanoparticles on Biocompatibility of Carrier Red Blood Cells. PLoS One, 2016. 11(3): p. e0152074). In brief, human RBCs obtained from retained segments of donor RBCs were suspended in PBS at 5% hematocrit prior to incubation with various concentrations of antibodies or fusion proteins. The RBCs were then washed and exposed to osmotic or mechanical stress. Osmotic stress was induced by incubation in 64 mM NaCl solution, conditions that give approximately 50% hemolysis of normal RBCs. The suspensions were then centrifuged at 13,400 g and the resulting supernatants were assayed for hemoglobin content by measuring absorbance at 540 nm. Hemolysis of equivalent concentrations of RBCs in water was taken as 100% hemolysis. To measure mechanical stress, RBCs were similarly treated with antibodies and fusion proteins, resuspended at 1% hematocrit, and rotated in the presence of 8x4mm glass beads (Corning Pyrex, Corning, NY) for 1 hour at 37 C. The RBC suspension supernatants were then similarly analyzed spectrophotometrically for hemolysis.

Ekacytometry

Ektacytometry was performed using a RheoScan AnD system (Rheo Meditech, Seoul, Republic of Korea). In a typical experiment, 50 μL of 5% RBC or 5 μL of whole blood was suspended in 700 μL of a 5.5% (w/v) solution of 360 kDa poly-vinylpyrrolidine (Sigma Aldrich, St. Louis, MO) in PBS. A 500 μL sample within each microfluidic chamber was then analyzed per the manufacturer's protocol. The elongation indices at the corresponding shear stresses were then input into statistical software (Prism, GraphPad, San Diego, CA) and the data were fit using non-linear regression and a Streekstra-Bronkhost model (Baskurt, O.K. and H.J. Meiselman, Data reduction methods for ektacytometry in clinical hemorheology. Clin Hemorheol Microcirc, 2013. 54(1): p. 99-107) to derive the maximal elongation indices (EImax) and shear stress at half-maximal deformation (SS1/2).

Example 2: Synthesis of targeting ligands

Using antibody phage display, we identified non-human-primate Fab antibody fragments to antigenic determinants on human RBCs. By panning phage libraries on human RBCs, we produced a Fab/phage preparation with $>10^7$ RBC-specific clones capable of agglutinating human RBCs. By performing binding assays against rare RBC types lacking highly conserved antigens and epitopes, we identified the target antigens of >30 of these clones. At least 34 clones bound the Wright b (Wrb) epitope formed by a Band 3/GPA interaction, present on the RBCs of essentially 100% of the human population. The Wrb epitope, determined by the protein sequence of Band 3, is a site of association between Band 3 with GPA, and GPA expression is simultaneously required for its presence on the membrane (Huang CH, Reid ME, Xie SS, Blumenfeld OO. Human red blood cell Wright antigens: a genetic and evolutionary perspective on glycophorin A-band 3 interaction. Blood. 1996;87(9):3942-3947). At least 3 other clones bound to a highly-conserved epitope Rh17(Hr0) on human RhCE protein, also present on essentially 100% of the human population. Both these targets are specific for erythroid lineage (Rojewski MT, Schrezenmeier H, Flegel WA. Tissue distribution of blood group membrane proteins beyond red cells: evidence from cDNA libraries. Transfus Apher Sci. 2006;35(1):71-82; Huang CH, Reid ME, Xie SS, Blumenfeld OO. Human red blood cell Wright antigens: a genetic and evolutionary perspective on glycophorin A-band 3 interaction. Blood. 1996;87(9):3942-3947;

and Chou ST, Westhoff CM. The Rh and RhAG blood group systems. Immunohematology. 2010;26(4):178-186). We assessed the extent of humanness of the variable chains using T20 scores44; scores of 79.8 for VH and 93.5 for VL framework regions were calculated for the anti-Rh17(aRh17), and 86.0 for VH and 85.4 for VL framework regions were calculated for anti-Wrb (aWrb). These scores are comparable with 'humanized' antibodies (Gao SH, Huang K, Tu H, Adler AS. Monoclonal antibody humanness score and its applications. BMC Biotechnol. 2013;13:55) and therefore are encouraging with respect to potential lack of immunogenicity of derivatives of these ligands.

Example 3: Binding of ligands and cargoes to RBCs

The sequences of the variable fragment genes (amino acid sequences shown in Tables 2 and 5, nucleic acid sequences shown in Tables 3 and 6) were cloned into plasmids to produce single chain variants (scFv) of the parent Fab, as well as fusions of the scFv antibodies with human thrombomodulin (hTM-scFv). These scFvs and hTM-scFvs were produced with high purity as characterized by SDS gel electrophoresis and size-exclusion HPLC, with peaks consistent with the expected molecular weights (FIG. 1A and FIG. 1B). We then performed direct binding assays with radio-labeled and fluorescently-labeled scFv antibody fragments and fusion proteins (see Example 1). The aRh17 and aWrb scFvs and their corresponding TM fusions demonstrated similar binding affinities (KD 21-53 nM, FIG. 1C and FIG. 1D, and Table 7), as did both radio-iodinated and fluorescently-labeled proteins (FIG. 7A – FIG. 7D). The scFvs and fusion proteins bound to conserved epitopes on human, but not mouse, rat, or pig RBCs (FIG. 7A – FIG. 7D), and binding parameters (Kd, Bmax) were consistent between multiple donors. Binding saturated at the expected level of target expression (Bmax of 100,000 to 160,000 copies/RBC for aRh17 and 750,000 to 900,000 copies/RBC for aWrb) (Lomas-Francis C, Olsson ML. The blood group antigen factsbook: Elsevier/Academic Press; 2012). The dissociation rates were similar for both scFvs alone and their corresponding fusions, with >50% of the ligands remaining bound after 4 hours at 37 degrees (FIG. 1E and FIG. 1F, Table 7). We also examined effects of shear stress on scFv binding and the potential for ligand exchange onto unbound RBCs in whole blood under constant mixing (FIG. 8A – FIG. 8E and FIG. 9A – FIG. 9C). These experiments demonstrated that short periods of low (5 dyne/cm^2) and high (200 dyne/cm^2) shear in whole

blood did not alter scFv binding and that similar dissociation kinetics were seen in the presence of whole blood containing mostly unbound RBCs (with gradual exchange onto the unbound RBC population). Hemagglutination by an anti-TM secondary antibody was seen when hTM-scFv fusions were added at concentrations estimated to generate ~1000 copies of TM per RBC based on the calculated affinities (FIG. 1G). The fusion proteins alone did not induce aggregation or agglutination of RBCs in the absence of secondary anti-TM. Morphology of fusion protein loaded RBCs was confirmed on Wright-Giemsa stained peripheral blood smears and no morphologic abnormalities in the RBCs were noted (FIG. 10).

Table 7. Anti-Band3 and anti-RHCE antibody clones from phage library. scFv produced as H_2N-VH-(GGGGS)3-VL-FLAGx3-COOH.

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

Table 8. 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.

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)

Example 4: Effect of ligands and cargoes on RBC function

Having characterized the binding of the antibody fragments and fusion proteins to human RBCs, we then investigated how the binding of these ligands may affect several

parameters of RBC integrity including osmotic fragility, mechanical resistance, membrane deformability, exposure of phosphatidylserine, and generation of reactive oxygen species. These experiments were conducted at 5% hematocrit and with ligand:RBC ratios calculated to yield 10,000 and 100,000 copies/RBC for both ligands based on their affinity and the known concentration of RBC targets. These copy numbers are below saturation for both Wrb and Rh17.

We found that the two scFvs (and their corresponding thrombomodulin fusions) had significantly different effects on target RBCs. Targeting of Wrb, but not Rh17, by the antibody fragments induced a left-shift in osmotic fragility curves (EC50 122 vs 128 mOsm, $p<0.05$) with a pattern suggesting a whole population change rather than just a subset (FIG. 2A and FIG. 2B). We tested the dose-dependence of the observed changes in osmotic resistance using the EC50 of naïve RBCs (128 mOsm) (FIG. 2C and FIG. 2E) and again found that aRh17 did not produce changes in osmotic hemolysis at this osmolarity, while aWrb again decreased hemolysis. The changes in osmotic resistance were paralleled by an increase in hemolysis following mechanical stress for aWrb (FIG. 2D), but similarly, no change was seen after treatment with aRh17 (FIG. 2F). While the mechanical stress assay (Pan D, Vargas-Morales O, Zern B, et al. The Effect of Polymeric Nanoparticles on Biocompatibility of Carrier Red Blood Cells. PLoS One. 2016;11(3):e0152074) does not directly represent a pathophysiologic scenario, it is intended to reflect overall integrity of the RBC membrane architecture. Nearly identical effects were observed after treatment with the scFvs alone or with their corresponding TM fusions (FIG. 3A and FIG. 3B).

We then used ektacytometry to test whether effects on osmotic and mechanical fragility were mirrored by alterations in membrane deformability. In this technique, a decrease in the maximal elongation index (EImax) or an increase in the shear stress to reach half-maximal deformation (SS1/2) reflects an increase in RBC rigidity. As we expected, when ligands were bound to Wrb, there was a dose-dependent increase in RBC rigidity (FIG. 4), reflected in both increased SS1/2 (FIG. 4B and FIG. 4D) or decreased EImax (FIG. 11). This rigidifying effect was identical for TM-scFv fusions and scFvs alone, again demonstrating that the ligand, and not the TM cargo, induced these changes. Consistent with the mechanical and osmotic stress assays, binding of fusions or scFvs to RhCE did not

change ektacytometric curves or indices (FIG. 4) and the behavior of aRh17 treated RBCs was consistently identical to naïve donor RBCs.

The target-dependent effect of these ligands on membrane deformability raised the question of how targeting other RBC epitopes (particularly on GPA, given its ubiquity as an erythroid specific target) might affect RBC physiology. To probe this question, we produced anti-GPA antibodies and Fab fragments from a commercially available hybridoma, YTH89.146 (FIG. 12). After incubating human RBCs with the anti-GPA IgG antibodies or their monovalent Fabs, we observed similar rigidifying effects to those seen with aWrb ligands. Monovalent Fab induced a slight dose-dependent change in ektacytometric indices, while the parent antibody induced more marked changes in red cell rigidity (FIG. 13A – FIG. 13D). The Fab also induced a slight increase in hemolysis under mechanical stress, while also inducing a slight increase in hemolysis under hypo-osmolar conditions. Because prior studies loading drugs onto murine RBCs have largely relied on Ter119 or other GPA-associated ligands as the targeting agent, we also examined the effects of a scFv-TM fusion of this antibody on mouse RBCs (Zaitsev S, Kowalska MA, Neyman M, et al. Targeting recombinant thrombomodulin fusion protein to red blood cells provides multifaceted thromboprophylaxis. Blood. 2012;119(20):4779-4785). As with targeting of human Wrb or glycophorin A, Ter119-TM fusions decreased deformability of murine RBCs (increased SS1/2, decreased EImax) as a monovalent fusion protein (Ter119-mTM), and markedly so as the parent IgG antibody (FIG. 14A – FIG. 14D). As with the human ligands, these changes in deformability were accompanied by changes in susceptibility to osmotic and mechanical stress.

To address the generalizability of the observed deformability effects of the Band 3, GPA, and RhCE ligands, we also compared the ektacytometric effects of a range of full-length IgG antibodies covering different epitopes on these membrane targets. For this purpose, we used BRIC69 (anti-RHCE, mouse IgG1), BRAD2 (anti-D, human IgG1), BRAD3 (anti-D, human IgG3), FOG1 (anti-D, human IgG1), BIRMA84b (anti-Wrb, mouse IgG3), BRIC14 (anti-Wrb, mouse IgG2a), YTH89.1 (anti-GPA, rat IgG2b), BRIC256 (anti-GPA, mouse IgG1), and BRIC200 (anti-Band3, mouse IgG1). In agreement with prior studies[26-28,31], we found that all IgGs tested against epitopes on GPA and Band3 induced decreases in deformability, while antibodies to RhCE and RhD (on serologically confirmed

RHD positive RBC donors) showed minimal change from naïve RBCs (FIG. 5A – FIG. 5C). Although all IgGs were added at a ratio of approximately 104 mAbs per RBC (10 nM mAb in a 5% RBC suspension), the differences in affinities of these clones would likely result in different numbers of bound copies and, therefore, the relative degrees of rigidification as a function of bound copy numbers remained uncertain. To address this, we selected representative anti-RhCE (BRIC69) and anti-Wrb (BRIC14) IgG antibodies and performed additional dose-titration experiments to show that when the anti-RhCE antibodies were added at ratios below saturation and which resulted in similar total numbers of bound IgG as anti-Wrb antibodies (FIG. 5D – FIG. 5F), no change in SS1/2 was seen for anti-RhCE while anti-Wrb showed significant, dose-dependent rigidification.

Additional characterization of the effects of the scFvs and fusions on RBCs included assays of PS surface exposure, as measured by annexin V binding, and ROS generation. Binding of both scFvs and hTM-scFv fusions did not lead to detectable increase in PS exposure (FIG. 15A). None of the scFv ligands examined demonstrated detectable induction of ROS generation by a dihydrorhodamine-based assay (FIG. 15B).

Example 5: Therapeutic effectiveness of RBC cargoes

Having examined the effects on aWrb and aRh17 scFvs and their respective TM fusion proteins on human RBC physiology, we next compared the enzymatic activity and therapeutic efficacy of these fusions. In solution, fusion proteins demonstrated APC generative capacity identical to soluble TM in the presence of human protein C and thrombin (FIG. 16). Fusion proteins were then pre-bound to human RBCs at saturating concentrations and their capacity to generate APC was measured as a function of RBC concentration. The fusions generated a RBC-dose dependent increase in APC generation by carrier RBCs (FIG. 6A). Using a standard curve generated with soluble TM, the Wrb-coupled RBC-TM generated roughly 100,000 soluble TM ‘equivalents’ per loaded RBC at saturation while the RhCE-coupled RBC-TM generated 50,000. Therefore, although Wrb-coupled TM would be predicted to carry 5- to 10-fold more copies of the fusion per RBC at saturation, the APC generating capacity was only 2-fold higher. We then reversed these conditions such that RBCs and target epitopes were at excess (50 nM fusion in 20% Hct, approximately 10,000 copies/RBC), which would drive fusions to be essentially completely RBC-bound. At these

high concentrations of RBCs, comparable to the circulatory environment, APC generation was similar for both fusion proteins and comparable to that seen for soluble TM, although a slight reduction was seen for hTM-aBand3/GPA and not hTM-aRhCE (FIG. 6B). These results confirm that the fusions maintain their enzymatic activity when coupled to RBCs, and suggest that RhCE-coupled TM may better conserve specific activity.

We then tested the therapeutic activity of hTM/scFv fusions bound to human RBC in a microfluidic model of microvascular inflammatory thrombosis that permits assessment of human-targeted therapeutics in whole blood in a system simulating human vessels[37]. In this model, fully endothelialized micro-channels are activated with an inflammatory mediator (e.g. TNF-α), inducing leukocyte and platelet adhesion and widespread fibrin generation when the channels are exposed to flowing human whole blood. We hypothesized that if the fusions maintain their activity in whole blood, they would significantly reduce fibrin and platelet deposition in response to inflamed endothelium. To do so, we added 200 nM of each fusion protein (and soluble TM as a control) to whole blood (a ratio of approximately 25,000 copies of TM per RBC at normal RBC counts). Both fusions significantly reduced fibrin deposition (measured by red fluorescence) in response to TNF-α activation (FIG. 6C). Channels exposed to Wrb-targeted fusions, as compared to RhCE-targeted, showed a slight increase in mostly platelet-associated fibrin deposition at the end of the perfusion period (20 minutes), but both remained significantly reduced compared to untreated controls and similar to soluble TM (data not shown). Additional analysis of fluorescence from calcein AM labeling (leukocytes and platelets) demonstrated that RhCE targeted hTM-scFv was more effective than the Wrb targeted fusion at reducing platelet and leukocyte adhesion (FIG. 6D), with efficacy of hTM-aRhCE again similar to soluble TM. Hypothesizing that the increase in calcein signal and late fibrin generation in hTM-aWrb compared to hTM-aRhCE was a result of rigidifying effects of the aWrb, we performed additional experiments in this model using the aWrb and aRHCE scFvs alone (not fused to TM), and demonstrated that after 15 minutes of flow, activated channels exposed to aWrb treated blood showed greater platelet and leukocyte accumulation compared to that treated with aRh17 (FIG. 6E), suggesting the difference in efficacy of hTM-aRHCE and hTM-aWrb is due to aWrb promotion of leukocyte and platelet adhesion rather than a loss of efficacy of the appended TM. We also confirmed

that RBC rigidification was seen at this ratio of scFv to RBC in whole blood (FIG. 15A – FIG. 15B).

Example 6:

As a critical step in the translation of RBC-targeted therapeutic fusion proteins to clinical practice, we designed human RBC-specific fusion proteins based on scFvs derived from non-human-primate antibody phage-display libraries. Using this technique, we generated antibodies against highly conserved, erythroid-specific epitopes on Band3/GPA (Wrb) and RhCE (Rh17) proteins. Both epitopes are on multi-pass transmembrane proteins and exist predominantly within discrete multiprotein complexes. While Wrb is more widely distributed between Band3/ankrin complexes, junctional complexes, and free forms, Rh17 (as part of RhCE) exists largely within Band/ankrin complexes[47]. Wrb has been localized to a juxtamembrane site of interaction between GPA and Band3, but the precise epitope for Rh17, which is defined serologically, is unknown. Both antibody fragments and their respective TM fusions showed affinities sufficient to drive rapid, complete binding in whole blood, where concentrations of their targets are >1 μM. While only a slight increase in off-rate was noted for TM fusion proteins, interaction with TM binding partners (thrombin, PF4, protein C) may promote dissociation in whole blood under flow, which was not directly assessed this in the present study. The primate origin of these ligands is expected to confer less immunogenicity than non-engineered murine monoclonal antibodies or foreign peptides, but further data would be required to support this.

Targeting of Band3/GPA (Wrb) led to changes in RBC membrane deformability, mechanical resistance, and osmotic resistance, while RhCE-targeted fusions and antibody fragments did not perturb any of the physiologic parameters assessed in this study. Membrane effects were shared, to varying extents, by other GPA and Band 3 ligands against human and murine RBCs, including Ter119, particularly for bivalent IgG ligands. In contrast, antibodies against RhD and RhCE failed to demonstrate significant rigidification of human RBCs. Antibodies against GPA and RhD also produce markedly different effects on different subsets of phagocytic cells[32], and while the authors hypothesized that copy number was critical, the current findings suggest that altered deformability may have also been contributory. The precise function of RhCE has been difficult to define[48] and a large diversity of

polymorphisms have been described[43]. Individuals expressing RhD but not RhCE (rare D--phenotype) show modest alteration of membranes without overt RBC or clinical phenotypes[49]. While homologous proteins participate in ammonia/ammonium transport and acid/base balance, RhCE and RhD do not [50,51]. Band 3 and GPA are highly expressed membrane proteins important for structural membrane complexes and ion exchange, and carriage of sialoglycoproteins, respectively. In this context, the apparent "unresponsiveness" of RBCs bound by RhD/RhCE-targeted ligands is consistent with a lack of recognized function in mature RBCs.

As a representative therapeutic, we coupled TM to both scFvs. TM shows promise in the treatment of sepsis[52] and RBC-coupled TM has demonstrated superiority to soluble TM in mouse models[20,21]. Coupling TM to either epitope resulted in efficacious RBC drug carriers as measured by enzymatic activity and in a humanized microfluidic model of inflammatory thrombosis. However, RhCE-coupled TM showed higher specific activity in vitro and improved efficacy in our microfluidic model. The reasons for the difference in enzymatic activity may reflect spatial localization, as the Wrb epitope is immediately adjacent to the RBC membrane which may limit substrate accessibility, while the precise Rh17 epitope localization is unknown. The difference in efficacy in our humanized microfluidic model was unexpected, but because cellular rigidity has significant effects on margination of red cells, white cells, and platelets within the vascular lumen, and decreased RBC deformability can drive increased platelet adhesion[53,54], we speculate that the difference in efficacy reflects the observed difference in membrane effects. Our observation of higher platelet adhesion after treatment with Wrb-targeted scFv is consistent with this phenomenon. The potential for drug or antibody loading of RBCs to affect their intravascular distribution and margination of cellular components, and how this distribution affects their therapeutic efficacy, warrants further investigation.

RBCs can respond to their environment in diverse ways including dynamic changes in linkage of membrane protein complexes[55], phosphorylation of membrane and cytoskeletal components[56-59], calcium influx[60,61], PS exposure[29,62], and oxidative stress responses[30]. In targeting RBCs for delivery of therapeutics, the present findings suggest that dose and target dependent changes in membrane physiology, and ultimately, circulatory behavior should be carefully considered [24-29,63]. As increases in RBC rigidity can result in an override of the

CD47/SIRPA interaction[64], these factors may also play a role in RBC interactions with host defenses and immune response. This is especially important because RBC drug carriers are drawing increased attention for their apparent ability to modulate immune responses and even induce immune tolerance[13-15]. However, while ligands to murine RBCs have been explored (e.g. Ter119, ERY1) in this approach, application to human RBCs has not been well-developed.

Based on the current findings, RhCE (on Rh17) may be a particularly attractive target for surface-loading of RBCs given its erythroid specificity, high copy number, apparent lack of adverse impact on RBC physiology, and presence on the RBCs of essentially 100% of the human population. The therapeutic efficacy of hTM targeted to human RBCs on either epitope was comparable to soluble TM, and was optimal when coupled to RhCE. The ligands described in the present study offer a new set of biochemical tools for optimizing the delivery of therapeutics by human RBCs.

Example 7: Red blood cell targeting of liposomes provides markedly enhanced circulation

Liposomes and other nanoparciles are limited by rapid reticuloendothelial system uptake and poor circulation. Red blood cells are natural long-circulating (~120 days in humans) carriers. Targeting liposomes to red blood cells may offer the ability to prolong their circulation. Red blood cell targeting must be carefully controlled with respect to target epitopes, binding affinities and loading ratios to maximize biocompatibility.

RBC-targetable liposomes were synthesized to include site-specifically modified RBC-targeting antibody fragments (scFv). Copper-independent click chemistry coupling allowed for precise control of ligand loading. Targeting via scFv and IgG was compared. Radiolabeled liposomes were loaded onto mouse RBCs bod in vivo by direct intravenous injection and ex vivo onto isolated RBCs before transfusion. Biocompatibility was assessed by agglutination assays and ektacytometry to determine membrane disruptive effects.

RBC-targeted liposomes are maintained in circulation significantly longer than conventional ‘stealth’ liposomes. Whole animal biodistribution of Ter119-liposomes (100-200 scFv:liposome) loaded onto RBCs in vivo by direct injection into the blood stream (blue) or unconjugated PEGylated liposomes (red) (FIG. 19A). For in vivo loading liposomes were

injected at a ratio of approximately 50 liposomes per RBC. Blood PK curves demonstrate that the large majority of both in vivo loaded Ter119-liposomes (blue) are maintained in circulation at 3 hours and gradually drop off over 24 hours (FIG. 19B). Compared to traditional "stealth" liposomes (red), there is approximately a 2-fold increase in area under the curve ($p<0.05$). Ter-119 liposomes are found mostly (>80%) in the RBC pellet of collected blood and gradually clear this compartment while free liposomes are largely in the plasma fraction (FIG. 19C).

These data demonstrate that RBC-targeted liposomes markedly prolonged the circulation of liposomes compared to traditional "stealth" technology (FIG. 19A – FIG. 19C). Circulation of ex vivo liposome loaded RBCs is dependent on the number of loaded nanocarriers. (FIG. 20A and FIG. 20B). RBC-bound liposomes circulate predominantly on the RBC surface over the initial 12 hours after which they are gradually cleared. Mechanisms of clearance remain uncertain. High loading induces RBC agglutination (FIG. 21A and FIG. 21B). Circulation is dependent on low loading ratios. scFv-liposomes provide superior circulation (FIG. 22) and better preserve normal RBC membrane physiology compared to IgG-liposomes (FIG. 23). Normal membrane deformability is both loading-ratio and target dependent.

Example 8: Rh17 recognizes a linear epitope in human RhCE.

A Western blot was performed to assess the binding of Rh17 to proteins extracted from mouse and human erythrocyte ghosts (FIG. 25). Because proteins were denatured in reducing SDS-PAGE buffer prior to gel electrophoresis, the presence of binding is due to interaction with linear, and not conformational, epitopes. This is in contrast to anti-RhCE mAbs described by other groups, which recognize conformational epitopes.

Rh17 recognizes an epitope present in the 6th extracellular loop of human RhCE. Flow cytometry was used to assess the binding of Rh17 to human erythrocytes in the presence and absence of linear peptides corresponding to the amino acid sequence 6th extracellular loop of human RhD (negative control) and human RhCE (FIG. 26). A decrease in binding signal only in the presence of the RhCE-derived peptide demonstrates that the 6th extracellular loop of RhCE is involved in the binding of Rh17 to human erythrocytes.

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All publications cited in this specification are incorporated herein by reference in their entireties as is US Provisional Patent Application No. 62/594,909, filed December 5, 2017. Similarly, the SEQ ID NOs which are referenced herein and which appear in the appended Sequence Listing are incorporated by reference. While the invention has been described with reference to particular embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

Table 1: Anti-RHCE antibody CDRs

	VH						VL					
Clone	CDR1	SID NO.	CDR2	SID NO.	CDR3	SID NO.	CDR1	SID NO.	CDR2	SID NO.	CDR3	SID NO.
KP3-11	GASISNYW	1	IDGSTYST	2	AREGQDPLAPTLATSGSGLDS	3	ENVNNY	4	AAS	5	QHSYGTPLT	6
KP3-14	GASISNYW	7	IDGSTYST	8	AREGQDPLAPTLATSGSGLDS	9	QDIYSN	10	GAS	11	QEVHRNPFT	12
KP3-17	GAPISNYW	13	IDGSIYTT	14	AREGQNPLVPTYGSTGFGLDF	15	QGISSW	16	KAS	17	QQYSSSPRT	18

Table 2: Anti-RHCE antibody heavy and light variable chain protein sequences

Clone	VH	SID	VL	SID
KP3-11	EVQLLESGPGLVKPSETLSLTCGVSGASISNYWWSWIRQSPGKGL EWIGEIDGSTYSTHYNPSLKGRVTISKDASKNQLSLRLTSVTAADT AVYYCAREGQDPLAPTLATSGSGLDSWGRGLVVSVSS	19	AAELQMTQSPSSLSASLGDRVTITCRASENVNNYLH WYQQKPGKAPKLLIYAASTLQSGVPSRFSGSGSGTD FTLTISSLQPEDVATYYCQHSYGTPLTFGGGTKVEIK	20
KP3-14	EVQLLESGPGLGKPSETLSLTCGVSGASISNYWWSWIRQSPGKGL EWIGEIDGSTYSTHYNPSLKGRVTISKDASKNQLSLRLTSVTAADT AVYYCAREGQDPLAPTLATSGSGLDSWGRGLVVTVSS	21	AAELQMTQSPSALSASVGDRVTISCRASQDIYSNLA WYQQKPGKAPKLLIYGASRLQSGIPSRFSASGAGTE FTLTISGLQPEDSAVYYCQEVHRNPFTFGPGTKLDIK	22
KP3-17	EVQLLESGPGLLKPSETLSLTCAVSGAPISNYWWSWIRQSPGKGL EWIGEIDGSIYTTYYNPSLKSRVAISKDTSKNRLSLKLTSVTAADTAV YYCAREGQNPLVPTYGSTGFGLDFWGHGLAVTVSS	23	AAELTQSPSSLSASVGDRVTITCQASQGISSWLAWY QQKPGKAPKLLIYKASSLQSGVPSRFSGSGSGTDFTL TISSLQSEDFATYYCQQYSSSPRTFGQGTKVEIK	24

Table 3: Anti-RHCE antibody heavy and light variable chain coding sequences

Clone	VH	SEQ ID	VL	SEQ ID
KP3-1-11	GAGGTGCAGCTGCTCGAGTCAGGTCCAGGACTGGTGAAGCCTTCGG AGACCCTGTCCCTCACCTGCGGTGTCTCTGGTGCCTCCATCAGTAATT ACTGGTGGAGTTGGATCCGCCAGTCCCCAGGGAAGGGACTGGAGT GGATTGGGGAGATCGATGGTAGTACTTATAGCACCCACTACAACCC CTCCCTCAAGGGTCGAGTCACCATTTCAAAAGACGCGTCCAAGAATC AGTTGTCCCTGAGGCTGACCTCTGTGACCGCCGCGGACACGGCCGT GTATTATTGTGCGAGAGAGGGACAGGATCCTTTAGCGCCTACCCTT GCCACGTCGGGTTCGGGGTTGGATTCCTGGGGCCGAGGGCTCGTCG TCTCCGTCTCCTCC	2 5	GCGGCCGAGCTCCAGATGACCCAGTCTCCATCCTCCCTATCTGCATC GCTGGGAGACAGAGTCACCATCACTTGCAGGGCAAGTGAGAACGTT AACAACTATTTACATTGGTATCAGCAGAAACCAGGGAAAGCCCCTA AGCTCCTGATCTATGCTGCATCCACTTTGCAAAGTGGGGTCCCATCA AGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCA GCAGCCTGCAGCCTGAAGATGTTGCAACTTATTACTGTCAGCATAGT TATGGTACCCCGCTCACTTTCGGCGGAGGGACCAAGGTGGAGATCA AACGA	26

KP314-1	GAGGTGCAGCTGCTCGAGTCAGGCCCAGGACTGGGGAAGCCTTCG GAGACCCTGTCCCTCACCTGCGGTGTCTCTGGTGCCTCCATCAGCAA TTACTGGTGGAGCTGGATCCGCCAGTCCCCAGGGAAGGGACTGGA GTGGATTGGGGAGATCGATGGTAGTACTTATAGCACCCACTACAAC CCCTCCCTCAAGGGTCGAGTCACCATTTCAAAAGACGCGTCCAAGAA TCAGTTGTCCCTGAGGCTGACCTCTGTGACCGCCGCGGACACGGCC GTGTATTATTGTGCGAGAGAGGGACAGGATCCTTTAGCGCCTACCC TTGCCACGTCGGGTTCGGGGCTGGATTCCTGGGGCCGAGGCCTCGT CGTCACCGTCTCCTCC	27	GCGGCCGAGCTCCAGATGACCCAGTCTCCATCTGCCTTGTCTGCATC TGTAGGAGACAGAGTCACCATCTCTTGCCGGGCAAGTCAGGACATT TATAGTAATTTAGCGTGGTATCAACAGAAACCAGGGAAAGCCCCTA AGCTCCTGATCTATGGCGCATCCAGATTGCAAAGTGGGATTCCCTCT CGGTTCAGTGCTAGCGGAGCTGGGACAGAATTCACTCTCACCATCA GCGGCCTGCAACCTGAAGATTCTGCAGTATATTACTGTCAAGAGGTT CATCGTAACCCATTCACTTTCGGCCCCGGGACCAAACTGGATATCAA ACGA	28

KP3-117	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGCTGAAGCCATCG GAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTGCCCCCATCAGTAA CTACTGGTGGAGTTGGATTCGTCAGTCCCCAGGGAAGGGACTGGAG TGGATTGGGGAGATCGATGGTAGTATATACTACTACTACAACCC CTCCCTCAAGAGTCGAGTCGCCATTTCAAAGGACACGTCCAAGAACC GGCTGTCCCTGAAACTGACCTCTGTGACCGCCGCGGACACGGCCGT CTATTATTGTGCGAGAGAGGGCCAGAACCCTCTAGTGCCTACATATG GTTCGACGGGATTCGGATTGGATTTCTGGGGCCATGGACTCGCCGT CACCGTCTCGTCA	29	GCGGCCGAGCTCACCCAGTCTCCATCTTCCTTGTCTGCATCTGTAGG AGACAGAGTCACCATCACTTGCCAAGCCAGTCAGGGTATTAGCAGC TGGTTAGCCTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCC TGATCTATAAGGCATCCAGTTTGCAAAGTGGGGTCCCATCAAGGTTC AGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCCT GCAGTCTGAAGATTTTGCAACTTATTACTGTCAACAGTATAGCAGTA GCCCTCGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA	30

Table 4: Anti-BAND 3 antibody CDRs

	VH						VL					
Clone	CDR1	SID	CDR2	SID	CDR3	SID	CDR1	SID	CDR2	SID	CDR3	SID
KP2-01	GDSISSGLG	31	IGGSRGNT	64	ARRAPYWGYSYLDY	97	QSIGSS	130	SAY	163	QQYNDLLPLT	196
KP2-02	GGSLSGGYD	32	IYDSRWTT	65	ARRGGYGASYFDL	98	QSIGSH	131	SVS	164	QQYNDLLPLT	197
KP2-04	GYSLSSAYG	33	IGGSRDNV	66	VRRATYGNSYFDS	99	QSVGSH	132	SAY	165	QQYNDLLPLT	198
KP2-06	GSSLSSAYG	34	IGGSRDNT	67	AQRGAYGYSYFDY	100	QSVGSS	133	SIS	166	QQYNDFFPLT	199
KP2-07	GDSISSGYG	35	IGGSRGTT	68	ARDSGYSFRYFDF	101	QSVGSN	134	SAY	167	QQYNDLLPLT	200
KP2-08	GYSISSGYG	36	IGGSRDNT	69	ARDGGYGSRYMDS	102	QSIGTS	135	SAY	168	QQYNDLLPLT	201
KP2-09	GYSISSGYG	37	IGGSRGNT	70	ARDSGYNTRYFDY	103	QSVGSR	136	GAS	169	QQYNDLLPLT	202
KP2-11	GSSLSSAYG	38	IGGSRDNT	71	AQRGAYGYSYFDY	104	QSLGSR	137	GAS	170	QQYNDFPPLT	203
KP2-13	GGSISGGYD	39	IYDSRGTT	72	ARRAGYGSAYFDY	105	QSIGTN	138	TAY	171	QQYNDLLPLT	204
KP2-14	GSSLSSAYG	40	IGGSRDNT	73	ARRGAFGNSYFDY	106	ESVGSS	139	SAS	172	QQYNDLLPLT	205
KP2-15	GGSISGGYD	41	IYDSRGTT	74	ARRAGYGSAYFDY	107	QTVGRN	140	SAH	173	CQQYNDLLPLTF	206
KP2-17	GYSISSGYG	42	IGGSRGNA	75	ARDGGYGERYLEF	108	QSIGSS	141	FAS	174	HQSSSFPWT	207
KP2-18	GNSISSGYG	43	IGGSRSNT	76	ARDWGYGYRYLDY	109	QSIGSS	142	YAS	175	QQSSSFPFT	208

KP2-19	GGSINGGYD	44	IYGSRGT	77	AKRVGYGNSYFDS	110	QSVSSR	143	DAS	176	CQQYNDLLPLTF	209
KP2-20	GGSISGGYD	45	IYDSRGT	78	ARRAGYGSAYFDY	111	QSVGSN	144	SGS	177	QQYNDLLPLT	210
KP2-22	GDSISSGYG	46	IGGSRGNT	79	ARRAPYWGYSYLDY	112	QSIGTN	145	SAY	178	QQYNDLLPLT	211
KP2-23	GSSLSSAYG	47	IGGSRDNT	80	AQRGAYGYSYFDY	113	QTVGRN	146	SAY	179	QQYNDLLPLT	212
KP2-24	GYSISSGYG	48	FGGSRGNT	81	ARDSGYSRRWVDY	114	QSVGTN	147	SAY	180	QQYNDLLPLT	213
KP3-01	GFSISSDYG	49	IGGSRGNT	82	ARDWGYGYRYFDF	115	QSVGSN	148	YAS	181	QQTNTFPWT	214
KP3-02	YSISSGYG	50	IGGSRGNT	83	ARDSGYNTRYFDY	116	QSVGSN	149	SAY	182	QQYNDLLPLT	215
KP3-03	GSSLSSAYG	51	IGGSRDNT	84	AQRGAYGYSYFDY	117	QSVGSY	150	GAY	183	QQYNDLLPLT	216
KP3-05	GSSLSSAYG	52	IGGSRDNT	85	AQRGAYGYSYFDY	118	QSVGSS	151	SAY	184	HQYNDLLPLT	217
KP3-06	GGSISSAS	53	ISGSGSPT	86	ARRGGYGNRYFDY	119	QSVGSS	152	SAY	185	QQYNDLLPLT	218
KP3-07	GSSLSSAYG	54	IGGSRDNT	87	AQRGAYGYSYFDY	120	QSIGSN	153	SAN	186	QQYNDFLPLT	219
KP3-08	GSSLSSAYG	55	IGGSRDNT	88	AQRGAYGYSYFDY	121	QSLGGR	154	GAS	187	QQYNDFLPLT	220
KP3-09	SLSLSSGFA	56	IGGSRDNV	89	VTIHGYRNWYLDH	122	QSIGTS	155	SAY	188	QQYNDLLPLT	221
KP3-12	GNSISSAYG	57	IGGSRGTT	90	ARDSGYSFRYFDF	123	QSIGTN	156	SAY	189	QQYNDLLPLT	222
KP3-13	GGSLSGGYD	58	IYDSRGTT	91	ARRGGYGASYFDL	124	QSVGSN	157	SAS	190	QQYNDFFPLT	223
KP3-15	GSSLSSAYG	59	IGGNRDNT	92	AQRGAYGYSYFDY	125	QTVGRN	158	SAH	191	QQYNDLLPLT	224

KP3-16	GSSLSSAYG	60	IGGSRDNT	93	AQRGAYGYSYFDY	126	QSLGSR	159	GAS	192	QQYNDFLPLT	225
KP3-18	GYSLSSAYG	61	IGGSRDNV	94	VRRATYGNSYFDS	127	QSVGSY	160	SAH	193	QQYNDLLPLT	226
KP3-19	GGSLSGGYD	62	IYDSRGTT	95	ARRVGYGATYFDL	128	QSVGSN	161	SAN	194	QQYNDLLPLT	227
KP3-20	GYSISSGFA	63	IGGSRDNT	96	ARRGAYGNSYFDF	129	QSVGSN	162	SAY	195	QQYNDLLPLT	228

Table 5: Anti-Band 3 antibody heavy and light variable chain protein sequences

Clone	VH	SID	VL	SID
KP2-01	EVQLLESGPGLVKPSETLSLTCAVSGDSISSGLGWSWIRQTPGKGLEWIGYIGGSRGNTNYNPSFKSRVTISRDTSKNQFSLRLSSMTAADTAVYYCARRAPYWGYSYLDYWGQGVLVTVSS	229	AAELTQSPATLSLSPGETATLSCRASQSIGSSLAWYQQRPGQAPKLLVHSAYFRAAGIPDRFSGSGSRTDFTLTISSLEPEDVGVYHCQQYNDLLPLTFGGGTKVELK	262
KP2-02	EVQLLESGPGLVKPSETLSLTCAVSGGSLSGGYDWSWIRQSSRKGLEWIGYIYDSRWTTNYNPSLKKRVTISIDTSKNQFSLNLKSVTAADTAVYYCARRGGYGASYFDLWGQGVLVTVSS	230	AAELTQSPATLSLFPGETATLSCRASQSIGSHLAWYQQKPGQAPKLLVHSVSFRATGIPDRFRGSGSRTDFTLTISSLEPEDVGVYHCQQYNDLLPLTFGGGTKVEIK	263
KP2-04	EVQLLESGPGLVKPSETLSLTCAVSGYSLSSAYGWNWIRQSPGKGLEWIGSIGGSRDNVNYNPSLKRRVTISKDTSTNHFSLRLSSVTAADTAVYYCVRRATYGNSYFDSWGQGVQVTVSS	231	AAELTLTQSPATLSLSPGETATLSCRASQSVGSHLAWYQQKPGQAPKLLVHSAYFRATGIPDRFSGSGSRTDFTLTISSLEPEDVGVYHCQQYNDLLPLTFGGGTKVEIK	264
KP2-06	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLEWIGSIGGSRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYYCAQRGAYGYSYFDYWGQGVLVAVSS	232	AAELTQSPATLSLSPGETATLSCRASQSVGSSLAWYQQKPGQAPKLLVHSISVRATGIPDRFSGSGSRTDFTLTITSLEPEDVGVYHCQQYNDFFPLTFGGGTKVEIK	265
KP2-07	EVQLLESGPGLVKPSETLSLTCAVSGDSISSGYGWHWIRQVPGRGLEWIGSIGGSRGTTNYNPSLKSRVTISEDTSKNQFSLSLRSVSAADTAVYFCARDSGYSFRYFDFWGQGVLVTVSS	233	AAELVMTQSPATLSLSPGETATLSCRASQSVGSNLAWYQQKPGQAPKLLVHSAYFRATGIPDRFSGSGSRTDFTLTISSLEPEDVGVYHCQQYNDLLPLTFGGGTKVEIN	266

KP2-08	EVQLLESGPGLVKPSETLSLTCAVSGYSISSGYGWNWIRQPPGKGLEWIGSIGGSRDNTNYNPSLKSRVTLSKDTSKNHFSLRLRSVTAADTAVYYCARDGGYGSRYMDSWGQGVLVAVSS	23 4	AAELTQSPATLSLSPGEAATLSCRASQSIGTSLAWYQQKPGQAPRLLVHSAYFRATGIADRFSGSGSRTDFTLTISSLEPEDVGVYYCQQYNDLLPLTFGGGTKVEIK	26 7
KP2-09	EVQLLESGPGLVRPSETLSLTCAVSGYSISSGYGWHWIRQPPGKGLESLGYIGGSRGNTNYNPSLKSRVTISTDTSKNQFSLKLRSVTAADTAVYYCARDSGYNTRYFDYWGQGVLVTVSS	23 5	AAELTQSPATLSLSPGERATLSCRASQSVGSRLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSRTDFTLTISSLEPEDVGVYHCQQYNDLLPLTFGGGTKVEIK	26 8
KP2-11	EVQLQLPGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLEWIGSIGGSRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYYCAQRGAYGYSYFDYWGQGVLVAVSS	23 6	AAELTLTQSPATLSLSPGETATLSCRASQSLGSRLAWYQQKPGQPPRLLIYGASTRATGIPDRFSGSGSRTDFTLTISSLEPEDVGVYHCQQYNDFPPLTFGGGTKVEIK	26 9
KP2-13	EVQLLESGPGLVKPSETLSLTCAVSGGSISGGYDWSWIRQSPGKGLEWIGYIYDSRGTTNYNPSLRKRVAISIDTSRNQFSLNLRSLTAADTAVYYCARRAGYGSAYFDYWGQGVLVTVSS	23 7	AAELTQSPATLSLAPGETATLSCRASQSIGTNLAWYHQKPGQPPKLLVHTAYVRATGIPNRFSGSGSRTDFTLTINSLQPEDVGVYHCQQYNDLLPLTFGGGTKIDIK	27 0
KP2-14	EVQLLESGPGLVKPSETLSLTCAVSGSSLSSAYGWNWIRQAPGKRLEWIGFIGGSRDNTNYNPSLRSRVTISKDTSKNHFSLKLTSVTAADTAVYFCARRGAFGNSYFDYWGQGVPVTVSS	23 8	AAELTQSPATLSLSPGETATLSCRASESVGSSLAWYHQKPGQAPRLLVHSASFRATGIPDRFSGSGSRTEFTLTVSSLEPEDVGVYHCQQYNDLLPLTFGGGTKVEIK	27 1
KP2-15	EVQLLESGPGLVKPSETLSLTCAVSGGSISGGYDWSWIRQSPGKGLEWIGYIYDSRGTTNYNPSLRKRVTISIDTSRNQFSLKLRSLTAADTAVYYCARRAGYGSAYFDYWGQGVLVTVSS	23 9	AAELTQSPATLSVSPGEAATLSCRASQTVGRNLAWYQQKPGQAPKLLVHSAHFRATGIPDRFSGSGSGTDFTLTISSLEPEDAGIYHCQQYNDLLPLTFGGGTKVEIK	27 2

KP2-17	EVQLLESGPGLVKPSETLSLTCAVSGYSISSGYGWTWIRQPPGKGLEW IGYIGGSRGNANYNPSLKSRVTISKDTSKNQFSLKLTSVTAADTAVYYC ARDGGYGERYLEFWGQGALVTVSS	24 0	AAELTQSPAFRSVTLKEKVTITCQASQSIGSSLHWYQQKPDQS PKLLIKFASQSISGVPSRFSGSGYGTDFTLTINSLEAEDAATYYC HQSSSFPWTFGQGTKVEIK	27 3
KP2-18	EVQLLESGPGLVRPSETLSLTCTVSGNSISSGYGWNWIRQPPGKGLELI GYIGGSRSNTNYNPSLKSRVTISIDTSKNQFSLKLRSVTAADTAVYYCA RDWGYGYRYLDYWGQGVLVTVSS	24 1	AAELTQSPAFRSVTLKEKVTITCQASQSIGSSLHWYQQKPDQS PKLLIKYASQSISGVPSRFSGSGSGTDFTLTINSLEAEDAATYYC QQSSSFPFTFGPGTKLDIK	27 4
KP2-19	EVQLLESGPGLVKPSETLSLTCAVSGGSINGGYDWTWIRQSPGKGLQ WIGWIYGSRGTTNYNPSLRNRVTISIDTSRNQFSLRLSSLTAADTAVYY CAKRVGYGNSYFDSWGQGVLVTVSS	24 2	AAELTLTQSPATLSLSPGERATLSCRASQSVSSRLAWYQQKPG QAPRLLIYDASSRVTGIPDRFSGSGSGTDFTLTISSLEPEDVGV YHCQQYNDLLPLTFGGGTKVEIK	27 5
KP2-20	EVQLLESGPGLVKPSETLSLTCAVSGGSISGGYDWSWIRQSPGKGLE WIGYIYDSRGTTNYNPSLRKRVTISIDTSRNQFSLKLRSLTAADTAVYYC ARRAGYGSAYFDYWGQGVLVTVSS	24 3	AAELTQSPATLSLSPGETATLSCRASQSVGSNLAWYQQKPGQ APKLLVHSGSVRATGIPDRFSGSGSRTDFTLIISSLEPEDVGVY HCQQYNDLLPLTFGGGTKVEIK	27 6
KP2-22	EVQLLESGPGLVKPSETLSLTCAVSGDSISSGYGWSWIRQTPGKGLEW IGYIGGSRGNTNYNPSLKSRVTISKDTSKNQFSLKLSSVTAADTAVYYC ARRAPYWGYSYLDYWGQGVLVTVSS	24 4	AAELTLTQSPATLSLAPGETATLSCRASQSIGTNLAWYHQKPG QSPKLLVHSAYVRATGIPDRFSGSGSRTDFTLTINSLQPEDVGV YHCQQYNDLLPLTFGGGTKVEIK	27 7
KP2-23	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLEWIG SIGGSRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYYCAQR GAYGYSYFDYWGQGVLVAVSS	24 5	AAELTLTQSPATLSLSPGETATLSCRASQTVGRNLAWYQQRPGQ APNLLVHSAYFRATGIPDRFSGSGSGTDFTLTISSLEPEDAGVYHC QQYNDLLPLTFGGGTKVEIK	27 8

KP2-24	EVQLLESGPGLVKPSETLSLTCTVSGYSISSGYGWGWIRQSPGKGLEWIGYFGGSRGNTNYNPSLKSRVTISQDTSKNQFSLKLKSVTAADTGIYYCARDSGYSRRWWDYWGQGVLVTVSS	24	AAELTQSPATLSLAPGETATLSCRASQSVGTNLAWYHQKPGQPPKLLVHSAYVRATGIPDRFSGSGSRTDFTLTINSLQPEDVGVYHCQQYNDLLPLTFGGGTKIDIK	279
KP3-01	EVQLLESGPGLVKPLETLSLTCDVSGFSISSDYGWSWIRQPPGKGLELIGYIGGSRGNTNYNPSLKSRVTISRDTSKNQFSLKLTSVTAADTAVXYCARDWGYGYRYFDFWGQGVLVTVSS	24	AAELTQSPAFRSVSLKETVTLTCQASQSVGSNLHWYQQKPAQSPKLLIKYASQSISGVPSRFSGTGSGTDFTLTINSLEAEDAATYYCCQQTNTFPWTFGQGTRVEIK	288
KP3-02	EVQLLESGPGLVRPSETLSLTCAVSGYSISSGYGWHWIRQPPGKGLESLGYIGGSRGNTNYNPSLKSRVTISTDTSKNQFSLKLRSVTAADTAVYYCARDSGYNTRYFDYWGQGVLVTVSS	24	AAELTQSPATLSLSPGETATLSCRASQSVGSNLAWYQQKPGQAPKLLVHSAYFRATGIPDRFSGSGSRTDFTLTISSLEPEDVGVYHCQQYNDLLPLTFGGGTKVEIK	281
KP3-03	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLEWIGSIGGSRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYYCAQRGAYGYSYFDYWGQGVLVAVSS	24	AAELTQSPATLSLSPGETATLSCRASQSVGSYLAWYQQKPGQAPKLLVHGAYFRAAGIPDRFTGSGSRTDFTLTISSLEPEDVGIYHCQQYNDLLPLTFGGGTKVEIK	282
KP3-05	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLEWIGSIGGSRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYYCAQRGAYGYSYFDYWGQGVLVAVSS	24	AAELTQSPATLSLSPGETATLSCRASQSVGSSLAWYQQKPGQAPKLLVHSAYFRATGIPDRFSGSGSRTDFTLTISSLEPEDVGVYHCHQYNDLLPLTFGGGTKVEIK	283
KP3-06	EVQLLESGPGLVRPSETLSVTCDVSGGSISSASWSWIRQAPGKRLEWIGAISGSGSPTNVNPSLKSRVTLSVDTSKNQLSLKLRSMTAADTAVYYCARRGGYGNRYFDYWGQGVAVTVSS	24	AAELTQSPATLSLSPGETATLSCRASQSVGSSLAWYQQKPGQAPKLLVHSAYFRATGIPDRFSGSGSRTDFTLTISSLEPEDVGVYHCQQYNDLLPLTFGGGTKVEIK	284

KP3-07	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLE WIGSIGGSRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYY CAQRGAYGYSYFDYWGQGVLVTVSS	25 22	AAELVMTQSPATLSLSPGETATLSCRASQSIGSNLAWYQQKPG QAPKLLVHSANIRATGIPDRFIGSGSRTDFTLTISSLEPEDVGVY HCQQYNDFLPLTFGGGTKVEIK	28 85
KP3-08	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLE WIGSIGGSRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYY CAQRGAYGYSYFDYWGQGVLVAVSS	25 23	AAELTLTQSPATLSLSPGETATLSCRASQSLGGRLAWYQQKPG QAPRLLIYGASTRATGIPDRFSGSGSRTEFTLTIAGLEPEDVGV YHCQQYNDFLPLTFGGGTKVEIK	28 86
KP3-09	EVQLLESGPGLVKPSETLSLTCAVSSLSLSSGFAWSWIRQPPGEGLEW IGSIGGSRDNVNYNPSLKSRVTISKDTSKNQFSLRLRSVTAADTAVYYC VTIHGYRNWYLDHWGQGVLVTVST	25 54	AAELTQSPAILSLSPGETATLSCRASQSIGTSLAWYQQKPGQA PKLLVHSAYYRATDIPERFSGSGSRTDFTLTISSLEPEDVGVYH CQQYNDLLPLTFGGGTKVEIK	28 87
KP3-12	EVQLLESGPGLVKPSETLSLTCAVSGNSISSAYGWHWIRQVPGKGLEW IGSIGGSRGTTNYNPSLKSRGTISEDTSKNQFSLRLRSVSAADTAVYFC ARDSGYSFRYFDFWGRGVLVTVSS	25 55	AAELTQSPATLSLAPGETATLSCRASQSIGTNLAWYHQKPGQP PKLLVHSAYVRATGIPNRFSGSGSRTDFTLTINSLQPEDVGVYH CQQYNDLLPLTFGGGTKIDIK	28 88
KP3-13	EVQLLESGPGLVRPSETLSLTCAVSGGSLSGGYDWSWIRQSPRKGLE WIGYIYDSRGTTNYNPSLKRRVTISIDTSKNQFSLNLKSVTAADTAVYYC ARRGGYGASYFDLWGQGVLVTVSS	25 56	AAELTQSPATLSLSPGETATLSCRASQSVGSNLAWYQQKPGQ APKLLVHSASVRATGIPDRFSGSGSRTDFTLTISSLEPEDVGVY HCQQYNDFFPLTFGGGTKVEIK	28 89
KP3-15	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLE WIGSIGGNRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYY CAQRGAYGYSYFDYWGQGVLVAVSS	25 57	AAELTQSPATLSVSPGEAATLSCRASQTVGRNLAWYQQKPGQ APKLLVHSAHFRATGIPDRFSGSGSGGTDFTLTISSLEPEDAGIYH CQQYNDLLPLTFGGGTKVEIK	29 00

KP3-16	EVQLLESGPGLVKPSETLSLTCTVSGSSLSSAYGWNWIRQPPGKGLE WIGSIGGSRDNTNYNPSLKRRVTISKDTSKNQFSLKLKSVTAADTAVYY CAQRGAYGYSYFDYWGQGVLVAVSS	25 28	AAELTQSPATLSLSPGETATLSCRASQSLGSRLAWYQQKPGQ APRLLIYGASTRATGIPDRFSGSGSGSRTDFTLTISSLEPEDVGVYH CQQYNDFLPLTFGGGTKVEIK	29 1
KP3-18	EVQLLEWGPGLVKPSETLSLTCAVSGYSLSSAYGWNWIRQSPGKGLE WIGSIGGSRDNVNYNPSLKRRVTISKDTSTNHFSLRLSSVTAADTAVYY CVRRATYGNSYFDSWGQGVQVTVSS	25 29	AAELTLTQSPATLSLSPGETATLSCRASQSVGSYLAWYQQKPG QAPKLLVHSAHFRATGIPDRFSGSGSGSRTDFTLTISSLEPEDVGV YHCQQYNDLLPLTFGGGTKVEIK	29 2
KP3-19	EVQLLESGPGLVKPSETLSLTCAVSGGSLSGGYDWYWIRQSPRKGLEY IGYIYDSRGTTNYNPSLKNRVTISIDTSKNHFSLNLKSVTAADTAVYYCA RRVGYGATYFDLWGQGVLVTVSS	26 00	AAELVMTQSPATLSLSPGETATLSCRASQSVGSNLAWYQQKP GQAPKLLVHSANFRATGISDRFSGSGSGSRTDFTLTISSLEPEDVG VYHCQQYNDLLPLTFGGGTKVEIK	29 3
KP3-20	EVQLLESGPGLVKPSETLSLTCAVSGYSISSGFAWNWIRQTPGKGLEWI GYIGGSRDNTNYNPSLKSRVTISKDTSKNQFSLKLTSMTAADTAMYYCA RRGAYGNSYFDFWGQGVPVTVSS	26 01	AAELTQSPATLSLSPGETATLSCRASQSVGSNVAWYQQKPGQ APKLLVHSAYYRATGIPDRFSGSGSGSRTDFTLTISSLEPEDVGVY HCQQYNDLLPLTFGGGTKVEIK	29 4

Table 6: Anti-Band 3 antibody heavy and light variable chain coding sequences

Clone	VH	SEQ ID	VL	SEQ ID
KP2-01	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTGACT CCATCAGCAGTGGTTTGGGCTGGAGCTGGATCCGCCAGACC CCAGGGAAGGGGCTGGAGTGGATTGGATACATCGGTGGTAG TAGGGGCAACACCAACTACAACCCCTCGTTCAAGAGTCGAGT CACCATTTCAAGGGACACGTCCAAGAACCAGTTCTCCCTGAG GCTGTCCTCTATGACCGCCGCGGACACGGCCGTCTATTACT GTGCGAGAAGGGCCCCGTATTGGGGTTATTCCTATCTTGACT ACTGGGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	2 9 5	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTATTGGC AGCTCCTTAGCCTGGTACCAGCAGAGACCTGGGCAGGCTCCCAAG CTCCTCGTCCATAGTGCATACTTCAGGGCCGCTGGCATCCCAGAC AGGTTCAGCGGCAGCGGGTCTAGGACAGACTTCACTCTCACCATT AGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCAG TATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGTG GAACTCAAGCGA	2 9 6
KP2-02	GAGGTGCAGCTGCTCGAGTCAGGTCCAGGACTGGTGAAGCC TTCAGAGACCCTGTCGCTCACCTGCGCTGTCTCTGGAGGCTC TCTCAGCGGTGGGTATGACTGGAGCTGGATCCGCCAGTCCT CAAGAAAGGGGCTGGAGTGGATTGGCTATATCTATGATAGTC GTTGGACCACCAACTACAACCCGTCCCTCAAGAAGCGCGTCA CCATTTCAATAGACACGTCCAAGAACCAGTTCTCCCTGAACC TCAAGTCTGTGACCGCCGCGGACACGGCCGTGTATTATTGTG CGAGACGAGGCGGCTACGGTGCCAGCTACTTTGACTTATGG GGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 2 9	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTTTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTATTGGC AGCCACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAAG CTCCTCGTCCATAGTGTATCCTTCAGGGCCACTGGCATCCCAGACA GGTTCCGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCATTA GCAGCCTGGAACCTGAAGATGTTGGAGTTTATCACTGTCAGCAGTA TAACGACTTACTTCCGCTCACTTTCGGCGGAGGGACCAAGGTGGA GATCAAACGA	2 9 7

KP2-04	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CATCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTTACT CCCTCAGCAGTGCTTATGGCTGGAACTGGATCCGACAGTCC CCCGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAG TAGGGATAATGTCAACTACAACCCCTCCCTCAAGAGGCGAGT CACCATTTCAAAAGACACGTCCACGAACCACTTCTCCCTGAG GCTGAGTTCTGTGACGGCCGCGGACACGGCCGTGTATTATT GTGTGAGACGCGCGACCTACGGTAACAGCTACTTTGACTCCT GGGGCCAGGGAGTCCAGGTCACGGTCTCTTCA	330	GCGGCCGAGCTCACACTCACGCAGTCTCCAGCCACCCTGTCTTTG TCTCCAGGGGAAACAGCCACCCTCTCGTGCAGGGCCAGTCAGAGT GTTGGCAGCCACTTAGCCTGGTACCAGCAGAAACCTGGACAGGCT CCCAAGCTCCTCGTCCATAGTGCGTACTTCAGGGCCACTGGCATC CCAGACAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTC ACCATTAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTC AGCAGTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCA AGGTGGAGATCAAACGA	298
KP2-06	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCC TTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTCCTC CCTCAGCAGTGCTTATGGGTGGAACTGGATCCGCCAGCCCC CAGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAGT AGGGATAACACCAACTATAATCCCTCCCTCAAGAGGCGAGTC ACCATTTCAAAGGACACGTCCAAGAACCAGTTCTCCCTGAAG CTGAAGTCTGTGACCGCCGCGGACACGGCTGTCTATTACTGT GCGCAGAGGGGTGCTTACGGTTATTCCTATTTTGACTACTGG GGACAGGGAGTCCTGGTCGCCGTCTCCTCA	331	GCGGCCGAGCTCACTCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CAGCTCCTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAA ACTCCTCGTCCATAGTATATCCGTCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT CACCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAACAA TATAACGACTTCTTTCCGCTCACTTTCGGCGGAGGGACCAAGGTG GAGATCAAACGA	299

KP2-07	GAGGTGCAGCTGCTCGAGTCTGGCCCGGGACTGGTGAAGCC TTCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTGACTC CATCAGCAGCGGCTATGGCTGGCACTGGATCCGCCAGGTCC CAGGGAGGGGGGCTGGAGTGGATTGGATCTATCGGTGGTAGT AGGGGTACGACCAACTACAATCCCTCCCTCAAGAGTCGAGTC ACCATTTCAGAAGACACGTCCAAGAACCAGTTCTCCCTGAGT CTGAGGTCAGTGTCCGCCGCGGACACGGCCGTGTATTTTCTG TGCGAGAGACAGCGGATATAGTTTCCGTTACTTTGACTTCTG GGGTCAGGGAGTCCTGGTCACCGTCTCCTCA	3 3 2	GCGGCCGAGCTCGTGATGACGCAGTCTCCAGCCACCCTGTCTTTG TCTCCAGGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGT GTTGGCAGTAACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCT CCCAAGCTCCTCGTCCATAGTGCATACTTCAGGGCCACTGGCATC CCAGACAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTC ACCATTAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTC AGCAGTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCA AGGTGGAGATCAATCGA	3 0 0
KP2-08	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTTACT CCATCAGCAGTGGTTATGGCTGGAACTGGATCCGCCAGCCC CCAGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGCGGTAG TAGGGATAACACCAACTACAACCCCTCCCTCAAAAGTCGAGT CACCCTTTCAAAAGACACATCCAAGAACCACTTCTCCCTGAG GCTGCGCTCTGTGACCGCCGCGGACACGGCTGTGTATTACT GTGCGAGAGATGGTGGGTACGGTTCCCGATACATGGACTCC TGGGGCCAGGGAGTCCTGGTCGCCGTCTCCTCT	3 3 3	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAGGCAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTATTGG CACCTCCTTAGCCTGGTACCAACAGAAACCTGGACAGGCTCCCAG GCTCCTCGTCCATAGTGCATACTTCAGGGCCACTGGCATCGCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATTACTGTCAGCAG TATAACGACTTGCTCCCGCTCACTTTCGGCGGAGGGACCAAGGTG GAGATCAAACGA	3 0 1

KP2-09	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAGGC CTTCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTTACT CCATCAGCAGTGGTTATGGCTGGCACTGGATCCGCCAGCCCC CCAGGGAAGGGGCTGGAGTCGCTTGGCTATATCGGTGGTAG TAGGGGTAACACCAACTACAACCCCTCCCTCAAGAGTCGAGT CACCATTTCAACAGACACGTCCAAGAACCAGTTCTCCCTGAA GCTGAGGTCTGTGACCGCCGCGGACACGGCCGTGTATTACT GTGCGAGAGATTCCGGATACAACACAAGATACTTTGACTACT GGGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 3 4	GCGGCCGAGCTCACACAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CAGCAGGTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAG GCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCA GTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGT GGAGATCAAACGA	3 0 2
KP2-11	GAGGTGCAGCTGCAGCTGCCTGGGCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTCCT CCCTCAGCAGTGCTTATGGGTGGAACTGGATCCGCCAGCCCC CCAGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAG TAGGGATAACACCAACTATAATCCCTCCCTCAAGAGGCGAGT CACCATTTCAAAGGACACGTCCAAGAACCAGTTCTCCCTGAA GCTGAAGTCTGTGACCGCCGCGGACACGGCCGTCTATTACT GTGCGCAGAGGGGTGCTTACGGTTATTCCTATTTTGACTACT GGGGACAGGGAGTCCTGGTCGCCGTCTCCTCA	3 3 5	GCGGCCGAGCTCACACTCACGCAGTCTCCAGCCACCCTGTCTTTG TCTCCAGGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGT CTTGGCAGCAGGTTAGCCTGGTACCAACAGAAACCTGGGCAGCCT CCCAGGCTCCTCATCTATGGTGCATCCACCAGGGCCACTGGCATC CCAGACAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTC ACCATTAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTC AGCAGTATAACGACTTCCCTCCGCTCACTTTCGGCGGAGGGACCA AGGTGGAGATCAAACGA	3 0 3

KP2-13	GAGGTGCAGCTGCTCGAGTCAGGCCCAGGACTGGTGAAGCC TTCAGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGAGGCTC TATCAGCGGTGGTTATGACTGGAGTTGGATCCGCCAGTCCCC AGGGAAGGGGCTGGAGTGGATTGGTTATATCTATGATAGTAG GGGGACCACCAACTACAACCCGTCCCTCAGGAAGCGGGTCG CCATTTCAATAGACACGTCCAGGAACCAGTTTTCCCTGAACC TGAGATCTCTGACCGCCGCGGACACGGCCGTCTATTACTGT GCGAGACGAGCCGGCTACGGTAGCGCCTACTTTGACTACTG GGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 3 6	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGGCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTATTGGC ACTAACTTAGCCTGGTATCACCAGAAACCTGGGCAGCCTCCCAAG CTCCTCGTCCATACTGCATATGTCAGGGCCACTGGCATCCCAAACA GGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCATTA ACAGCCTGCAGCCTGAAGATGTTGGCGTTTATCACTGTCAGCAATA CAACGACTTGCTTCCTCTCACTTTCGGCGGAGGGACCAAGATAGA CATCAAACGA	3 0 4
KP2-14	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCC TTCGGAGACCCTGTCCCTCACCTGCGCTGTCTGGTTCCTC CCTCAGCAGTGCTTATGGGTGGAACTGGATCCGTCAGGCTC CAGGGAAGCGCCTGGAGTGGATTGGGTTTATCGGTGGTAGT CGTGATAACACCAATTACAACCCCTCCCTCAGGAGTCGGGTC ACCATTTCAAAAGACACGTCCAAGAACCACTTCTCCCTGAAA CTGACTTCTGTGACCGCCGCGGACACGGCCGTGTATTTCTGT GCGAGAAGGGGGGGCCTTCGGTAACTCCTACTTTGACTACTG GGGCCAGGGAGTCCCGGTCACCGTCTCCTCA	3 3 7	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTGAGAGTGTTGG CAGCTCCTTAGCCTGGTACCACCAGAAGCCTGGGCAGGCTCCCAG GCTCCTCGTCCATAGTGCATCCTTCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGAGTTCACTCTCACCGT TAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCA GTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGT GGAGATCAAACGA	3 0 5

KP2-15	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCC TTCAGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGAGGCTC TATCAGCGGTGGTTATGACTGGAGTTGGATCCGCCAGTCCCC AGGGAAGGGACTGGAGTGGATTGGTTATATCTATGATAGCAG GGGGACCACCAACTACAACCCGTCCCTCAGGAAACGGGTCA CCATTTCAATAGACACGTCCAGGAACCAGTTCTCCCTGAAGC TGAGATCTCTGACCGCCGCGGACACGGCCGTCTATTACTGT GCGAGACGAGCCGGCTACGGTAGCGCCTACTTTGACTACTG GGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 3 8	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTGTGTCTCCA GGGGAAGCAGCCACCCTCTCCTGCAGGGCCAGTCAGACTGTTGG CAGAAACTTAGCCTGGTACCAGCAGAAGCCTGGGCAGGCTCCCAA GCTCCTCGTCCATAGTGCACACTTCAGGGCCACTGGCATCCCGGA CAGGTTCAGTGGCAGCGGGTCTGGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAAGACGCTGGAATTTATCACTGTCAGCA ATATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGT GGAGATCAAACGA	3 0 6
KP2-17	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTTACT CCATCAGCAGTGGTTATGGCTGGACCTGGATCCGCCAGCCC CCAGGGAAGGGGCTGGAGTGGATTGGCTATATCGGTGGTAG TAGGGGAAACGCCAACTACAACCCCTCCCTCAAGAGTCGAGT CACCATTTCAAAGACACGTCCAAGAACCAGTTCTCCCTGAA GCTGACCTCTGTGACCGCCGCGGACACGGCCGTGTATTACT GTGCGAGAGATGGGGGATACGGAGAGAGATACCTCGAATTC TGGGGCCAGGGCGCCCTGGTCACCGTCTCCTCC	3 3 9	GCGGCCGAGCTCACACAGTCTCCAGCCTTTCGGTCTGTGACTCTG AAGGAGAAAGTCACCATCACCTGCCAGGCCAGTCAGAGCATTGGT AGTAGCTTACACTGGTACCAGCAGAAACCGGATCAGTCTCCAAAAC TCCTCATCAAGTTTGCTTCCCAGTCCATTTCAGGGGTCCCCTCAAG GTTCAGTGGCAGTGGATATGGGACAGATTTCACCCTCACTATCAAT AGCCTGGAAGCTGAAGATGCTGCGACGTATTACTGTCATCAGAGTA GTAGTTTCCCGTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCA AACGA	3 0 7

KP2-18	GAGGTGCAGCTGCTCGAGTCAGGCCCAGGACTGGTGAGGC CTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTAACT CCATCAGCAGTGGTTATGGCTGGAACTGGATCCGCCAGCCCC CCAGGGAAGGGGCTGGAGTTGATTGGGTATATCGGTGGAAG TAGAAGTAATACCAACTACAACCCCTCCCTCAAGAGTCGAGT CACCATTTCAATAGACACGTCCAAGAACCAGTTCTCCCTGAA ACTGAGGTCTGTGACTGCCGCGGACACGGCTGTGTATTACT GTGCGAGAGATTGGGGCTACGGTTACAGATACCTTGACTACT GGGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 4 0	GCGGCCGAGCTCACTCAGTCTCCAGCCTTTCGGTCTGTGACTCTA AAGGAGAAAGTCACCATCACCTGCCAGGCCAGTCAGAGCATTGGT AGTAGCTTACACTGGTACCAGCAGAAACCGGATCAGTCTCCAAAG CTCCTCATCAAGTATGCTTCCCAGTCCATCTCAGGGGTCCCCTCAA GGTTCAGTGGCAGTGGATCTGGGACAGATTTCACCCTCACTATCAA TAGCCTGGAAGCTGAAGATGCTGCGACGTATTACTGTCAGCAGAG TAGTAGTTTCCCATTCACTTTCGGCCCCGGGACCAAACTGGATATC AAACGA	3 0 8
KP2-19	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCC TTCAGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGAGGCTC TATCAACGGTGGTTATGACTGGACCTGGATCCGCCAGTCCCC AGGGAAGGGGCTGCAGTGGATTGGGTGGATCTATGGTAGTA GGGGGACCACCAACTACAACCCGTCCCTCAGGAATCGAGTC ACCATTTCAATAGACACGTCCAGGAACCAGTTCTCCCTGAGG CTGAGCTCTCTGACCGCCGCGGACACGGCCGTCTATTACTG TGCGAAACGAGTCGGCTACGGTAACAGCTACTTTGACTCCTG GGGCCAGGGAGTCCTGGTCACCGTGTCCTCA	3 4 1	GCGGCCGAGCTCACACTCACGCAGTCTCCAGCCACCCTGTCTTTG TCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGT GTCAGCAGCAGGTTAGCCTGGTACCAGCAGAAACCTGGGCAAGCT CCCAGGCTCCTCATCTATGATGCATCCAGCAGGGTCACTGGTATC CCAGACAGGTTCAGTGGCAGCGGGTCTGGGACAGACTTCACTCTC ACCATCAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTC AGCAGTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCA AGGTGGAGATCAAACGA	3 0 9

KP2-20	GAGGTGCAGCTGCTCGAGTCAGGCCCAGGACTGGTGAAGCC TTCAGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGAGGCTC TATCAGCGGTGGTTATGACTGGAGTTGGATCCGCCAGTCCCC AGGGAAGGGACTGGAGTGGATTGGTTATATCTATGATAGCAG GGGGACCACCAACTACAACCCGTCCCTCAGGAAACGGGTCA CCATTTCAATAGACACGTCCAGGAACCAGTTCTCCCTGAAGC TGAGATCTCTGACCGCCGCGGACACGGCCGTCTATTACTGT GCGAGACGAGCCGGCTACGGTAGCGCCTACTTTGACTACTG GGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 4 2	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CAGCAACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAA GCTCCTCGTCCATAGTGGTTCCGTCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCATCATT AGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCAG TATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGTG GAGATCAAACGA	3 1 0
KP2-22	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTGACT CCATCAGCAGTGGTTATGGCTGGAGCTGGATCCGCCAGACC CCAGGGAAGGGGCTGGAGTGGATTGGATACATCGGTGGTAG TAGGGGCAACACCAACTACAACCCCTCCCTCAAGAGTCGAGT CACCATTTCAAAAGACACGTCCAAGAACCAGTTCTCCCTGAA GCTGAGCTCTGTGACCGCCGCGGACACGGCCGTGTATTACT GTGCGAGAAGGGCCCCGTACTGGGGTTATTCCTATCTTGACT ACTGGGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 4 3	GCGGCCGAGCTCACACTCACGCAGTCTCCAGCCACCCTGTCTTTG GCTCCAGGGGAAACAGCCACCCTCTCCTGTAGGGCCAGTCAGAGT ATTGGCACTAACTTAGCCTGGTATCACCAAAAACCTGGGCAGTCTC CCAAGCTCCTCGTCCATAGTGCATATGTCCGGGCCACTGGCATCC CAGACAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCA CCATTAACAGCCTGCAGCCTGAAGATGTTGGCGTTTATCACTGTCA GCAGTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGAACCAA GGTGGAGATCAAACGA	3 1 1

KP2-23	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCCT TCGGAGACCCTGTCCCTCACCTGCACTGTCTCTCTGGTTCCTCCCT CAGCAGTGCTTATGGGTGGGAATCCGCCAGCCCCCAGG GAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAGTAGGG ATAACACCAACTATAATCCCTCCCTCAAGAGGCGAGTCACCAT TTCAAAGGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAAG TCTGTGACCGCCGCGGACACGGCCGTCTATTACTGTGCGCAGA GGGGTGCTTACGGTTATTCCTATTTTGACTACTGGGGACAGGG AGTCCTGGTCGCCGTCTCCTCA	GCGGCCGAGCTCACACTCACGCAGTCTCCAGCCACCCTGTCTTTGTC TCCAGGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGACTGTT GGCAGAAACTTAGCCTGGTACCAGCAGAGGCCTGGGCAGGCTCCC AACCTCCTCGTCCATAGTGCATACTTCAGGGCCACTGGCATCCCGGA CAGGTTCAGTGGCAGCGGGTCTGGGACAGACTTCACTCTCACCATT AGCAGCCTGGAGCCTGAAGATGCTGGAGTTTATCACTGTCAGCAAT ATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGTGGA GATCAAACGA	3 1 2
		3 4 4		
KP2-24	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTACT CCATCAGCAGTGGTTATGGCTGGGGCTGGATCCGCCAGTCC CCAGGGAAGGGGCTGGAGTGGATTGGCTATTTTGGTGGTAG TAGAGGTAACACCAACTACAACCCCTCCCTCAAGAGTCGAGT CACCATTTCACAAGACACGTCCAAGAATCAGTTCTCCCTGAA ACTGAAGTCTGTGACCGCCGCGGACACGGGCATTTTATTACTG CGCGCGAGACAGCGGTTATTCCCGGCGTTGGGTTGACTACT GGGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGGCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CACTAACTTAGCCTGGTATCACCAGAAACCTGGGCAGCCTCCCAA GCTCCTCGTCCATAGTGCATATGTCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT TAACAGCCTGCAGCCTGAAGATGTTGGCGTTTATCACTGTCAGCAG TATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGATA GACATCAAACGA	3 1 3
		3 4 5		

KP3-01	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTTGGAGACCCTGTCCCTCACCTGCGATGTCTCTGGTTTCT CCATTAGTAGTGATTATGGCTGGAGCTGGATCCGCCAGCCCC CAGGGAAGGGGCTGGAGTTGATTGGCTATATCGGTGGTAGT CGTGGTAACACCAACTATAACCCCTCCCTCAAGAGTCGAGTC ACCATTTCAAGAGACACTTCCAAGAATCAGTTCTCCCTGAAG CTGACCTCTGTGACCGCCGCGGACACGGCCGTCTACTACTG TGCGAGAGATTGGGGCTACGGTTATAGGTACTTTGACTTCTG GGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 4 6	GCGGCCGAGCTCACTCAGTCTCCAGCCTTTCGGTCTGTGAGTCTG AAGGAGACAGTCACCCTCACCTGCCAGGCCAGTCAGAGCGTTGGT AGTAACTTACACTGGTACCAGCAGAAACCGGCTCAGTCTCCAAAAC TCCTCATCAAGTATGCTTCCCAGTCCATCTCAGGGGTCCCCTCAAG GTTCAGTGGCACTGGATCTGGGACAGATTTCACCCTCACTATCAAT AGTCTGGAAGCTGAAGATGCTGCGACATATTACTGTCAGCAGACTA ATACTTTCCCCGTGGACGTTCGGCCAAGGGACCAGGGTGGAAATCA AGCGA	3 1 4
KP3-02	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGGCCT TCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTTACTCCAT CAGCAGTGGTTATGGCTGGCACTGGATCCGCCAGCCCCCAGG GAAGGGGCTGGAGTCGCTTGGCTATATCGGTGGTAGTAGGG GTAACACCAACTACAACCCCTCCCTCAAGAGTCGAGTCACCAT TTCAACAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGG TCTGTGACCGCCGCGGACACGGCCGTGTATTACTGTGCGAGA GATTCCGGATACAACACAAGATACTTTGACTACTGGGGCCAG GGAGTCCTGGTCACCGTCTCCTCA	3 4 7	GCGGCCGAGCTCACTCAGTCTCCAGCCACCCTGTCTTTGTCTCCAGG GGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGGCAGC AACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAAGCTCC TCGTCCATAGTGCATACTTCAGGGCCACTGGCATCCCAGACAGGTTC AGTGGCAGCGGGGTCTAGGACAGACTTCACTCTCACCATTAGCAGCC TGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCAGTATAACGA CTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAA CGA	3 1 5

KP3-03	GAGGTGCAGCTGGAGGTGCAGCTGCTCGAGTCTGGCCCAG GACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACT GTCTCTGGTTCCTCCCTCAGCAGTGCTTATGGGTGGAACTGG ATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGT CTATCGGTGGTAGTAGGGATAACACCAACTATAATCCCTCCC TCAAGAGGCGAGTCACCATTTCAAAGGACACGTCCAAGAACC AGTTCTCCCTGAAGCTGAAGTCTGTGACCGCCGCGGACACG GCTGTCTATTACTGTGCGCAGAGGGGTGCTTACGGTTATTCC TATTTTGACTACTGGGGACAGGGAGTCCTGGTCGCCGTCTCC	3 4 8	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CAGCTACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAA GCTCCTCGTCCATGGTGCATACTTCAGGGCCGCTGGCATCCCAGA CAGGTTCACTGGCAGCGGGTCTCGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAAGATGTTGGAATTTATCACTGTCAGCAG TATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGTG GAGATCAAACGA	3 1 6
KP3-05	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTCCT CCCTCAGCAGTGCTTATGGGTGGAACTGGATCCGCCAGCCC CCAGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGGTAG TAGGGATAACACCAACTATAATCCCTCCCTCAAGAGGCGAGT CACCATTTCAAAGGACACGTCCAAGAACCAGTTCTCCCTGAA GCTGAAGTCTGTGACCGCCGCGGACACGGCCGTCTATTACT GTGCGCAGAGGGGTGCTTACGGTTATTCCTATTTTGACTACT GGGGACAGGGAGTCCTGGTCGCCGTCTCCTCA	3 4 9	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CAGCTCCTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAA GCTCCTCGTCCATAGTGCATACTTCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCACCAG TATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGTG GAGATCAAACGA	3 1 7

KP3-06	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGGC CTTCGGAGACCCTGTCTGTCACCTGCGATGTCTCTGGTGGCT CAATCAGCAGTGCTTCCTGGAGCTGGATCCGCCAGGCCCCA GGGAAGAGACTGGAGTGGATTGGGGCTATCTCTGGTAGTGG TAGTCCCACCAACGTCAACCCCTCCCTCAAGAGTCGAGTCAC CCTGTCAGTAGACACGTCCAAGAACCAGCTCTCCCTGAAGTT GAGGTCAATGACCGCCGCGGACACGGCCGTATATTACTGTG CAAGACGAGGGGGTTACGGTAATAGATACTTTGACTATTGGG GCCAGGGAGTCGCGGTCACCGTCTCCTCA	3 5 0	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CAGCTCCTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAA ACTCCTCGTCCATAGTGCATACTTCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCA GTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGT GGAGATCAAACGA	3 1 8
KP3-07	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTCCT CCCTCAGCAGTGCTTATGGGTGGAACTGGATCCGCCAGCCC CCAGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAG TAGGGATAACACCAACTATAATCCCTCCCTCAAGAGGCGAGT CACCATTTCAAAGGACACGTCCAAGAACCAGTTCTCCCTGAA GCTGAAGTCTGTGACCGCCGCGGACACGGCCGTCTATTACT GTGCGCAGAGGGGTGCTTACGGTTATTCCTATTTTGACTACT GGGGACAGGGAGTCCTGGTCACCGTCTCCTCA	3 5 1	GCGGCCGAGCTCGTGATGACACAGTCTCCAGCCACCCTGTCTTTG TCTCCAGGGGAAACAGCCACCCTTTCCTGCAGGGCCAGTCAGAGT ATTGGCAGCAACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCT CCCAAGCTCCTCGTCCATAGTGCAAACATCAGGGCCACTGGCATC CCAGACAGGTTCATTGGCAGCGGGTCTAGGACAGACTTCACTCTC ACCATTAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTC AGCAGTATAACGACTTCCTTCCGCTCACTTTCGGCGGAGGGACCA AGGTGGAGATCAAACGA	3 1 9

KP3-08	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAAGC CTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTCCT CCCTCAGCAGTGCTTATGGGTGGAACTGGATCCGCCAGCCCC CCAGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAG TAGGGATAACACCAACTATAATCCCTCCCTCAAGAGGCGAGT CACCATTTCAAAGGACACGTCCAAGAACCAGTTCTCCCTGAA GCTGAAGTCTGTGACCGCCGCGGACACGGCCGTCTATTACT GTGCGCAGAGGGGTGCTTACGGTTATTCCTATTTTGACTACT GGGGACAGGGAGTCCTGGTCGCCGTCTCCTCA	3 5 2	GCGGCCGAGCTCACACTCACGCAGTCTCCAGCCACCCTGTCTTTG TCTCCAGGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGT CTTGGCGGCAGGTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCT CCCAGGCTCCTCATCTATGGTGCATCCACCAGGGCCACTGGCATC CCAGACAGGTTCAGTGGCAGCGGGTCTAGGACAGAGTTCACTCTC ACCATTGCCGGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTC AGCAGTATAACGACTTCCTTCCGCTCACTTTCGGCGGAGGGACCA AGGTGGAGATCAAACGA	3 5 2
KP3-09	GAGGTGCAGCTGCTCGAGTCTGGGCCAGGACTGGTGAAGCC TTCGGAGACCCTGTCGCTCACCTGCGCTGTCTCTAGTCTGTC CCTCAGTAGTGGTTTTTGCCTGGAGCTGGATCCGCCAGCCCC CAGGAGAGGGACTGGAGTGGATTGGGTCTATCGGTGGTAGT CGTGACAACGTCAATTATAACCCCTCCCTCAAGAGTCGAGTC ACCATTTCGAAAGACACGTCCAAGAACCAGTTCTCCCTGAGG CTGCGTTCTGTGACCGCCGCGGACACGGCCGTGTATTACTG TGTGACCATTCATGGCTACCGTAACTGGTATCTTGACCACTG GGGCCAGGGAGTCCTGGTCACCGTCTCCACA	3 5 3	GCGGCCGAGCTCACGCAGTCTCCAGCCATCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGTAGGGCCAGTCAGAGTATTGGC ACGTCCTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAAG CTCCTCGTCCATAGTGCATACTACAGGGCCACTGACATCCCAGAG AGGTTCAGTGGCAGCGGATCTAGGACAGACTTCACTCTCACCATTA GCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCAGT ATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGTGG AGATCAAACGA	3 2 1

KP3-12	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCC TTCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTAACTC CATCAGCAGCGCCTATGGCTGGCACTGGATCCGCCAGGTCC CAGGGAAGGGGCTGGAGTGGATTGGATCTATCGGTGGTAGT AGGGGTACGACCAACTACAATCCCTCCCTCAAGAGTCGAGG CACCATTTCAGAAGACACGTCCAAGAACCAGTTCTCCCTGAG GCTGAGGTCAGTGTCCGCCGCGGACACGGCCGTGTATTTCT GTGCGAGAGACAGCGGATATAGTTTCCGTTACTTTGACTTCT GGGGTCGGGGAGTTCTGGTCACCGTCTCCTCA	3 5 4	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGGCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTATTGGC ACTAACTTAGCCTGGTATCACCAGAAACCTGGGCAGCCTCCCAAG CTCCTCGTCCATAGTGCATATGTCAGGGCCACTGGCATCCCAAACA GGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCATTA ACAGCCTGCAGCCTGAAGATGTTGGCGTTTATCACTGTCAACAGTA CAACGACTTGCTTCCTCTCACTTTCGGCGGAGGGACCAAGATAGA CATCAAACGA	3 2 2
KP3-13	GAGGTGCAGCTGCTCGAGTCGGGCCCAGGACTGGTGAGGC CTTCAGAGACCCTGTCGCTCACCTGCGCTGTCTCTGGAGGCT CTCTCAGCGGTGGTTATGACTGGAGCTGGATCCGCCAGTCC CCAAGAAAGGGGCTGGAGTGGATTGGCTATATCTATGATAGT CGTGGGACCACCAACTACAACCCGTCCCTCAAGAGGCGAGT CACCATTTCAATAGACACGTCCAAGAACCAGTTCTCCCTGAA CCTCAAGTCTGTGACCGCCGCGGACACGGCCGTGTATTATT GTGCGAGACGAGGCGGCTACGGTGCCAGCTACTTTGACTTA TGGGGCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 5 5	GCGGCCGAGCTCACACAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CAGCAACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAA GCTCCTCGTCCATAGTGCATCCGTCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT TAGTAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCAG TATAACGACTTCTTTCCGCTCACTTTCGGCGGAGGGACCAAGGTG GAGATCAAACGA	3 2 3

KP3-15	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCC TTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTCCTC CCTCAGCAGTGCTTATGGGTGGAACTGGATCCGCCAGCCCC CAGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAAT AGGGATAACACCAACTATAATCCCTCCCTCAAGAGGCGAGTC ACCATTTCAAAGGACACGTCCAAGAACCAGTTCTCCCTGAAG CTGAAGTCTGTGACCGCCGCGGACACGGCCGTCTATTACTG TGCGCAGAGGGGTGCTTACGGTTATTCCTATTTTGACTACTG GGGACAGGGAGTCCTGGTCGCCGTCTCCTCA	3 5 6	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTGTGTCTCCA GGGGAAGCAGCCACCCTCTCCTGCAGGGCCAGTCAGACTGTTGG CAGAAACTTAGCCTGGTACCAGCAGAAGCCTGGGCAGGCTCCCAA GCTCCTCGTCCATAGTGCACACTTCAGGGCCACTGGCATCCCGGA CAGGTTCAGTGGCAGCGGGTCTGGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAAGATGCTGGAATTTATCACTGTCAGCAA TATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGTG GAGATCAAACGA	3 2 4
KP3-16	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCC TTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTCCTC CCTCAGCAGTGCTTATGGGTGGAACTGGATCCGCCAGCCCC CAGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAGT AGGGATAACACCAACTATAATCCCTCCCTCAAGAGGCGAGTC ACCATTTCAAAGGACACGTCCAAGAACCAGTTCTCCCTGAAG CTGAAGTCTGTGACCGCCGCGGACACGGCCGTCTATTACTG TGCGCAGAGGGGTGCTTACGGTTATTCCTATTTTGACTACTG GGGACAGGGAGTCCTGGTCGCCGTCTCCTCA	3 5 7	GCGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTCTTGGC AGCAGGTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAG GCTCCTCATCTATGGTGCATCCACCAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTCAGCA GTATAACGACTTCCTTCCGCTCACGTTCGGCGGAGGGACCAAGGT GGAGATCAAACGA	3 2 5

KP3-18	GAGGTGCAGCTGCTCGAGTGGGGCCCAGGACTGGTGAAGC CATCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTGGTTACT CCCTCAGCAGTGCTTATGGCTGGAACTGGATCCGACAGTCC CCCGGGAAGGGGCTGGAGTGGATTGGGTCTATCGGTGGTAG TAGGGATAATGTCAACTACAACCCCTCCCTCAAGAGGCGAGT CACCATTTCAAAAGACACGTCCACGAACCACTTCTCCCTGAG GCTGAGTTCTGTGACGGCCGCGGACACGGCCGTGTATTATT GTGTGAGACGCGCGACCTACGGTAACAGCTACTTTGACTCCT GGGGCCAGGGAGTCCAGGTCACGGTCTCTTCA	3 5 8	GCGGCCGAGCTCACACTCACGCAGTCTCCAGCCACCCTGTCTTTG TCTCCAGGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGT GTTGGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCT CCCAAGCTCCTCGTCCATAGTGCACACTTCAGGGCCACTGGCATC CCAGACAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTC ACCATTAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTC AGCAGTATAACGACTTACTTCCCCTCACTTTCGGCGGAGGGACCAA GGTGGAGATCAAACGA	3 2 6
KP3-19	GAGGTGCAGCTGCTCGAGTCAGGCCCAGGACTGGTGAAGCC CTCAGAGACCCTGTCCCTCACCTGCGCGGTCTCTGGAGGCT CTCTCAGTGGTGGTTATGACTGGTACTGGATCCGCCAGTCCC CAAGAAAGGGCCTGGAGTATATTGGTTATATCTATGATAGTC GTGGGACCACCAACTACAACCCGTCCCTCAAGAATCGAGTCA CCATTTCAATAGACACGTCCAAGAACCACTTCTCCCTGAACCT CAAGTCTGTGACCGCGCCGCGGACACGGCCGTGTATTACTGTG CGAGACGAGTCGGGTACGGTGCCACCTATTTTGACTTATGGG GCCAGGGAGTCCTGGTCACCGTCTCCTCA	3 5 9	GCGGCCGAGCTCGTGATGACGCAGTCTCCAGCCACCCTGTCTTTG TCTCCAGGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGT GTTGGCAGCAACTTAGCCTGGTACCAGCAGAAACCTGGGCAGGCT CCCAAGCTCCTCGTCCATAGTGCAAACTTCAGGGCCACTGGCATC TCAGACAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTC ACCATCAGCAGCCTGGAGCCTGAAGATGTTGGAGTTTATCACTGTC AGCAGTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCA AGGTGGAGATCAAACGA	3 2 7

KP3-20	GAGGTGCAGCTGCTCGAGTCTGGCCCAGGACTGGTGAAGCC TTCGGAGACCCTGTCCCTCACCTGCGCTGTGTCTGGTTACTC CATCAGCAGTGGTTTTGCCTGGAACTGGATCCGCCAGACCC CAGGGAAGGGACTGGAGTGGATTGGGTATATCGGTGGTAGT CGTGATAACACCAACTACAACCCCTCCCTCAAGAGTCGAGTC ACCATTTCAAAAGACACGTCCAAGAACCAGTTCTCCCTTAAG CTGACTTCTATGACCGCCGCGGACACGGCCATGTATTACTGT GCGAGAAGGGGGGCCTACGGTAACTCCTACTTTGACTTCTG GGGCCAGGGAGTCCCGGTCACCGTCTCCTCA	360	GCGGCCGAGCTCACACAGTCTCCAGCCACCCTGTCTTTGTCTCCA GGGGAAACAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTGG CAGTAATGTAGCCTGGTACCAGCAGAAACCTGGGCAGGCTCCCAA GCTCCTCGTCCATAGTGCATACTACAGGGCCACTGGCATCCCAGA CAGGTTCAGTGGCAGCGGGTCTAGGACAGACTTCACTCTCACCAT TAGCAGCCTGGAGCCTGAGGATGTTGGAGTTTATCACTGTCAGCA GTATAACGACTTGCTTCCGCTCACTTTCGGCGGAGGGACCAAGGT GGAGATCAAACGA

Table 7

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)

WHAT IS CLAIMED IS:

1. An antibody or fragment thereof comprising at least one CDR selected from those of SEQ ID NO: 1 to SEQ ID NO: 18 or SEQ ID NO: 31 to SEQ ID NO: 228, wherein said antibody or fragment thereof specifically binds an erythrocyte.

2. An antibody or fragment thereof comprising at least a VH or VL sequence selected from those of SEQ ID NO: 19 to SEQ ID NO: 24 or SEQ ID NO: 229 to SEQ ID NO: 294, wherein said antibody or fragment thereof specifically binds an erythrocyte.

3. An antibody or fragment thereof comprising a VH and VL sequence selected from those of SEQ ID NO: 19 to SEQ ID NO: 24 or SEQ ID NO: 229 to SEQ ID NO: 294, wherein said antibody or fragment thereof specifically binds an erythrocyte.

4. An antibody or fragment thereof which specifically binds an epitope on an erythrocyte without causing significant adverse alteration of membrane deformability.

5. The antibody or fragment of claim 4, wherein said specific binding does not significantly alter resistance of said erythrocyte to physical, chemical, mechanical stress, or combinations thereof.

6. The antibody or fragment of claim 4 or 5, wherein at least 100,000 copies of the epitope are present on the erythrocyte.

7. The antibody of any of claims 4 to 6, comprising at least one CDR selected from those of SEQ ID NO: 1 to SEQ ID NO: 18.

8. The antibody of any of claims 4 to 7, comprising at least a VH or VL sequence selected from those of SEQ ID NO: 19 to SEQ ID NO: 24.

9. The antibody of any of claims 4 to 8, wherein the antibody comprises a variable heavy chain sequence of SEQ ID NO: 23 and a variable light chain sequence of SEQ ID NO: 24.

10. The antibody of any of claims 4 to 9, wherein the epitope is present in the 6^{th} extracellular loop of human RhCE.

11. The antibody of claim 10, wherein the epitope comprises at least 5 consecutive amino acids of SEQ ID NO: 361.

12. An antibody which competes for the binding site of the antibody of any of claims 4 to 11.

13. The antibody of any preceding claim, which is an scFv.

14. The antibody of any preceding claim, wherein the antibody comprises a variable heavy chain sequence of SEQ ID NO: 23 and a variable light chain sequence of SEQ ID NO: 24.

15. A plasmid comprising nucleic acid sequences encoding the antibody of any of claims 1 to 14.

16. The plasmid according to claim 15, wherein the antibody is encoded by any of the nucleic acid sequences of Table 3 or Table 6, or a sequence sharing at least 70% identity therewith.

17. A fusion protein comprising an anti-RHCE antibody according to any preceding claim fused to a pharmacological, therapeutic, prophylactic, imaging or diagnostic agent.

18. A fusion protein comprising an anti-Band 3 antibody according to any of claims 1 to 3 or 13 which binds to the Wrb epitope fused to a pharmacological, therapeutic, prophylactic, imaging or diagnostic agent.

19. A fusion protein comprising the antibody of any preceding claim fused to a pharmacological, therapeutic, prophylactic, imaging or diagnostic agent.

20. The fusion protein according to claim 19, wherein the therapeutic or prophylactic agent is an anticoagulant, anti-malarial, anti-hemolytic or fibrinolytic.

21. A plasmid comprising nucleic acid sequence encoding the fusion protein of any of claims 17 to 20.

22. A pharmaceutical composition comprising the antibody or fusion protein according to any one of the preceding claims and a pharmaceutically acceptable carrier, excipient, diluent and/or adjuvant.

23. A method for delivering an agent using red blood cells, said method comprising administering the composition of claim 22 to a subject in need thereof.

24. A method for preventing or reducing coagulation, said method comprising administering the composition of claim 22 to a subject in need thereof.

25. A method of treating or preventing thrombosis, tissue ischemia, acute myocardial infarction (AMI), non-segmented elevated AMI, deep vein thrombosis, ischemic stroke, hyperoxic injury, transient ischemic attack (TIA), cerebrovascular disease, disseminated intravascular coagulation (DIC), pulmonary embolism, ischemic peripheral vascular disease, inflammation, pulmonary edema, sepsis, malaria, SDC, PNH, hemolytic anemia, acute lung

injury (ALI), acute respiratory distress syndrome (ARDS), a bleeding disorder such as hemophilia, or aseptic systemic inflammation, comprising administering the composition of claim 22 to a subject in need thereof.

26. A method for prolonging circulation of a pharmacological, therapeutic, prophylactic, imaging or diagnostic agent, said method comprising administering the composition of claim 22 to a subject in need thereof.

27. The method according to any of claims 23 to 26, wherein the fusion protein an scFv fused to thrombomodulin.

28. The method according to any of claims 23 to 27, wherein said composition is administered intravenously.

29. The method according to any of claims 23 to 28, wherein said subject is a mammal.

30. The method according to claim 29, wherein said subject is a human.

FIG 1A

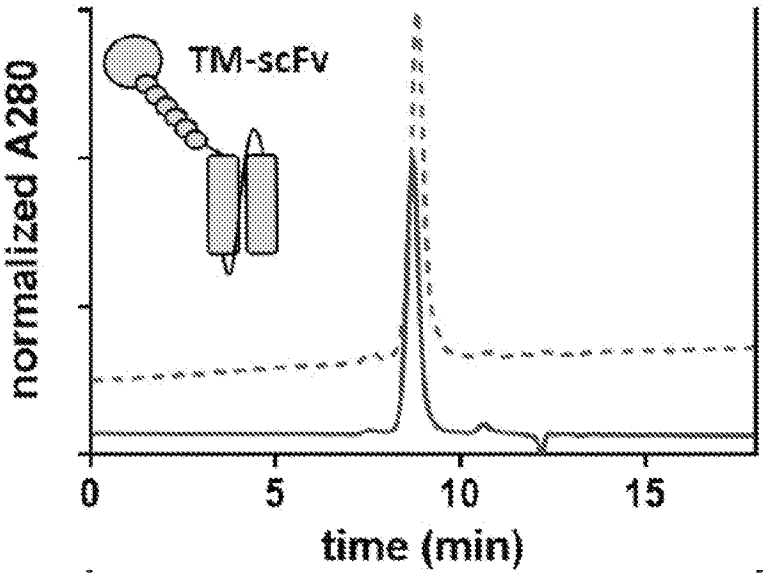


FIG 1C

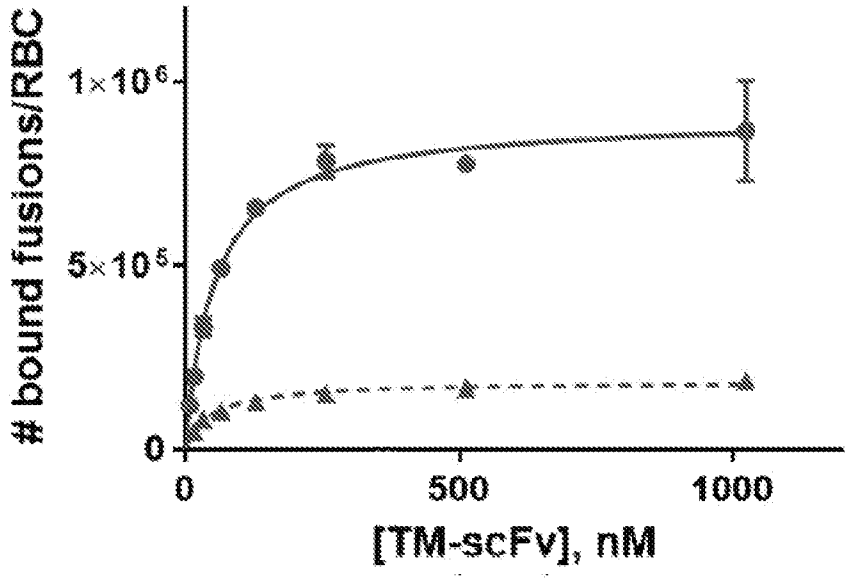


FIG 1B

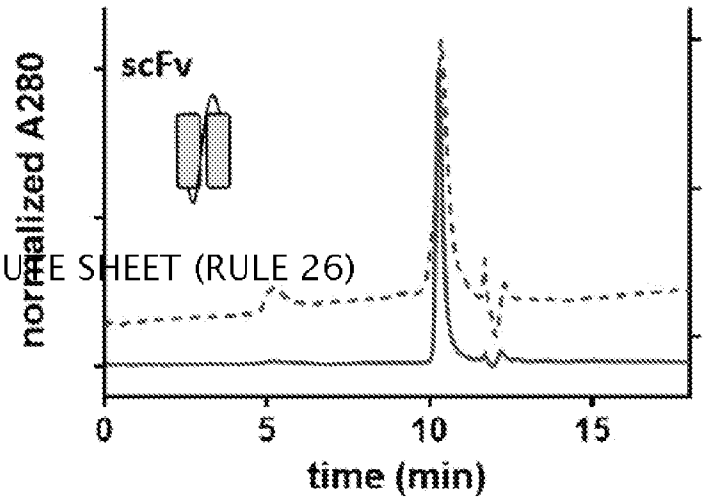


FIG 1D

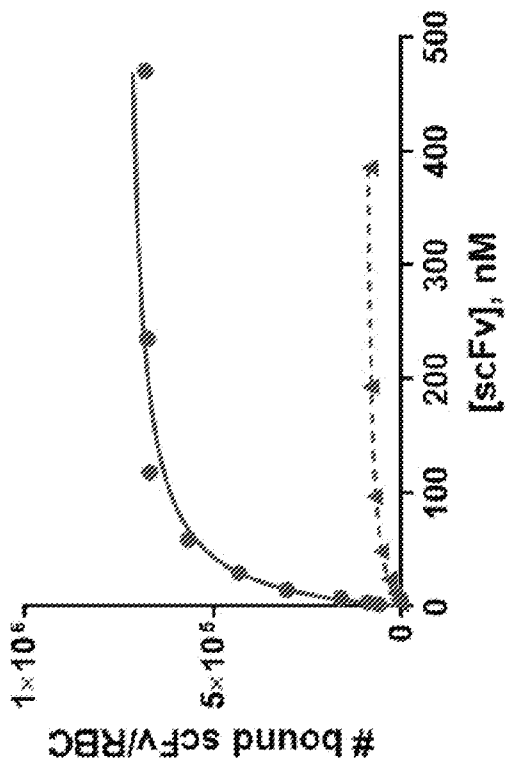


FIG 1E

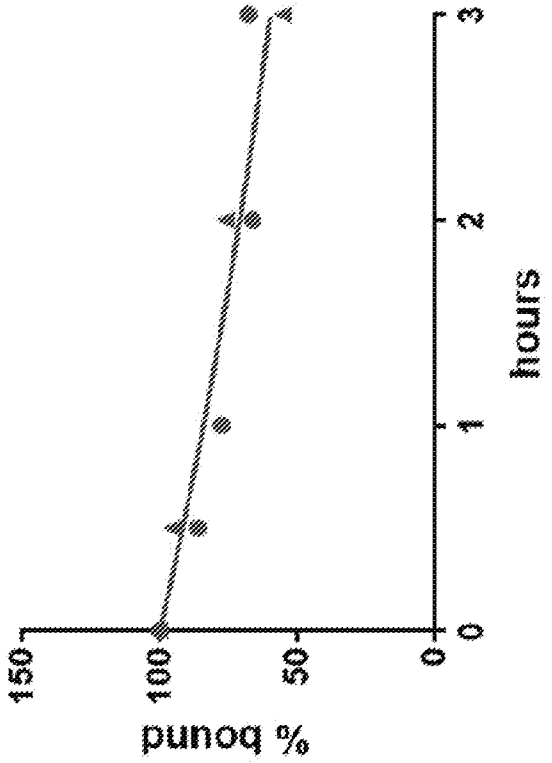


FIG 1F

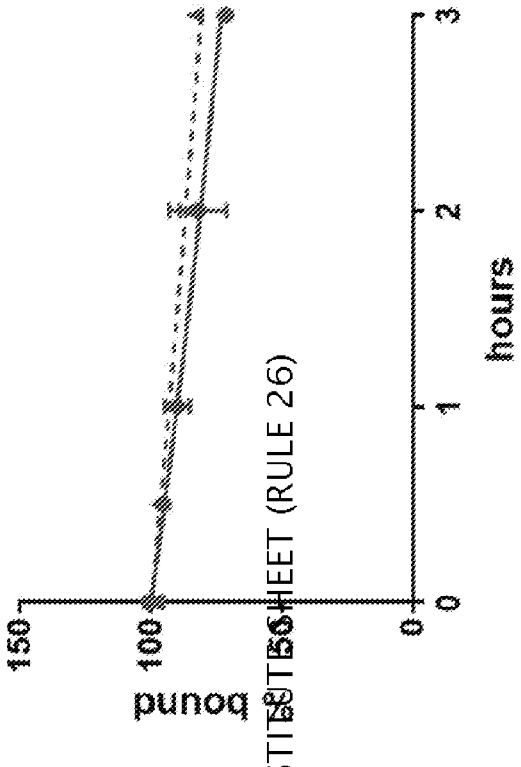
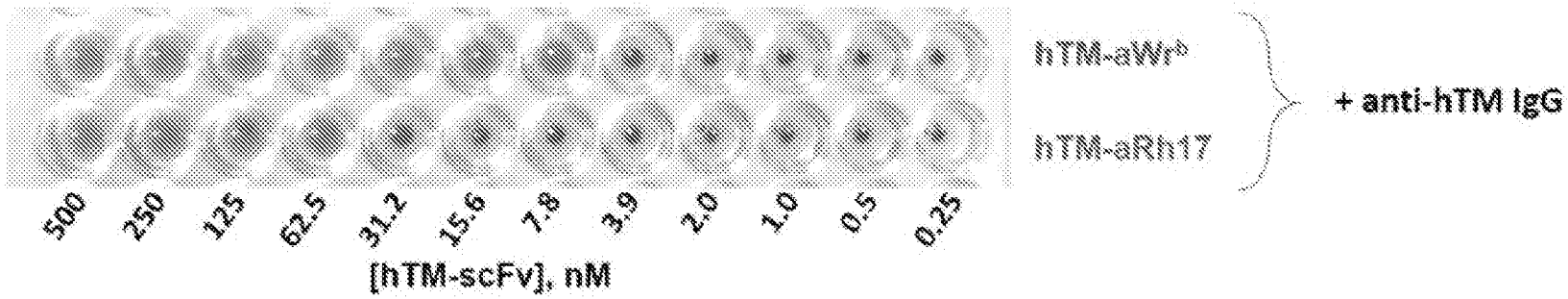


Fig 1G



SUBSTITUTE SHEET (RULE 26)

FIG 2A

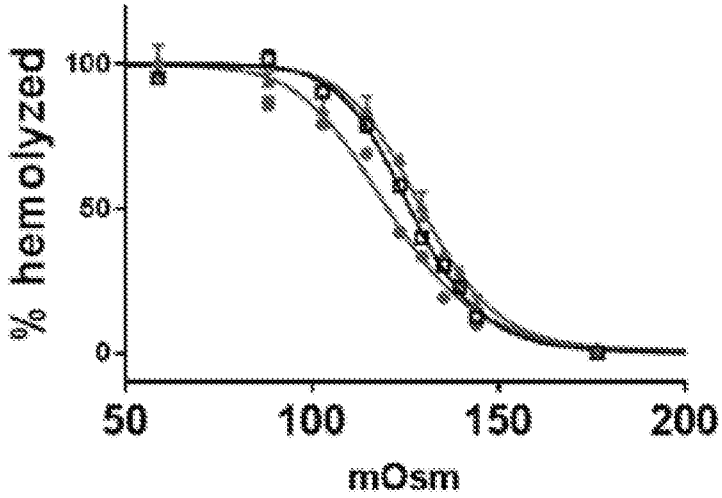

% hemolyzed
100
50
0
50
100
150
200
mOsm

FIG 2B

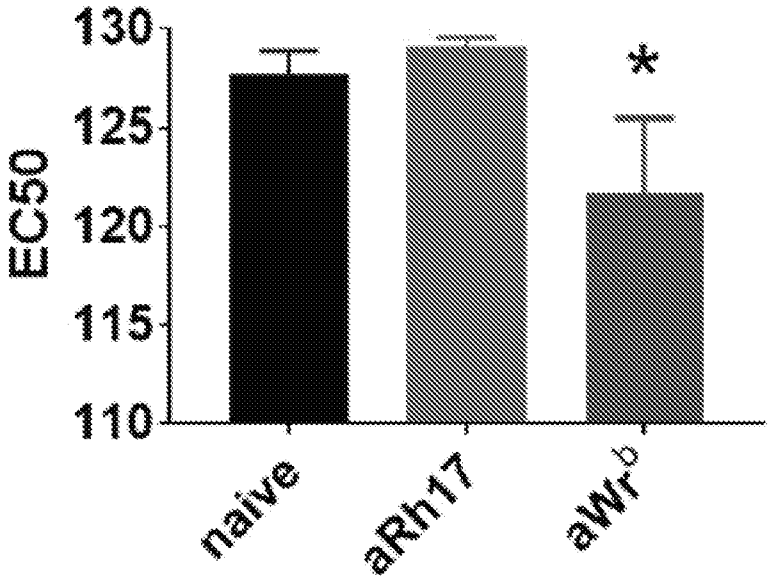

EC50
130
125
120
115
110
*
naive
aRh17
aWr^b

FIG 2C

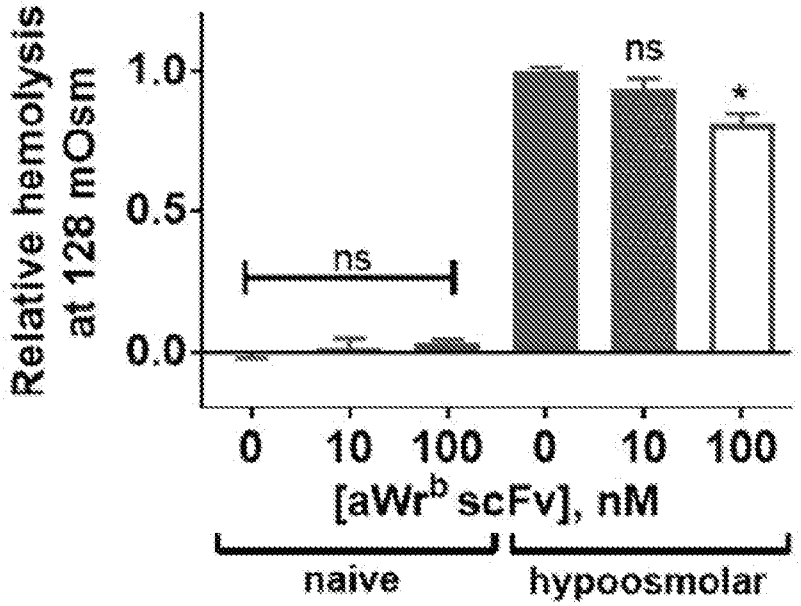

Relative hemolysis at 128 mOsm
1.0
0.5
0.0
ns
ns
*
0
10
100
0
10
100
[aWr^b scFv], nM
naive
hypoosmolar

FIG 2D

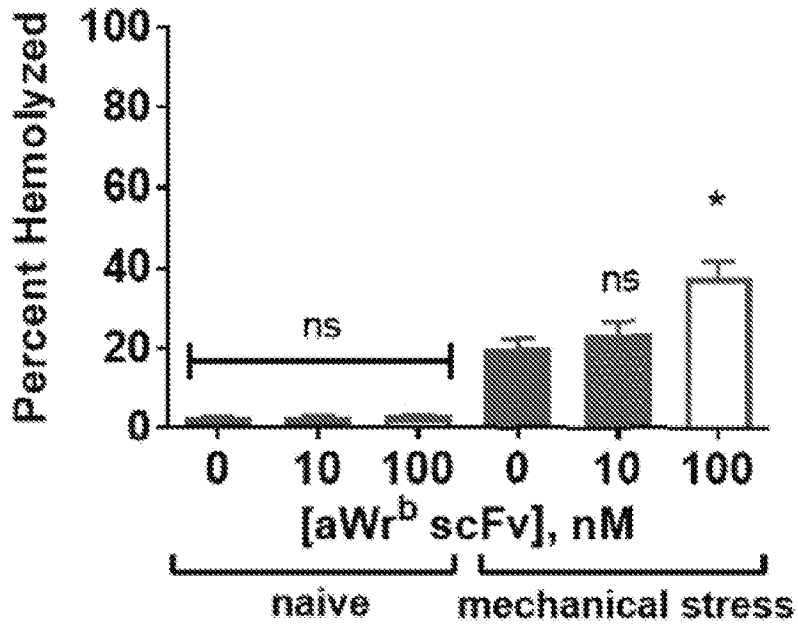

Percent Hemolyzed
100
80
60
40
20
0
ns
ns
*
0
10
100
0
10
100
[aWr^b scFv], nM
naive
mechanical stress

FIG 2E

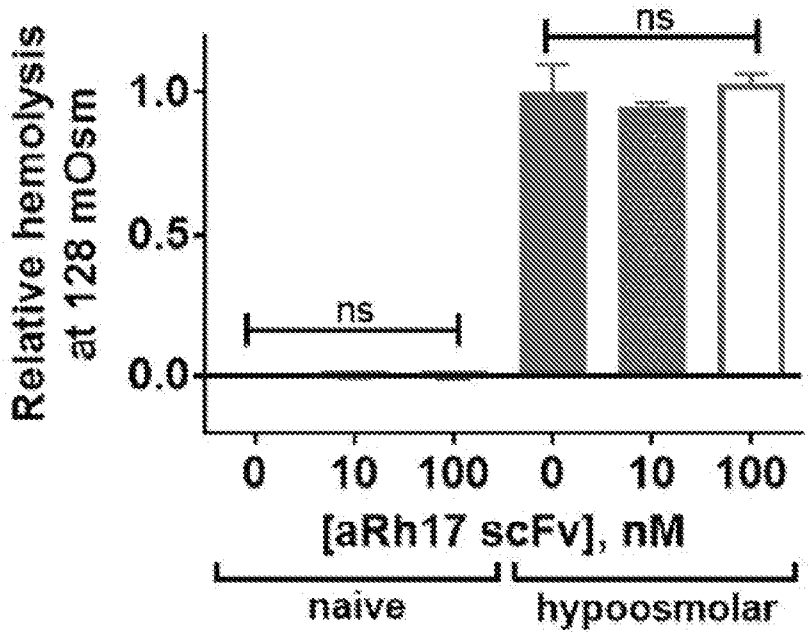

Relative hemolysis at 128 mOsm
1.0
0.5
0.0
ns
ns
0
10
100
0
10
100
[aRh17 scFv], nM
naive
hypoosmolar

FIG 2F

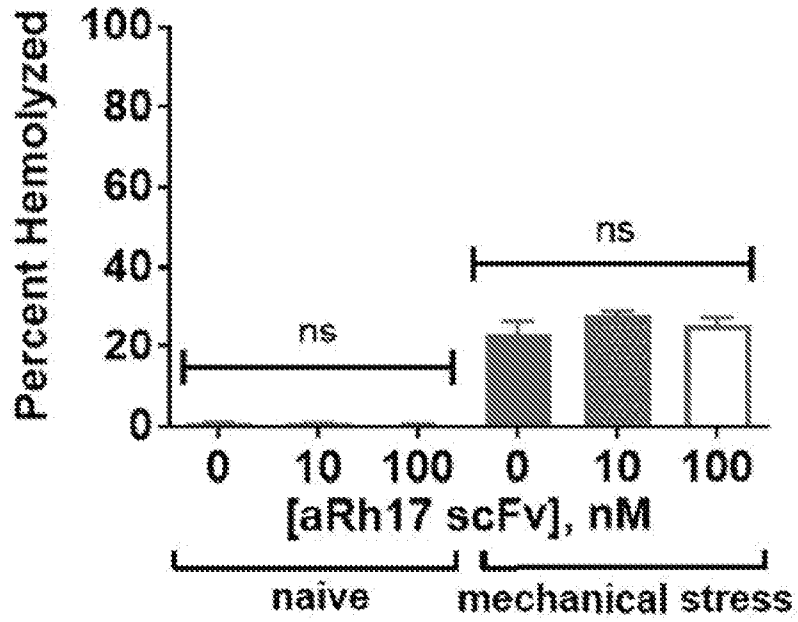

Percent Hemolyzed
100
80
60
40
20
0
ns
ns
0
10
100
0
10
100
[aRh17 scFv], nM
naive
mechanical stress

FIG 3A

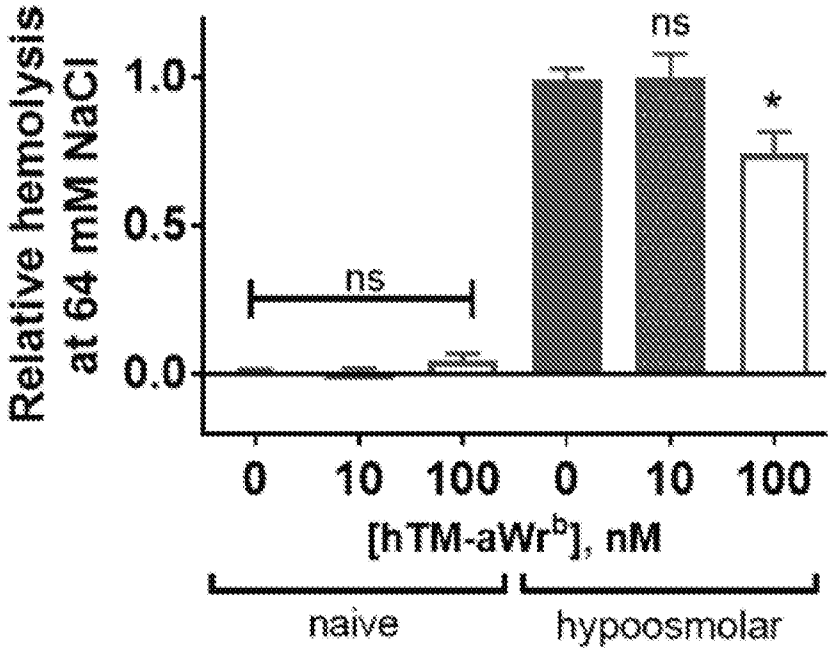

Relative hemolysis at 64 mM NaCl
1.0
0.5
0.0
ns
ns
*
0
10
100
0
10
100
[hTM-aWr^b], nM
naive
hypoosmolar

FIG 3A

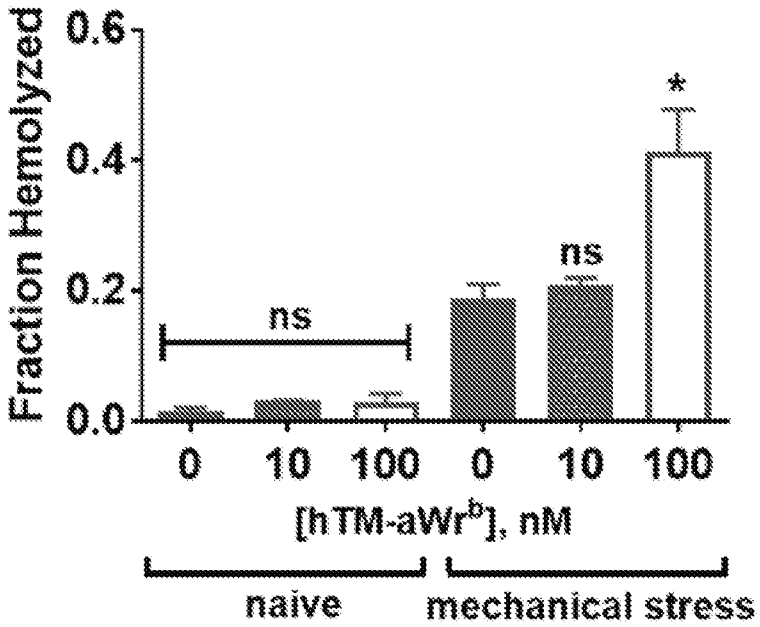

Fraction Hemolyzed
0.6
0.4
0.2
0.0
ns
ns
*
0
10
100
0
10
100
[hTM-aWr^b], nM
naive
mechanical stress

FIG 3C

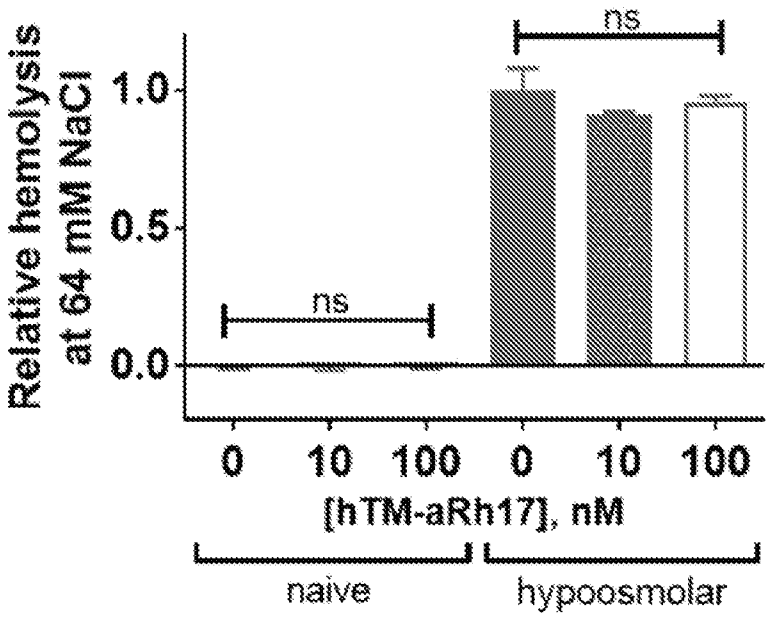

Relative hemolysis at 64 mM NaCl
1.0
0.5
0.0
ns
ns
0
10
100
0
10
100
[hTM-aRh17], nM
naive
hypoosmolar

FIG 3D

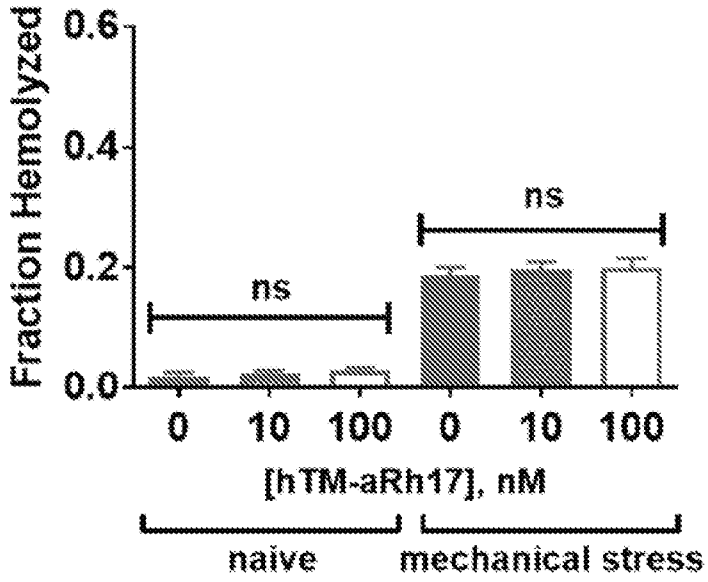

Fraction Hemolyzed
0.6
0.4
0.2
0.0
ns
ns
0
10
100
0
10
100
[hTM-aRh17], nM
naive
mechanical stress

FIG 4A

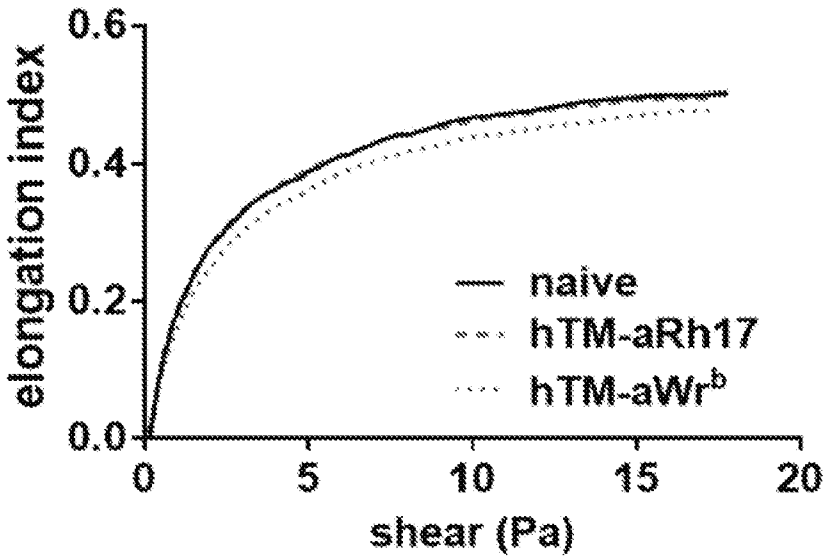

elongation index
0.6
0.4
0.2
0.0
0
5
10
15
20
shear (Pa)
naive
hTM-aRh17
hTM-aWr^b

FIG 4B

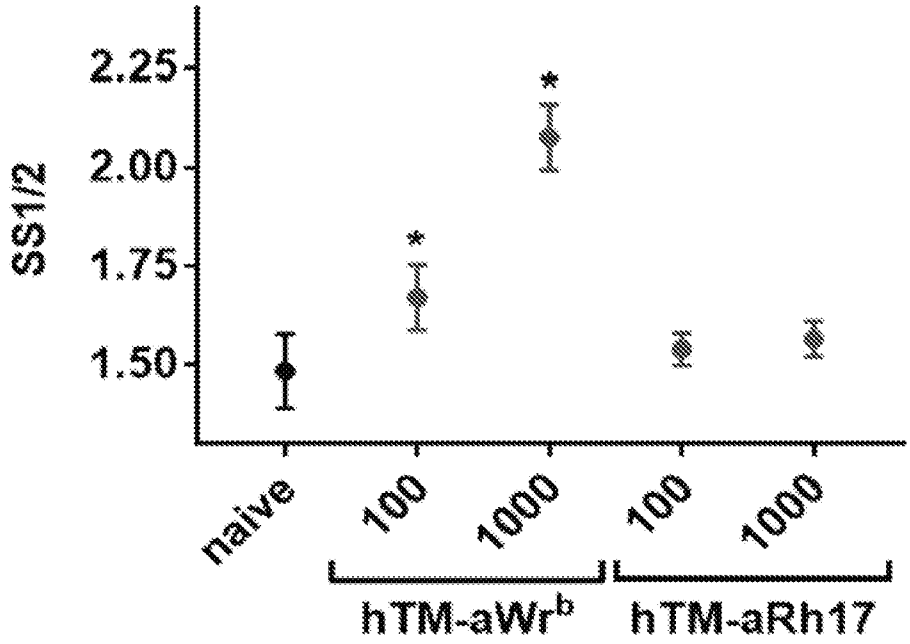

SS1/2
2.25
2.00
1.75
1.50
*
*
naive
100
1000
100
1000
hTM-aWr^b
hTM-aRh17

FIG 4C

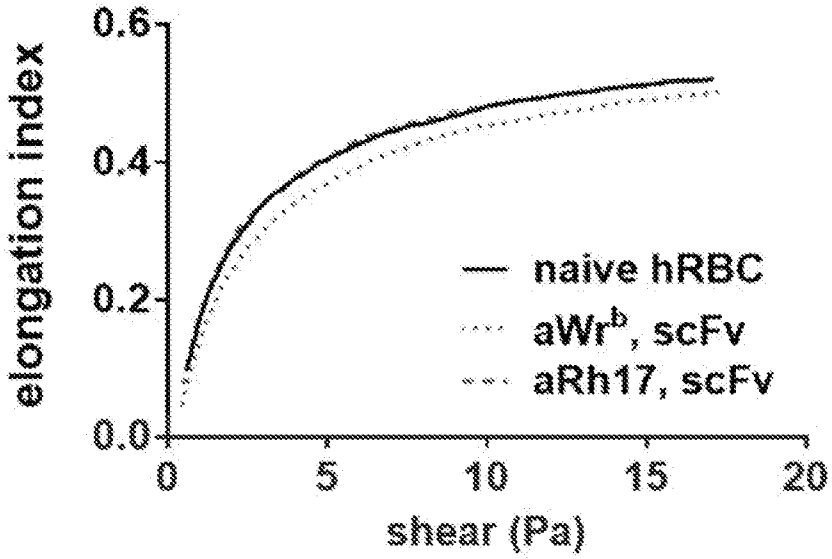

elongation index
0.6
0.4
0.2
0.0
0
5
10
15
20
shear (Pa)
naive hRBC
aWr^b, scFv
aRh17, scFv

FIG 4D

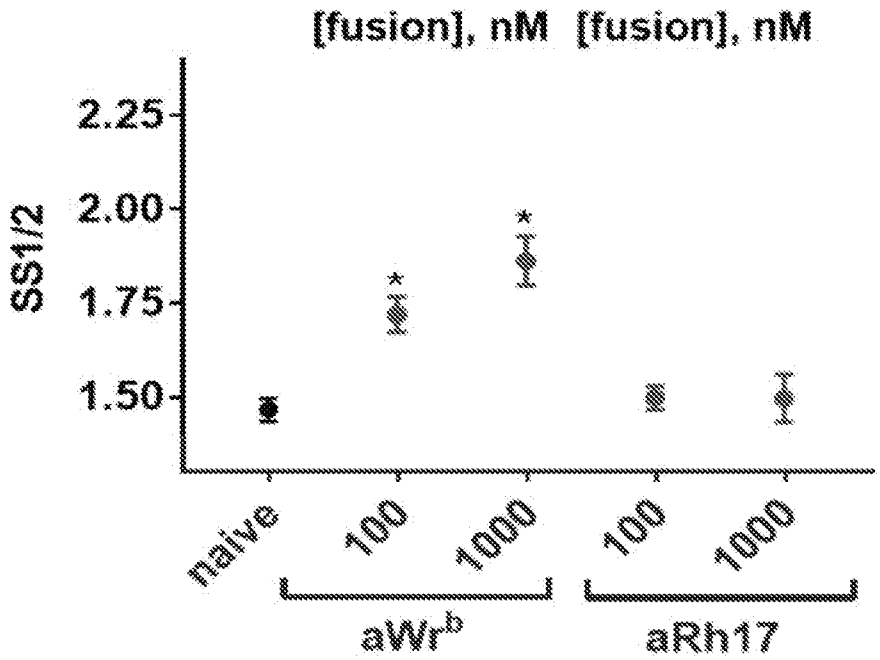

[fusion], nM [fusion], nM
SS1/2
2.25
2.00
1.75
1.50
naive
100
1000
100
1000
aWr^b
aRh17

FIG 5A

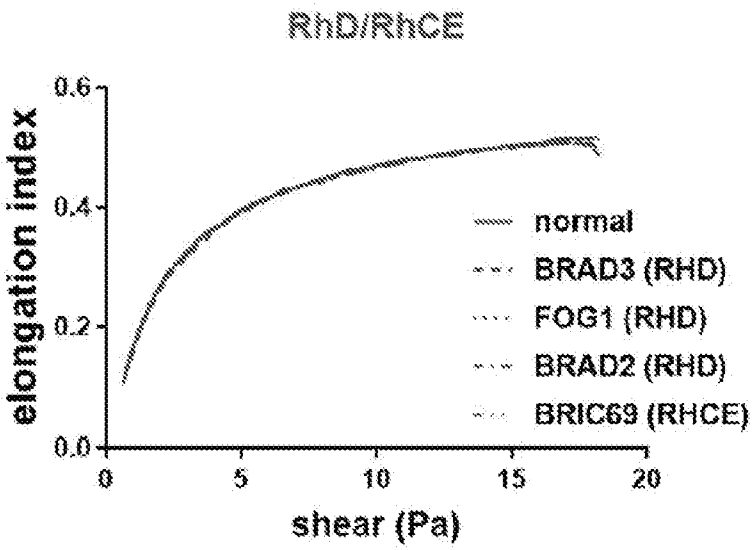


FIG 5B

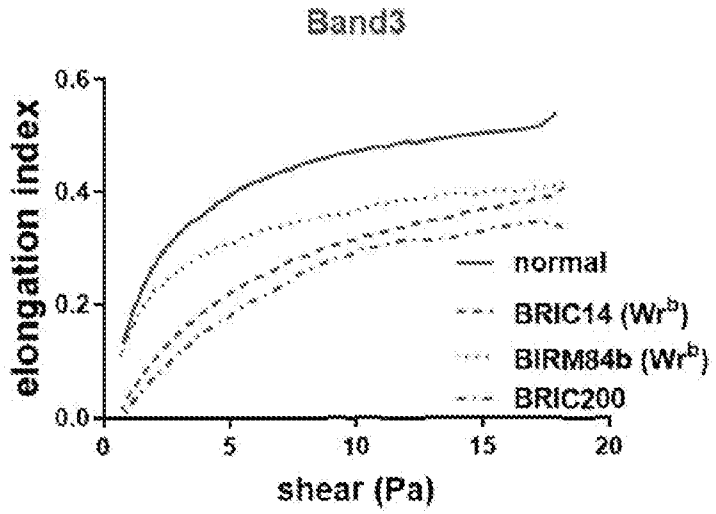


FIG 5C

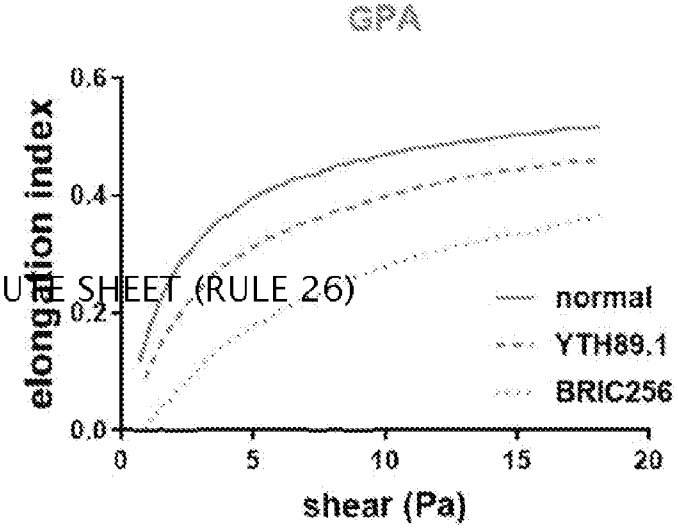


FIG 5D

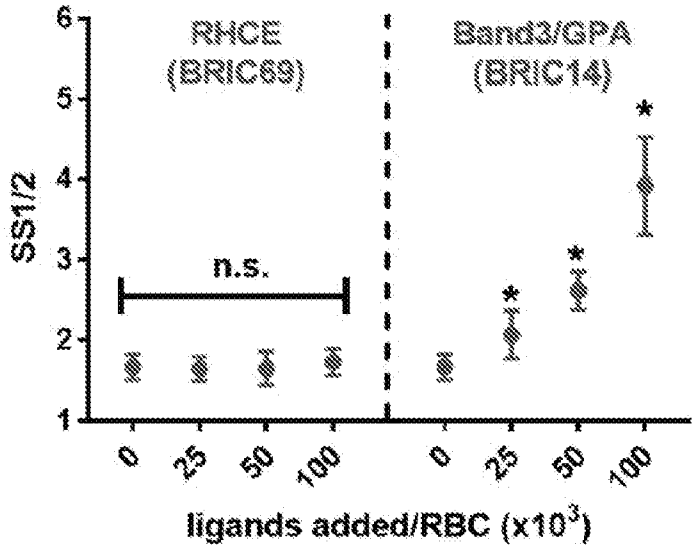
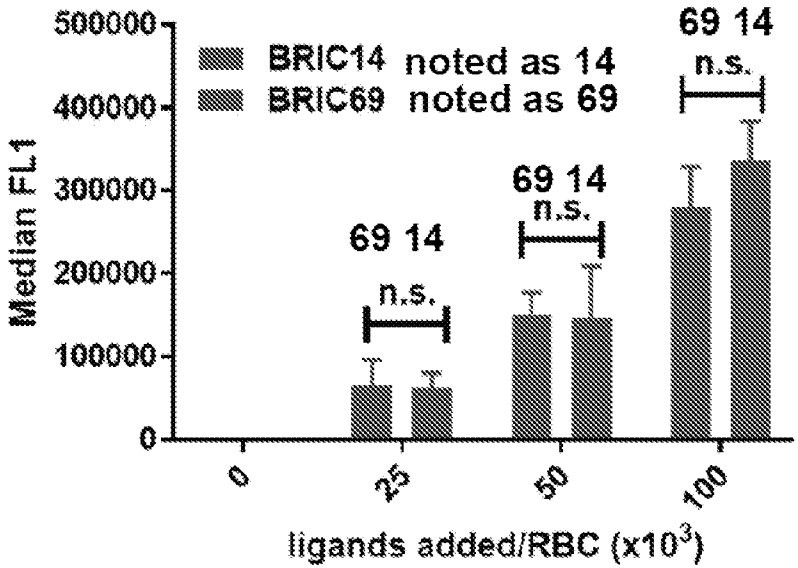
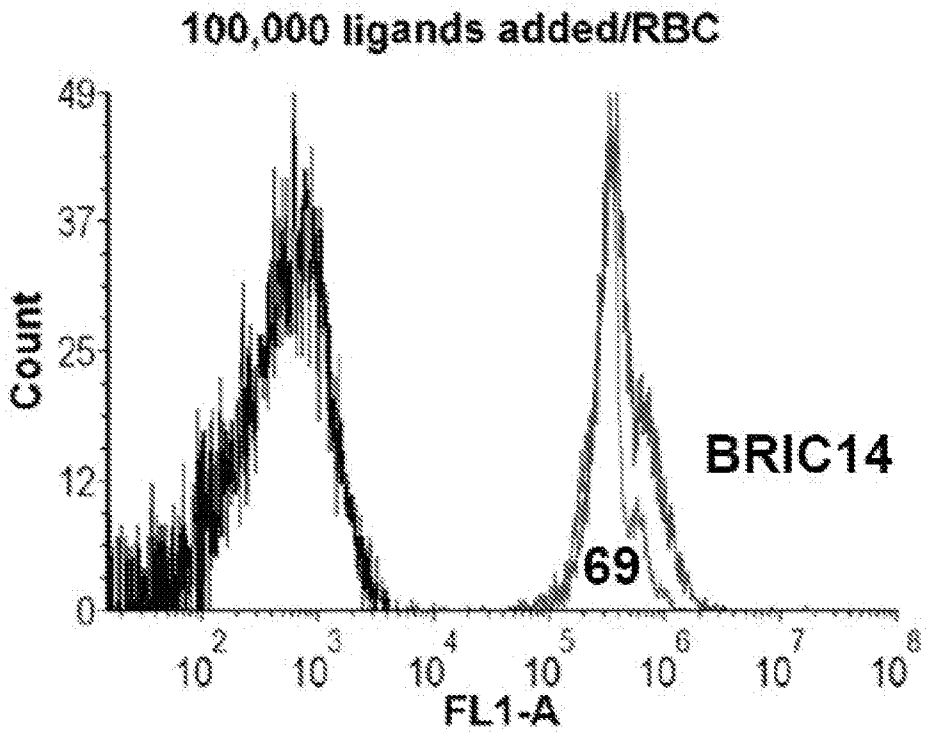


FIG 5E



SUBSTITUTE SHEET (RULE 26)

FIG 5F



SUBSTITUTE SHEET (RULE 26)

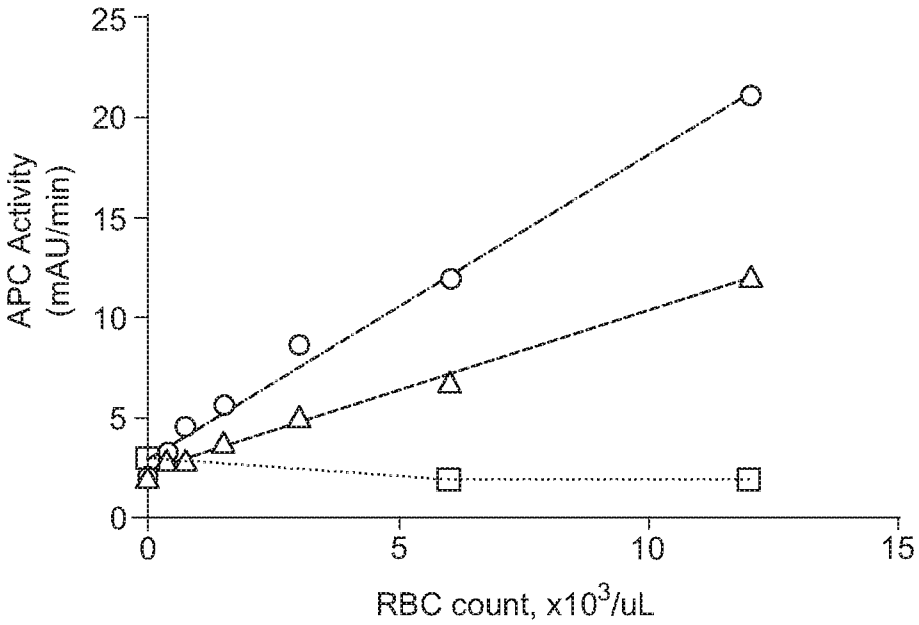


FIG. 6A

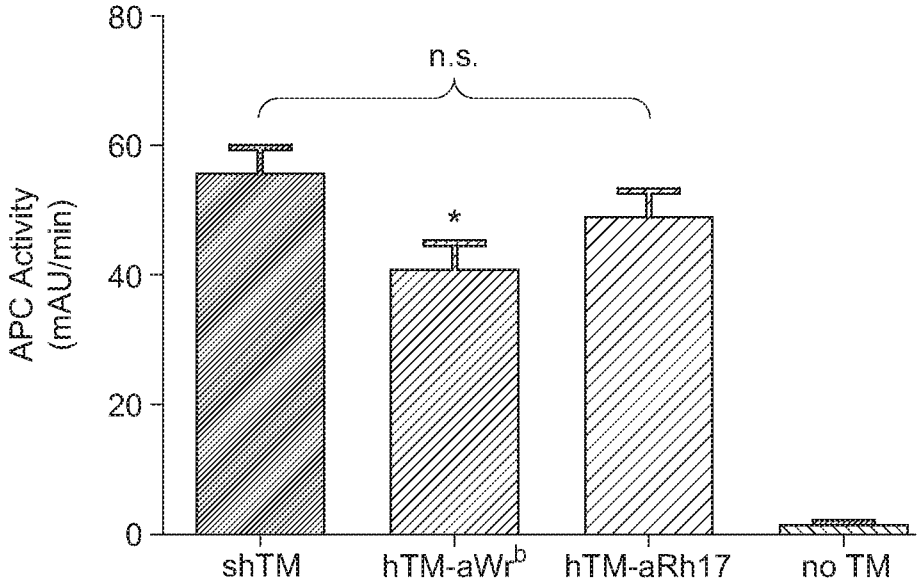

80
60
40
20
0
APC Activity
(mAU/min)
n.s.
*
shTM
hTM-aWr^b
hTM-aRh17
no TM

FIG. 6B

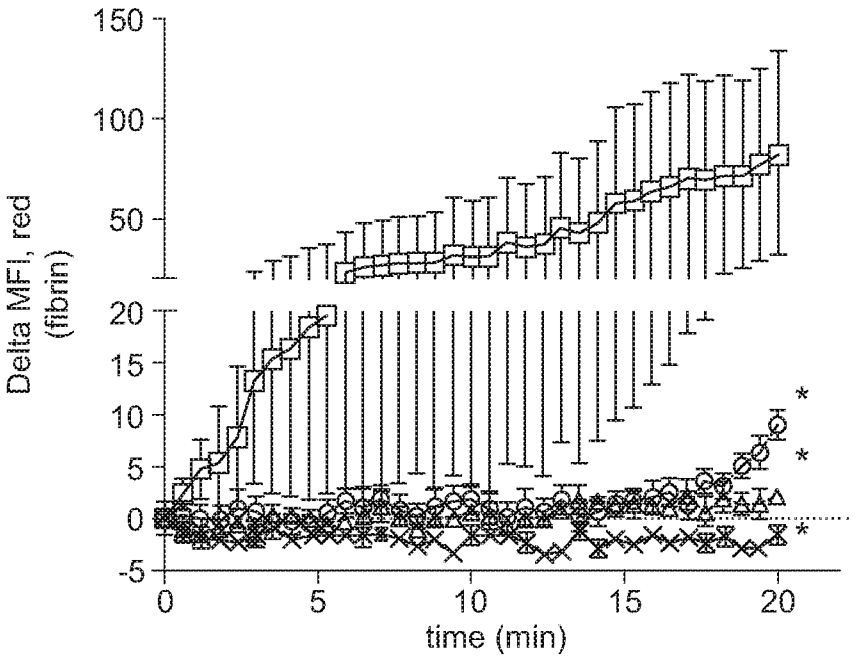


FIG. 6C

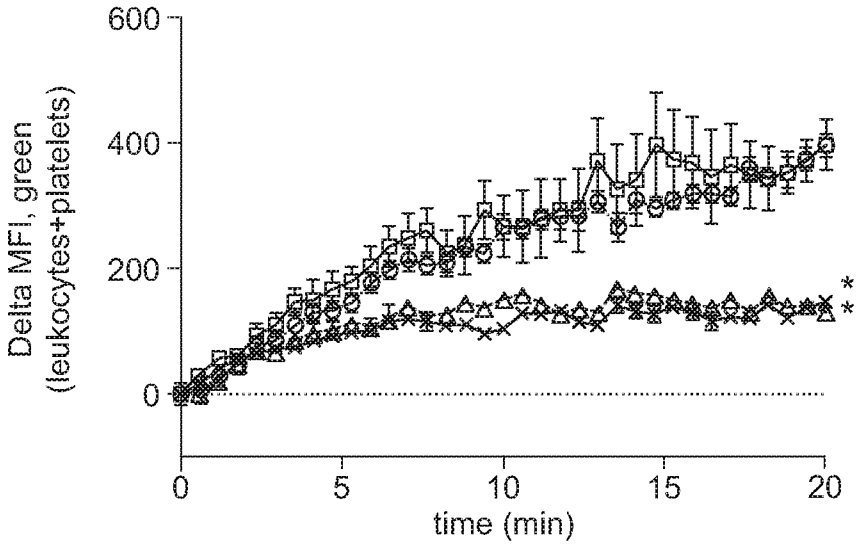

600
400
200
0
Delta MFI, green
(leukocytes+platelets)
0
5
10
15
20
time (min)
*
*

FIG. 6D

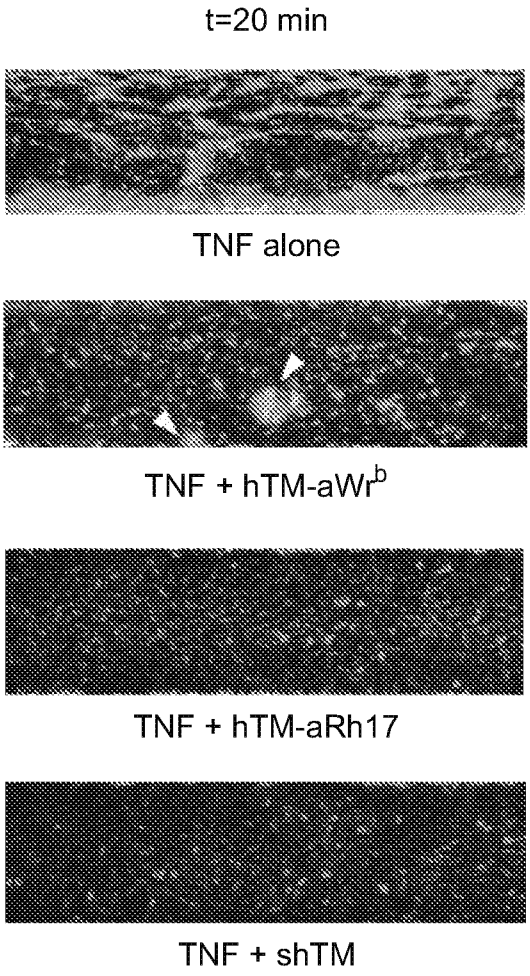


FIG. 6E

FIG 7A

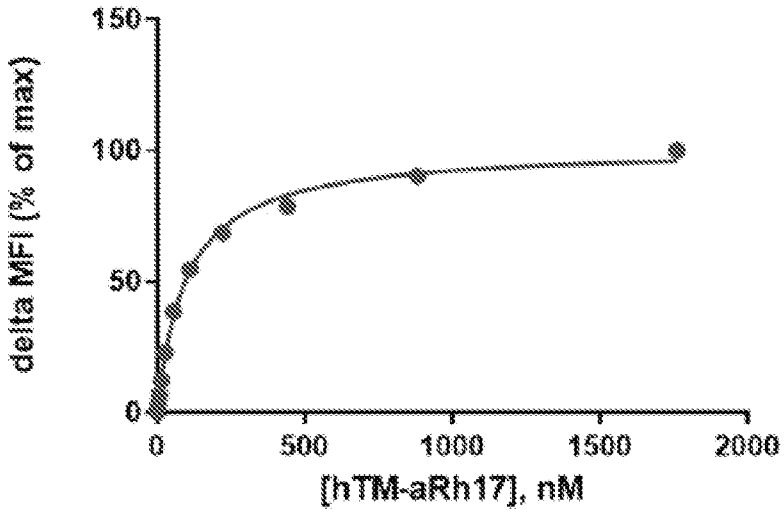

150
100
50
0
delta MFI (% of max)
0
500
1000
1500
2000
[hTM-aRh17], nM

FIG 7B

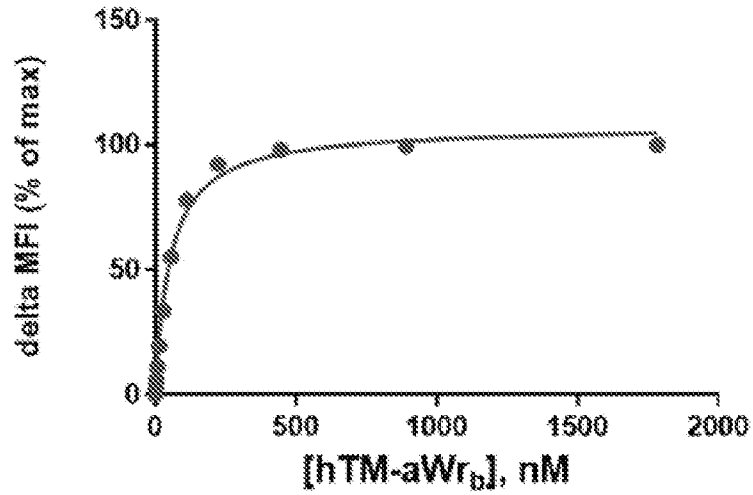

150
100
50
0
delta MFI (% of max)
0
500
1000
1500
2000
[hTM-aWrb], nM

FIG 7C

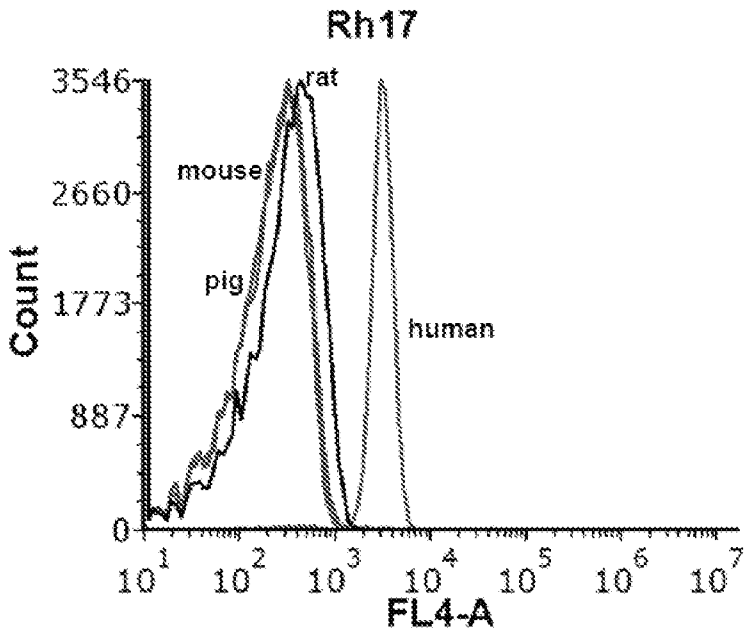

Rh17
3546
2660
1773
887
0
Count
rat
mouse
pig
human
10^1 10^2 10^3 10^4 10^5 10^6 10^7
FL4-A

FIG 7D

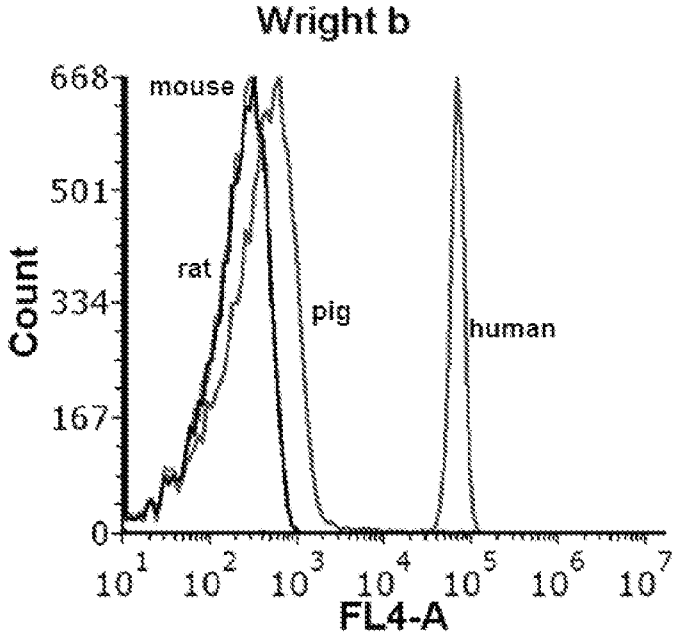

Wright b
668
501
334
167
0
Count
mouse
rat
pig
human
10^1 10^2 10^3 10^4 10^5 10^6 10^7
FL4-A

FIG 8A

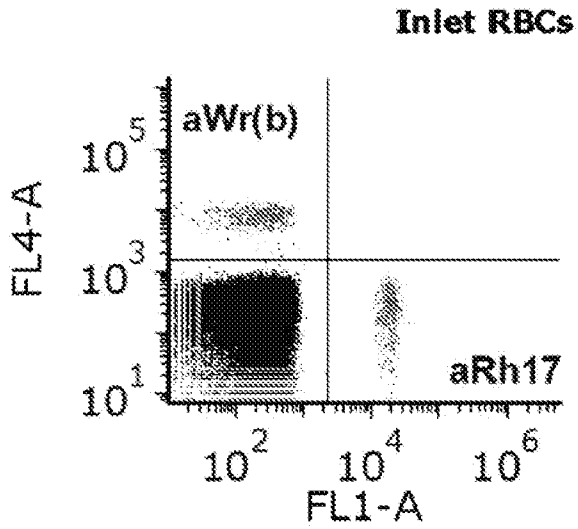


FIG 8B

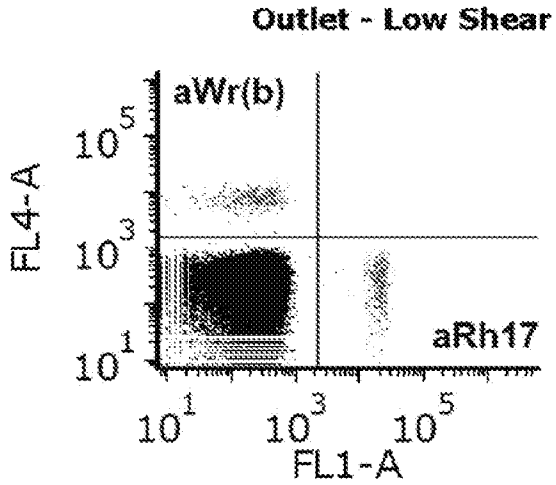


FIG 8C

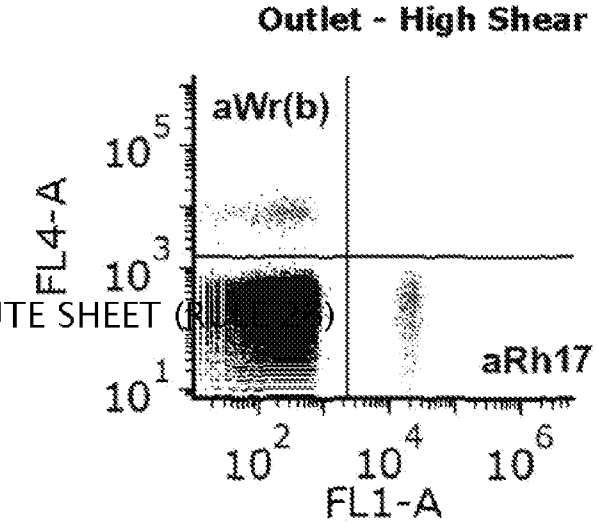


FIG 8D

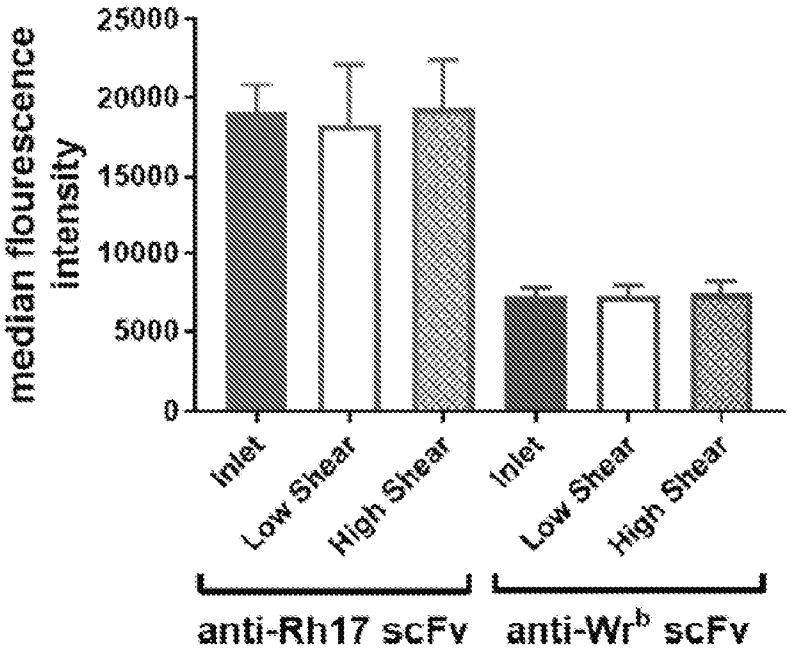
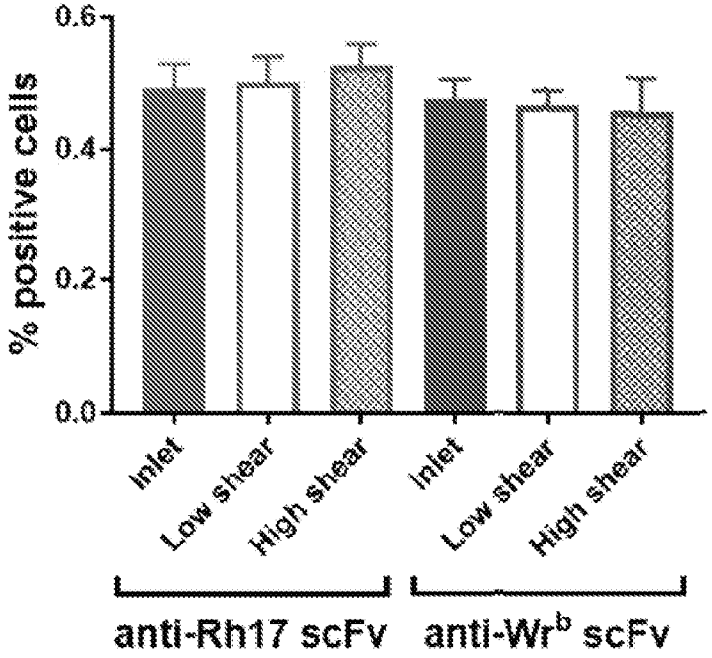


FIG 8E



SUBSTITUTE SHEET (RULE 26)

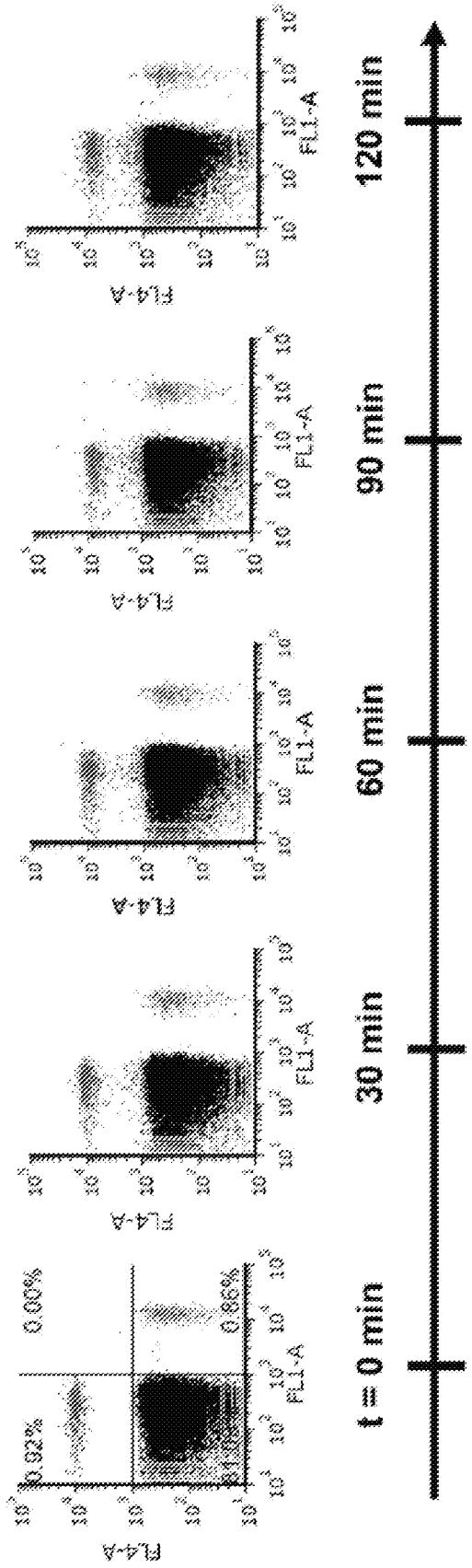


FIG 9A

SUBSTITUTE SHEET (RULE 26)

FIG 9B

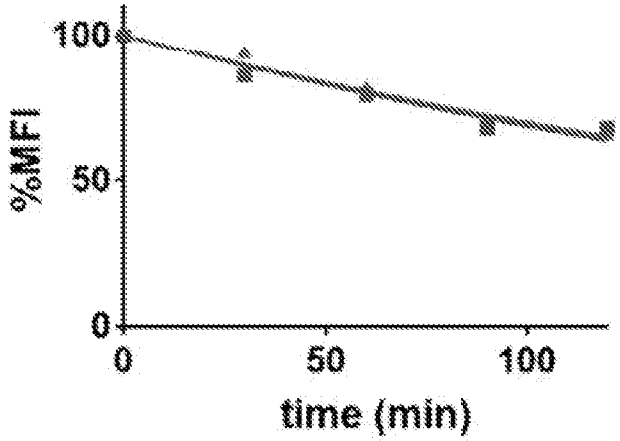


FIG 9C

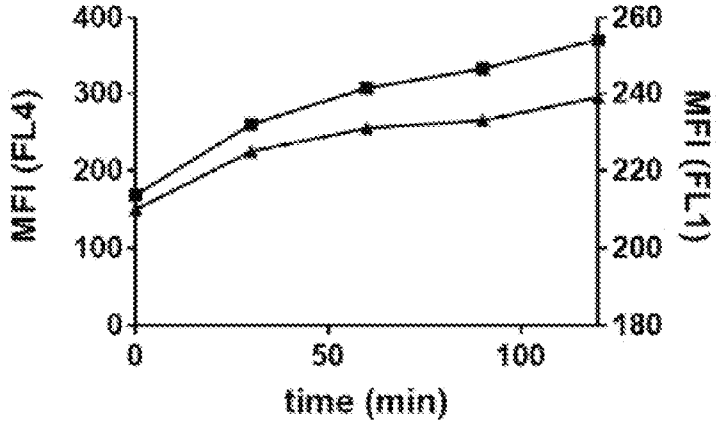


FIG 10

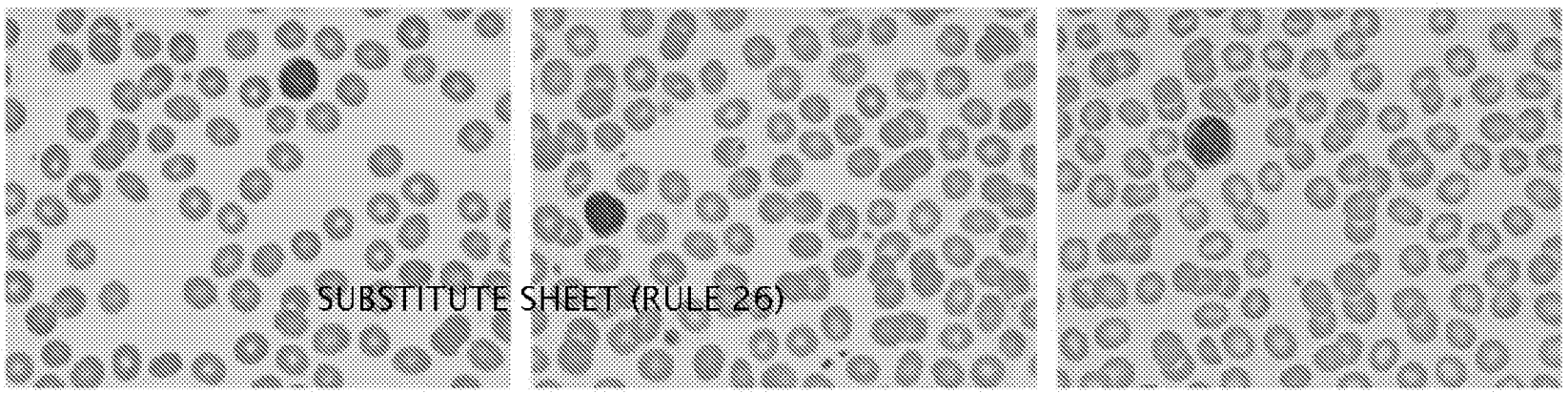


FIG 12

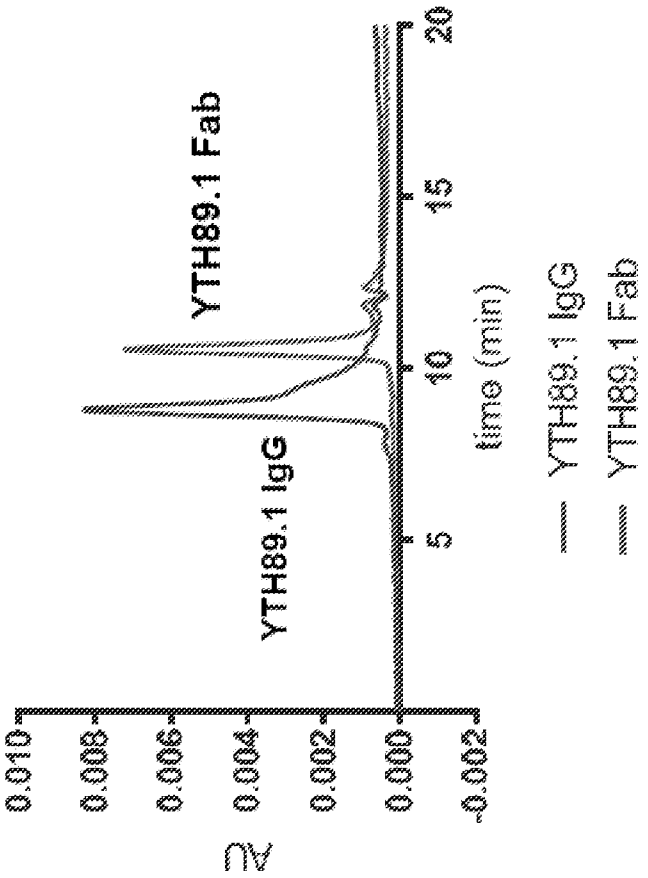
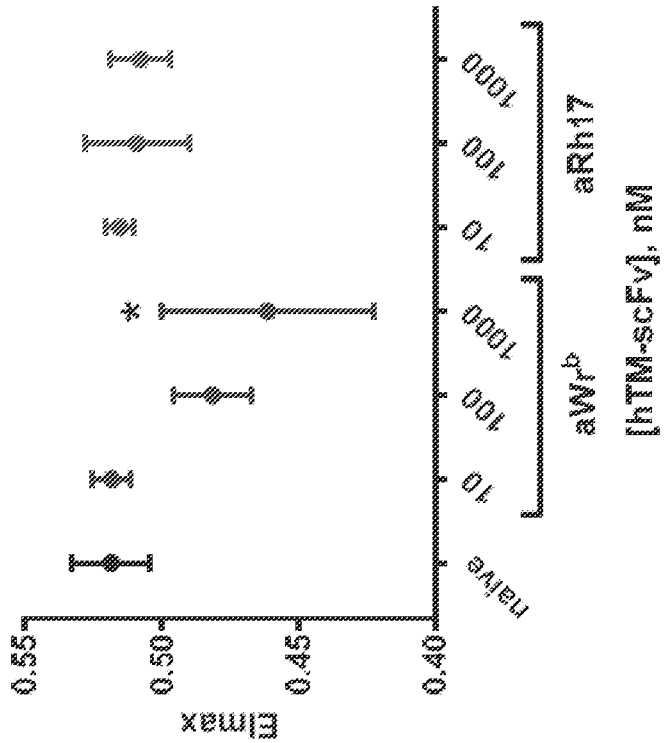


FIG 11



SUBSTITUTE SHEET (RULE 26)

FIG 13A

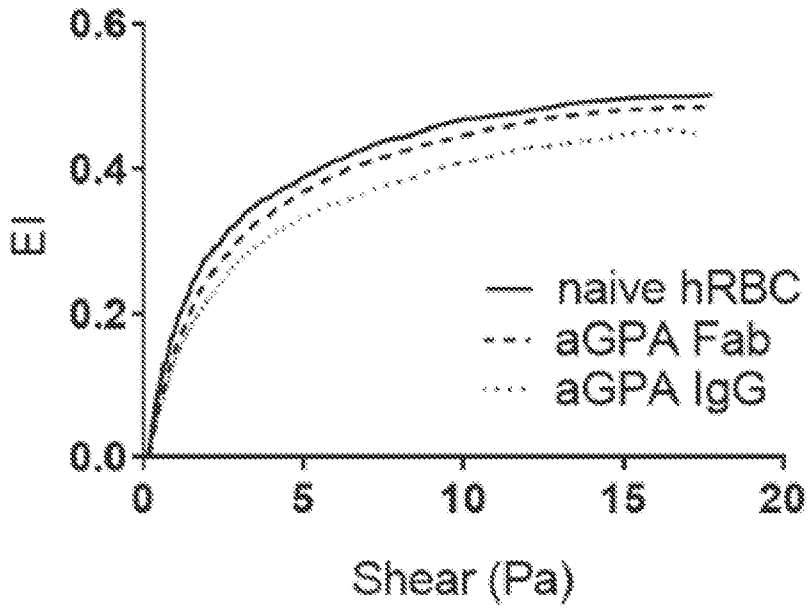


FIG 13B

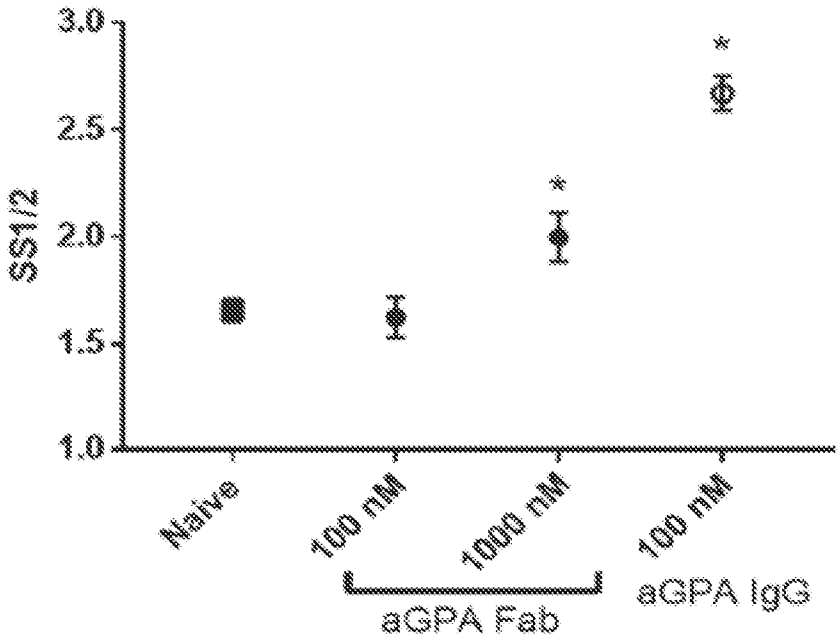


FIG 13C

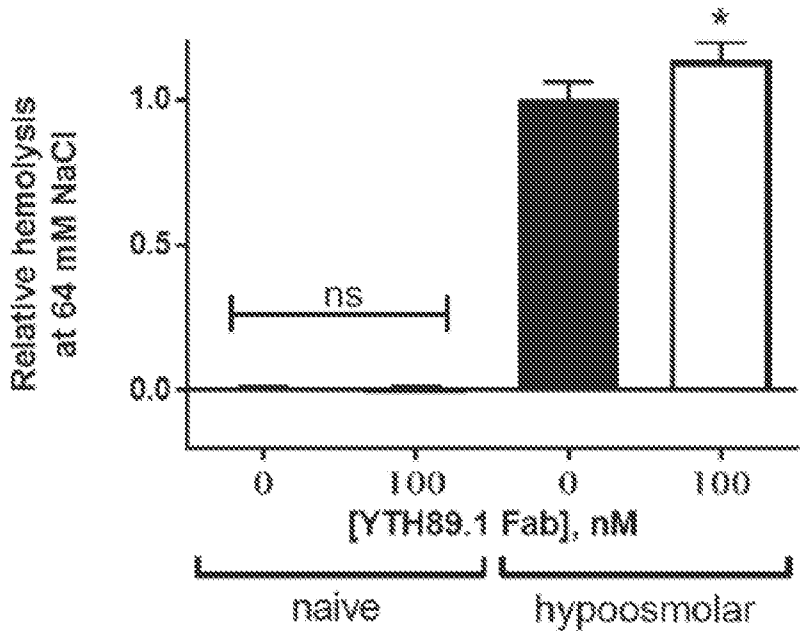


FIG 13D

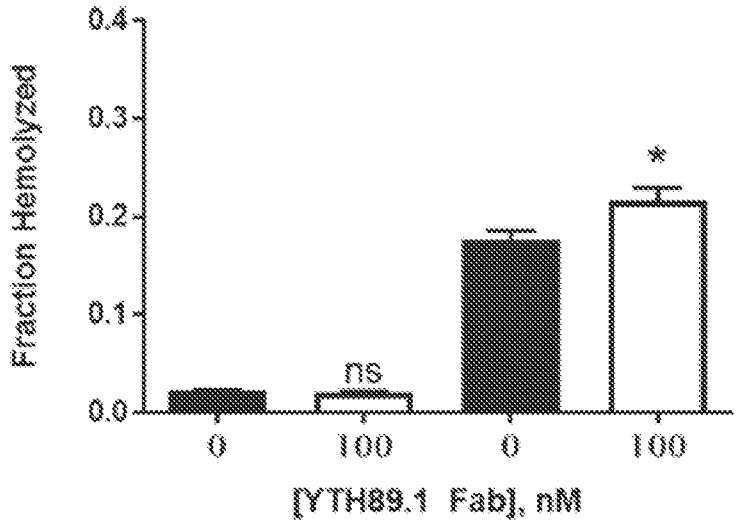


FIG 14A

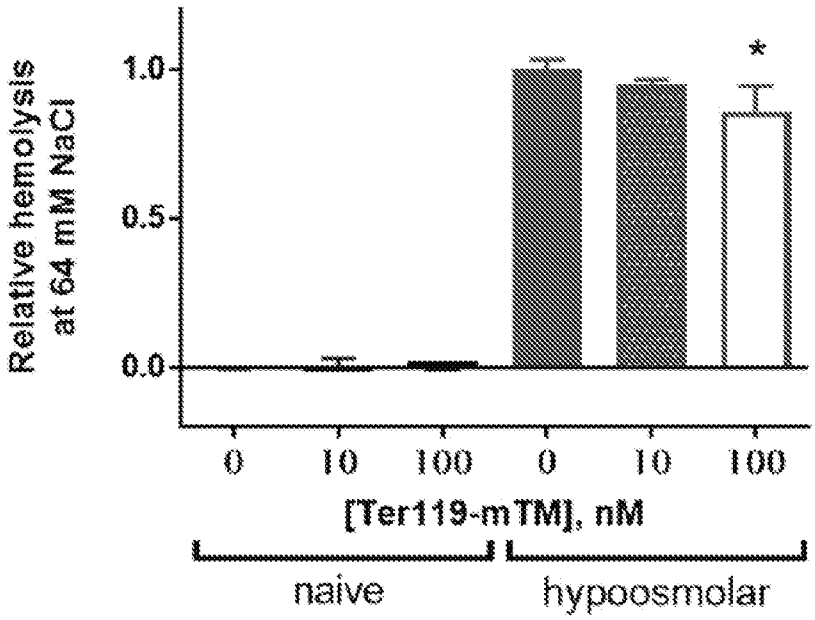


FIG 14B

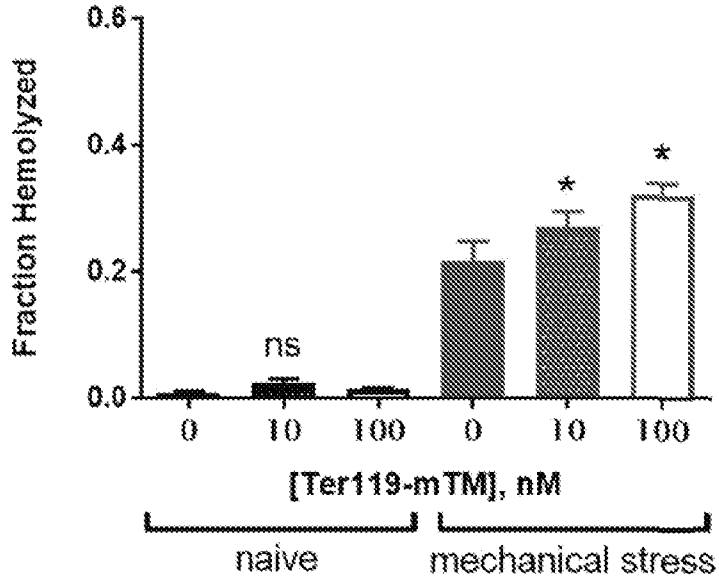


FIG 14C

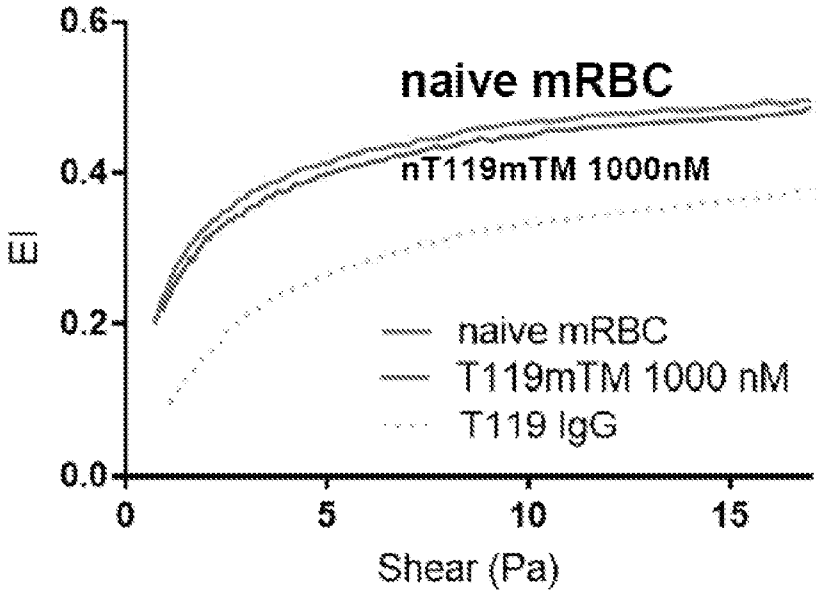


FIG 14D

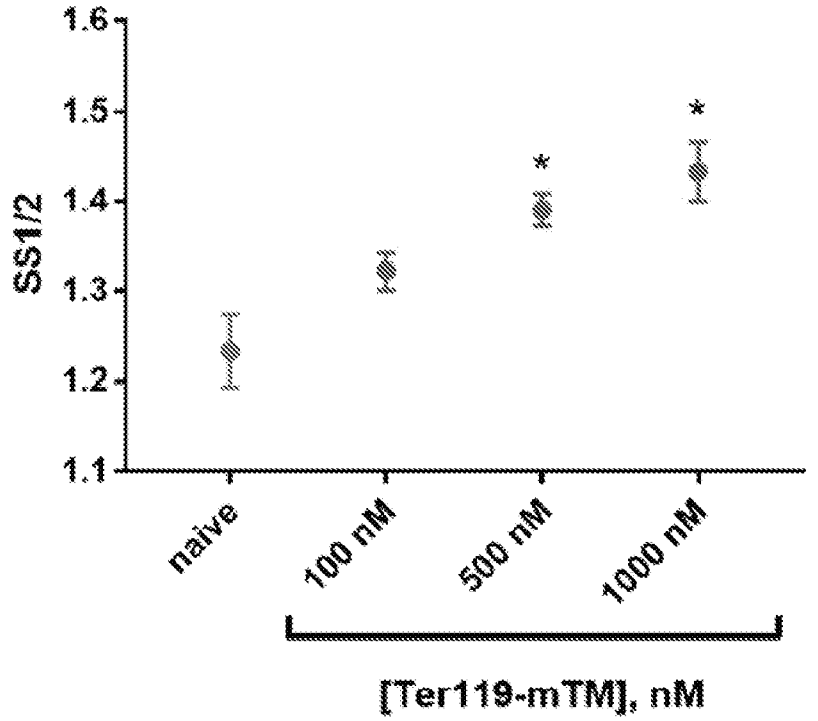


FIG 15A

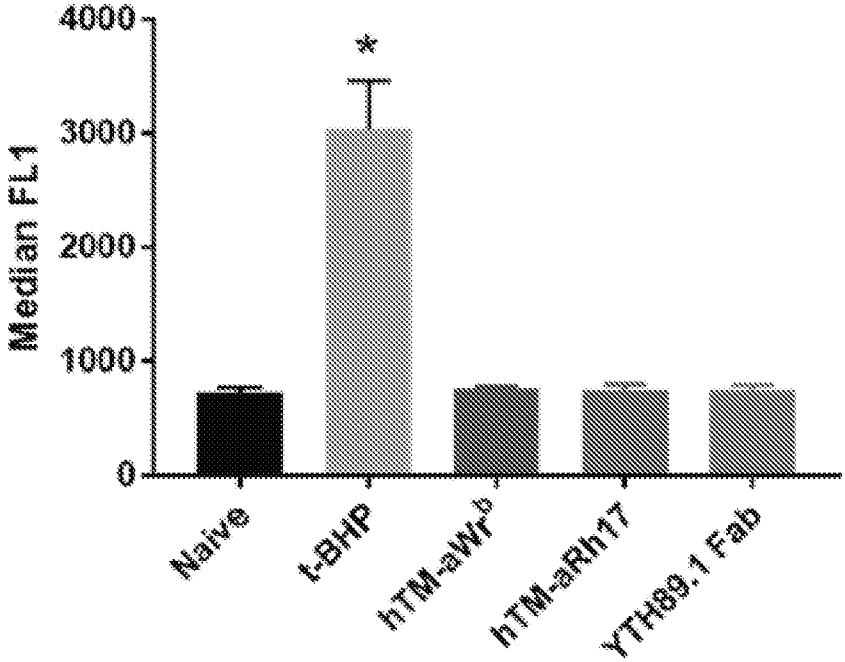


FIG 15B

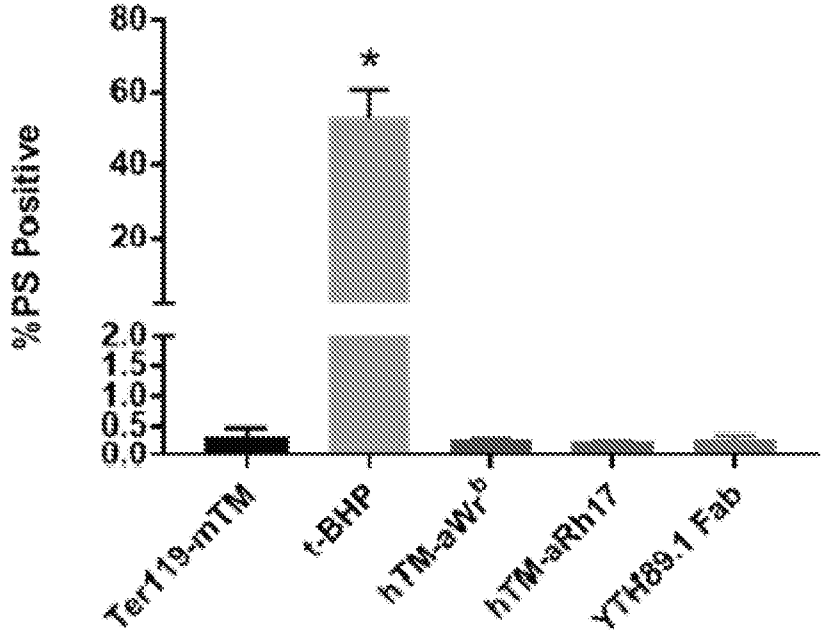


FIG 16

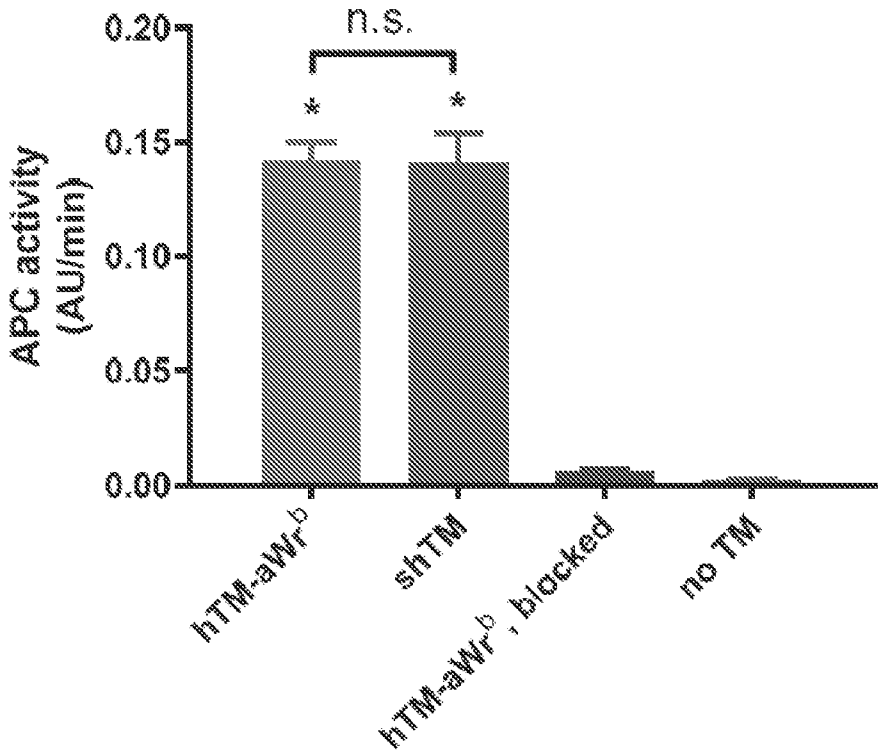

n.s.
*
*
APC activity (AU/min)
0.20
0.15
0.10
0.05
0.00
hTM-aWr^b
shTM
hTM-aWr^b, blacked
no TM

FIG 17

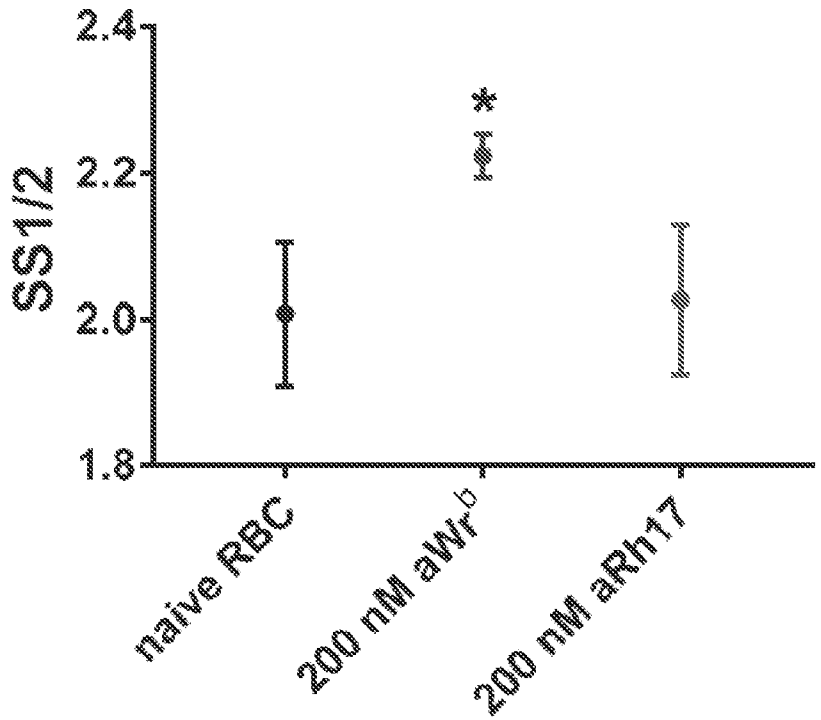

*
SS1/2
2.4
2.2
2.0
1.8
naive RBC
200 nM aWr^b
200 nM aRh17

FIG 18

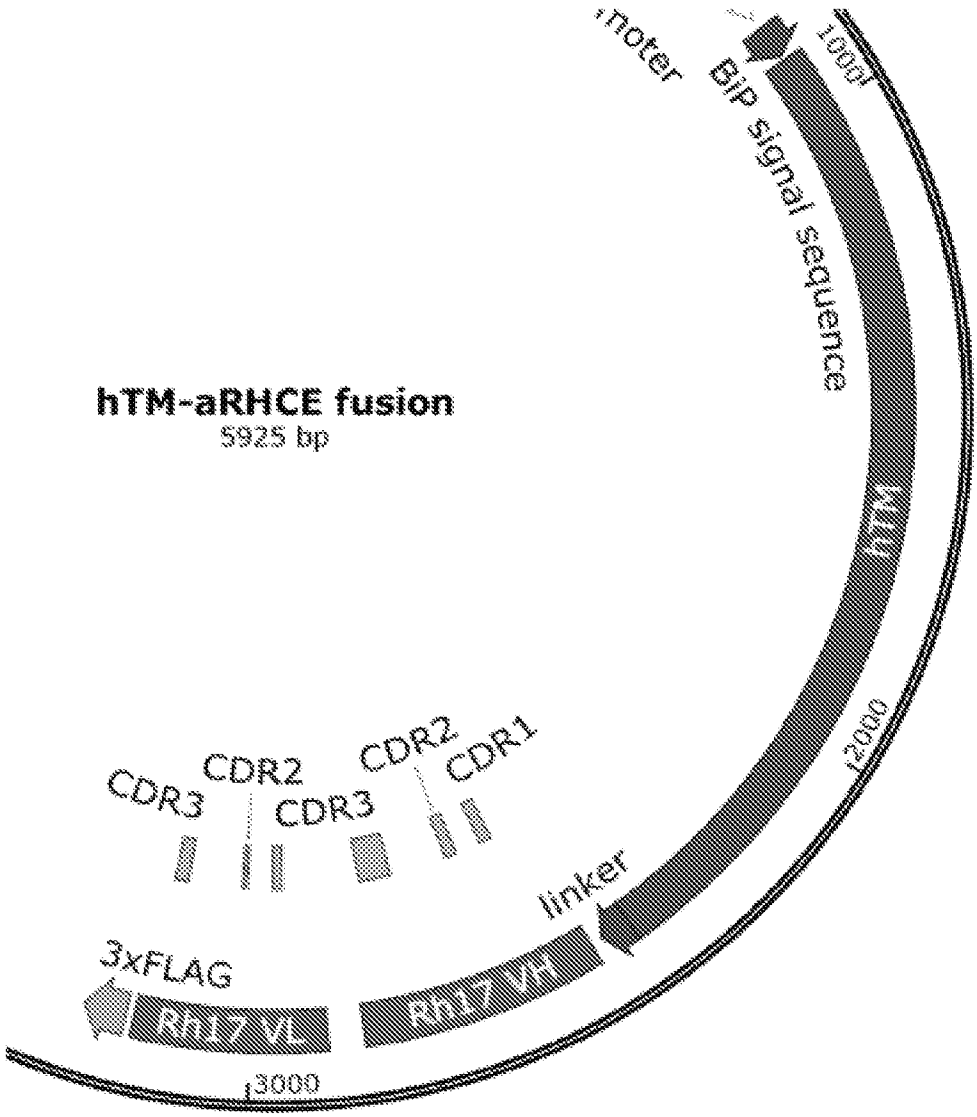
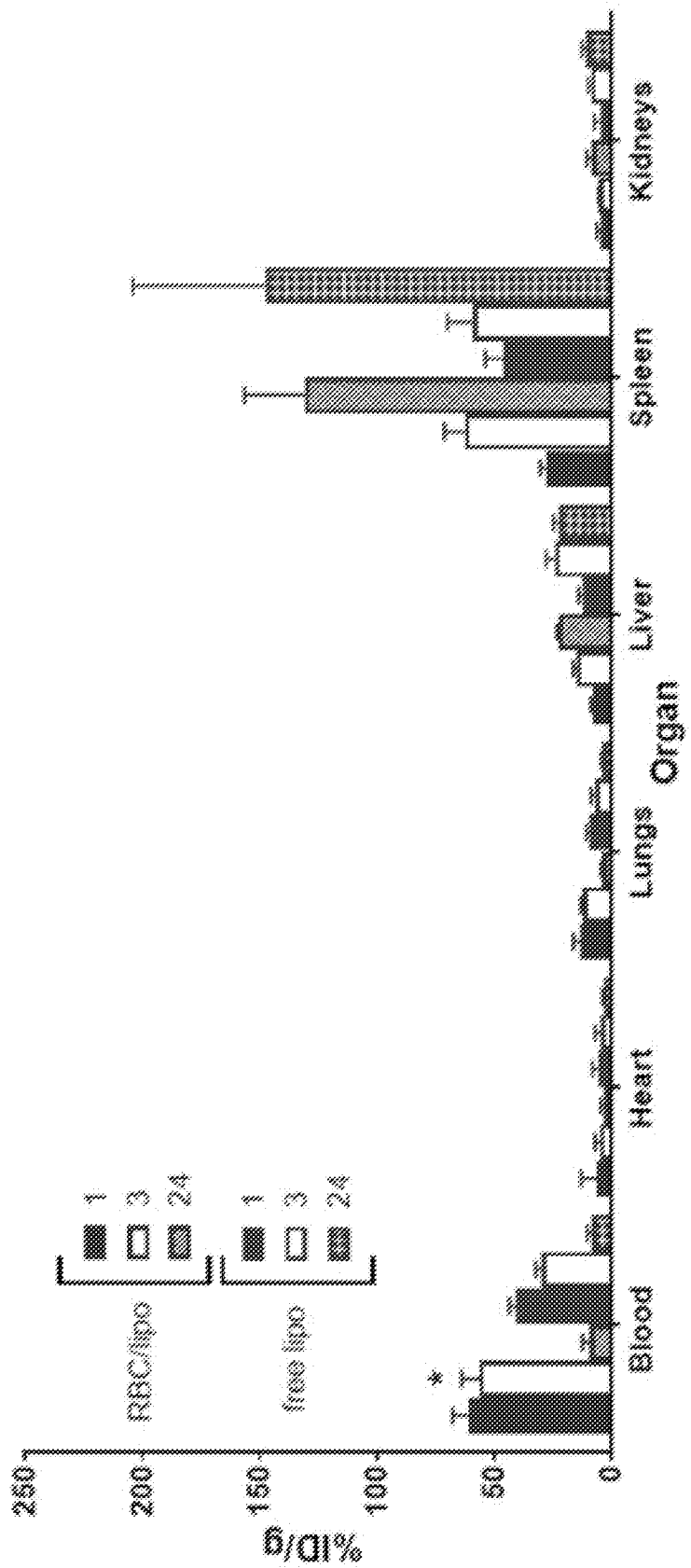

hTM-aRHCE fusion
5925 bp
1000
BiP signal sequence
hTM
2000
linker
Rh17 VH
Rh17 VL
3xFLAG
3000
CDR3
CDR2
CDR3
CDR2
CDR1

FIG 19A



SUBSTITUTE SHEET (RULE 26)

FIG 19C

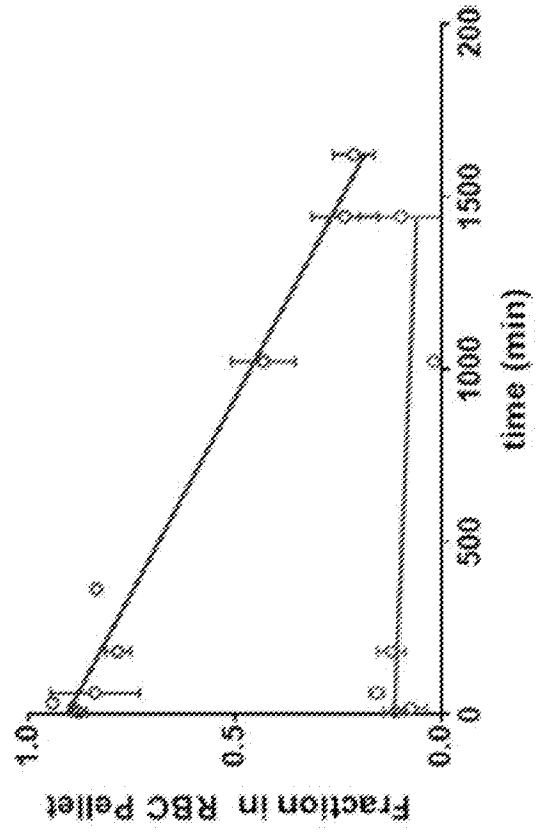
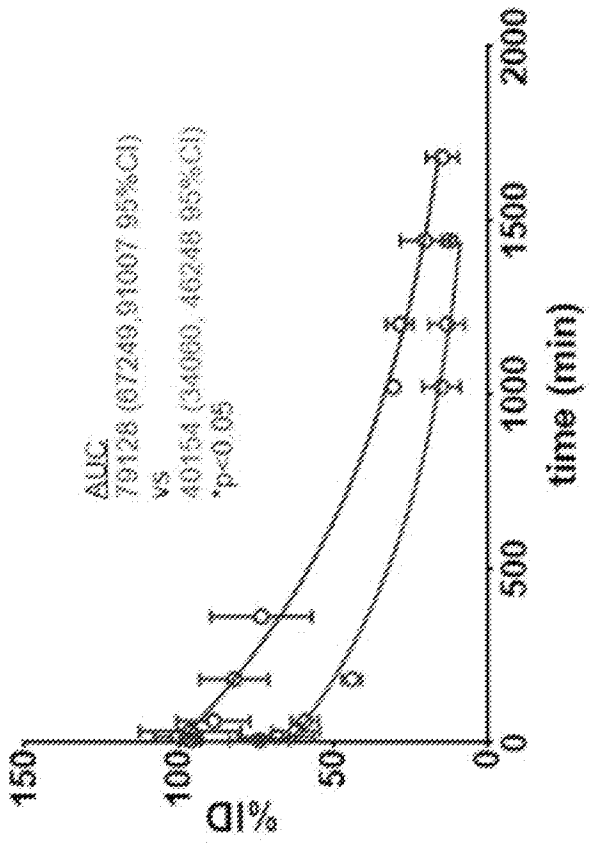


FIG 19B



SUBSTITUTE SHEET (RULE 26)

FIG 20A

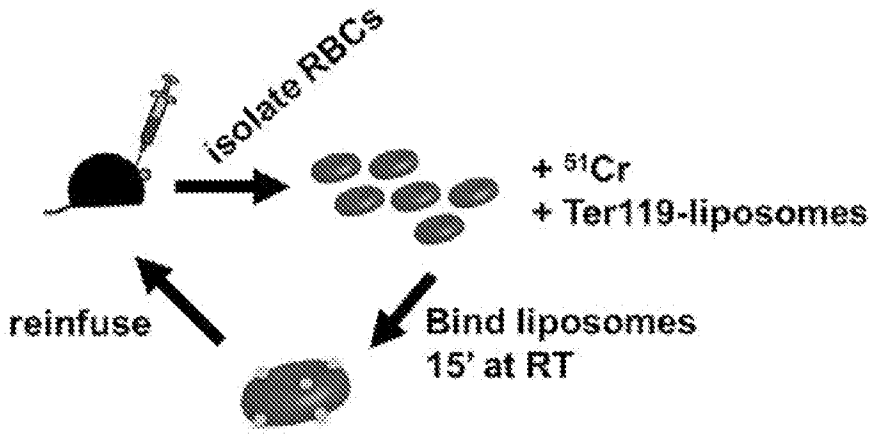
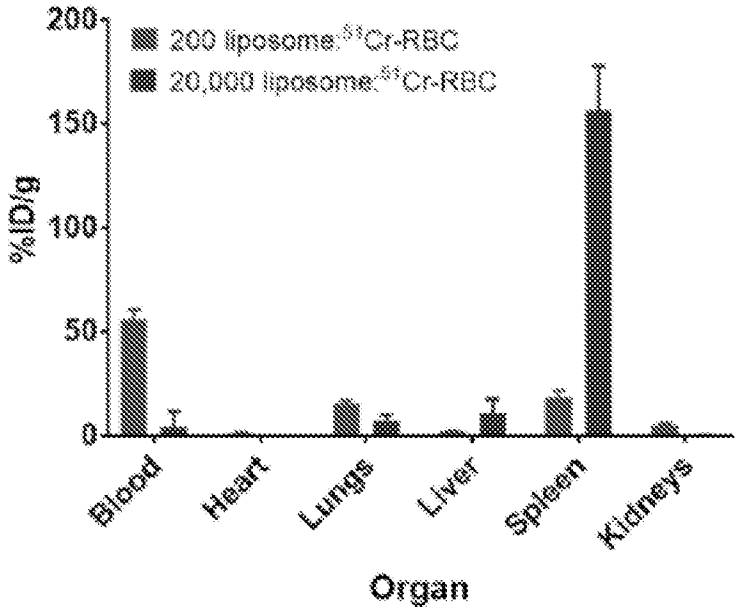


FIG 20B



SUBSTITUTE SHEET (RULE 26)

FIG 21A

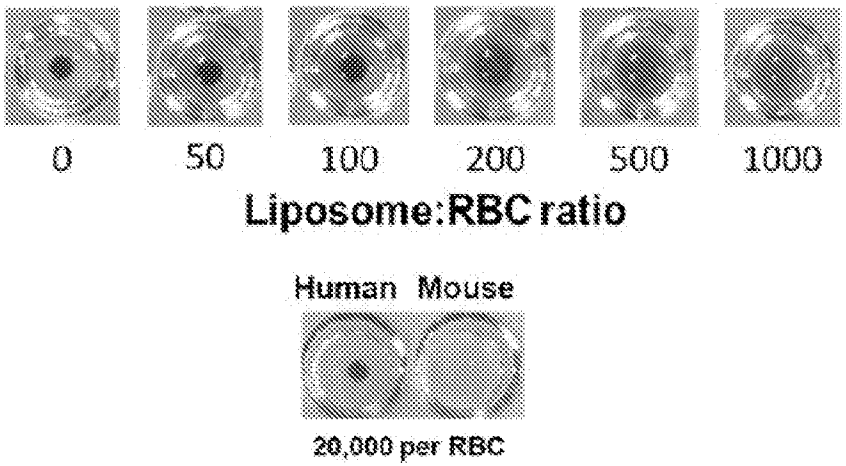
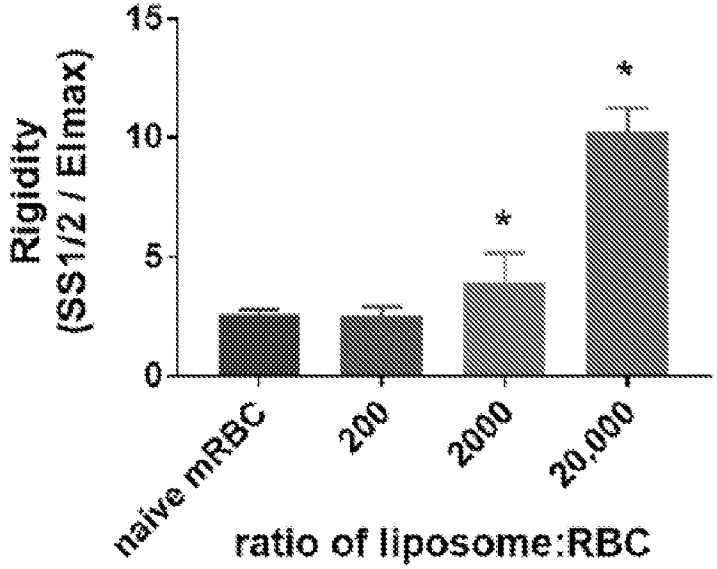


FIG 21B



SUBSTITUTE SHEET (RULE 26)

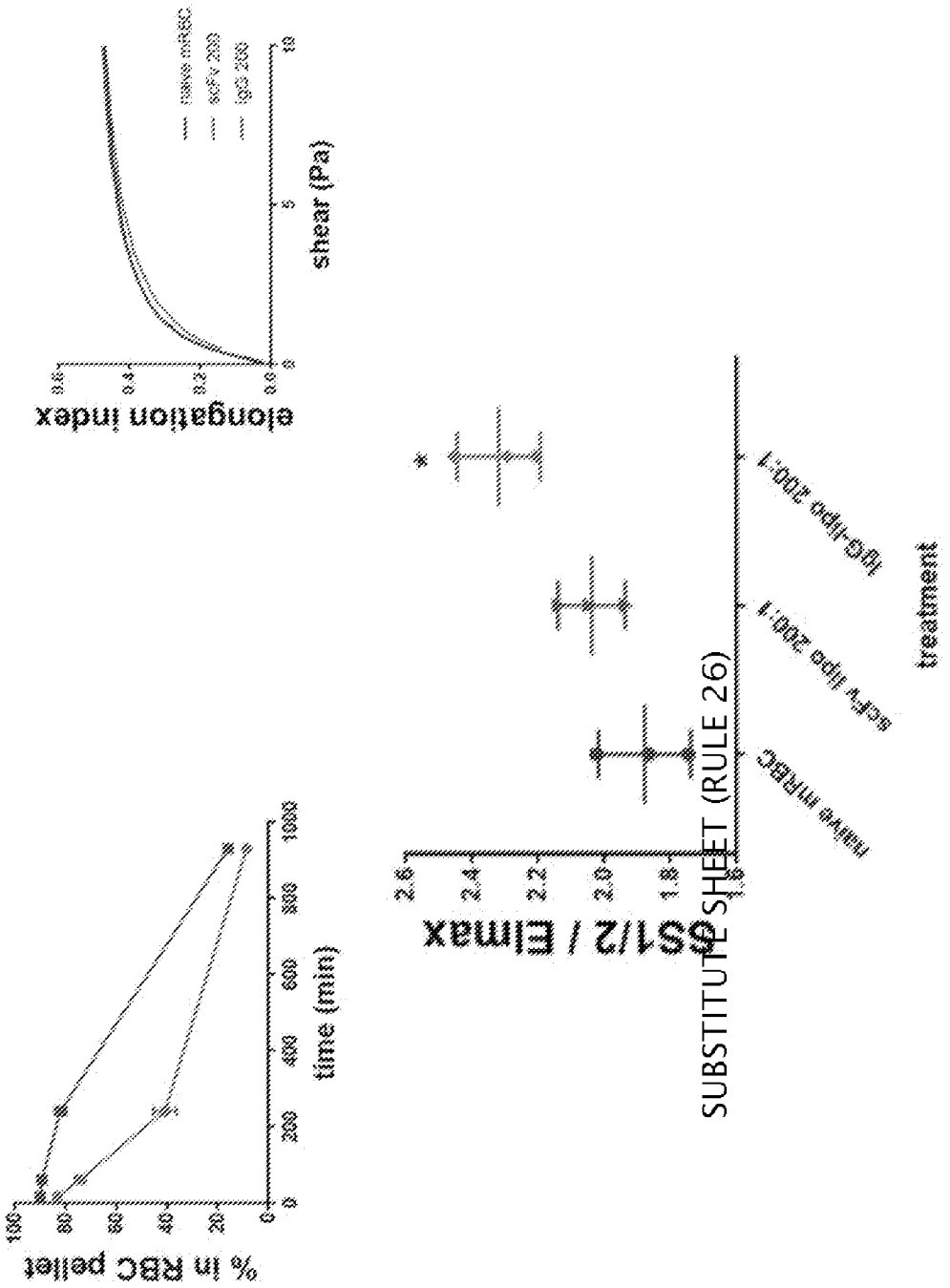

elongation index
shear (Pa)
native mRBC
scFv 200
IgG 200
% in RBC pellet
time (min)
GS1/2 / Elmax
native mRBC
scFv-lipo 200:1
IgG-lipo 200:1
treatment
*

FIG 22

FIG 23

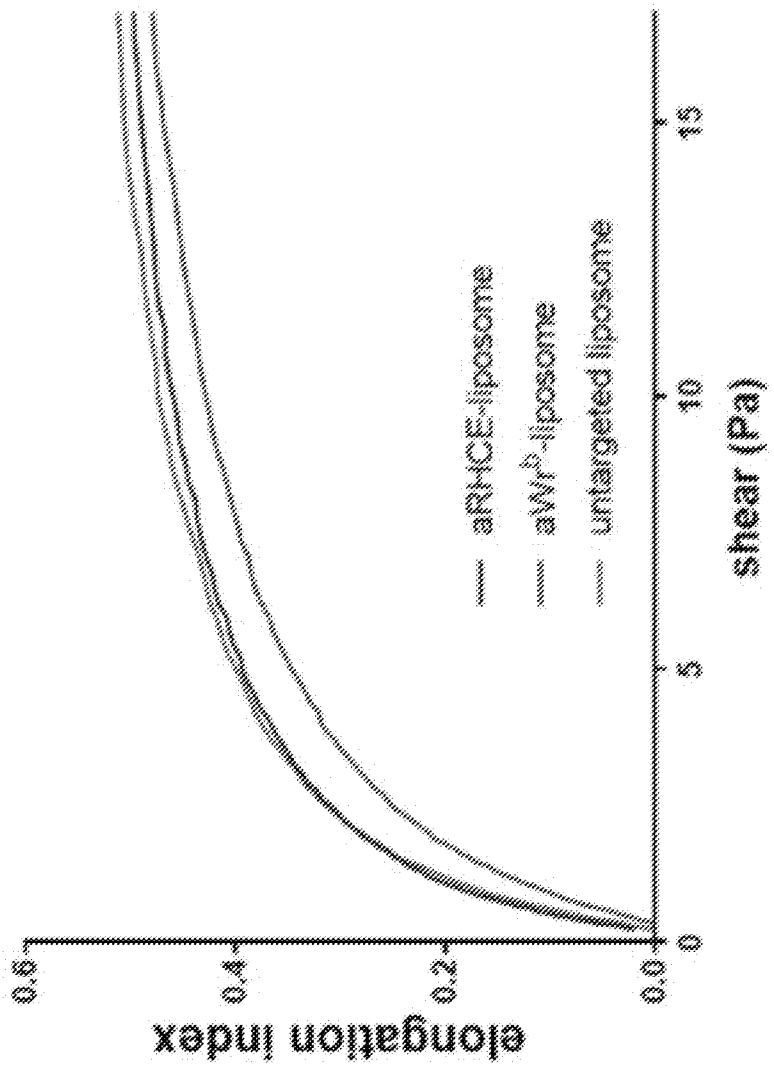

elongation index
0.0
0.2
0.4
0.6
0
5
10
15
shear (Pa)
aRHCE-liposome
aWP2-liposome
untargeted liposome

FIG 24A

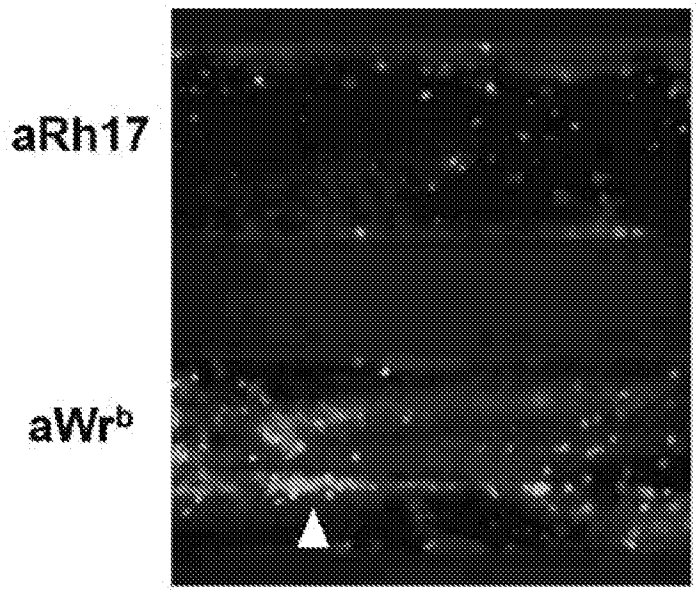


FIG 24B

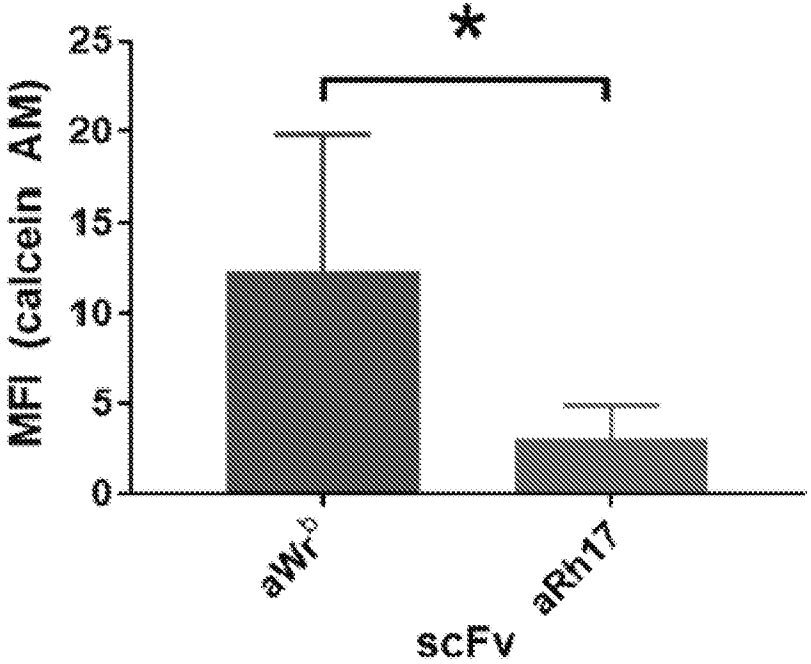


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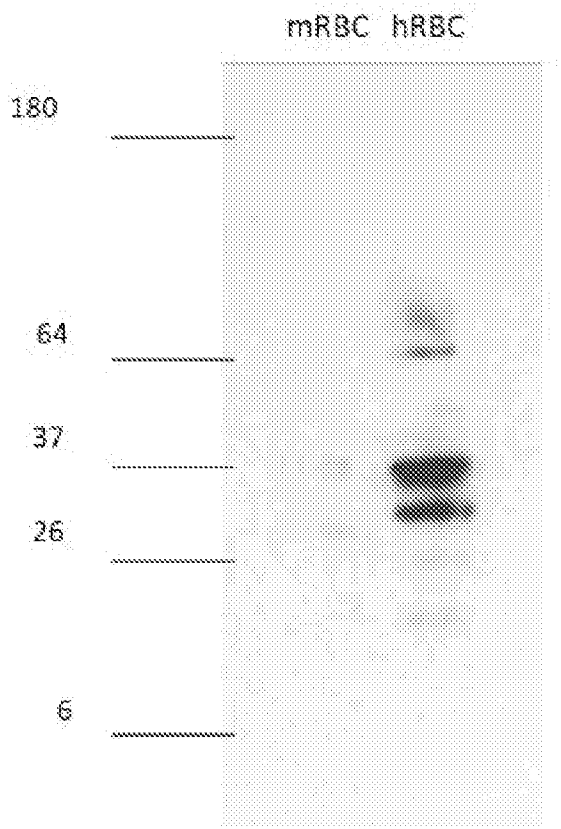
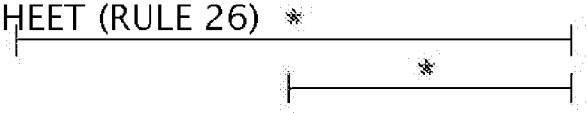
FIG 25





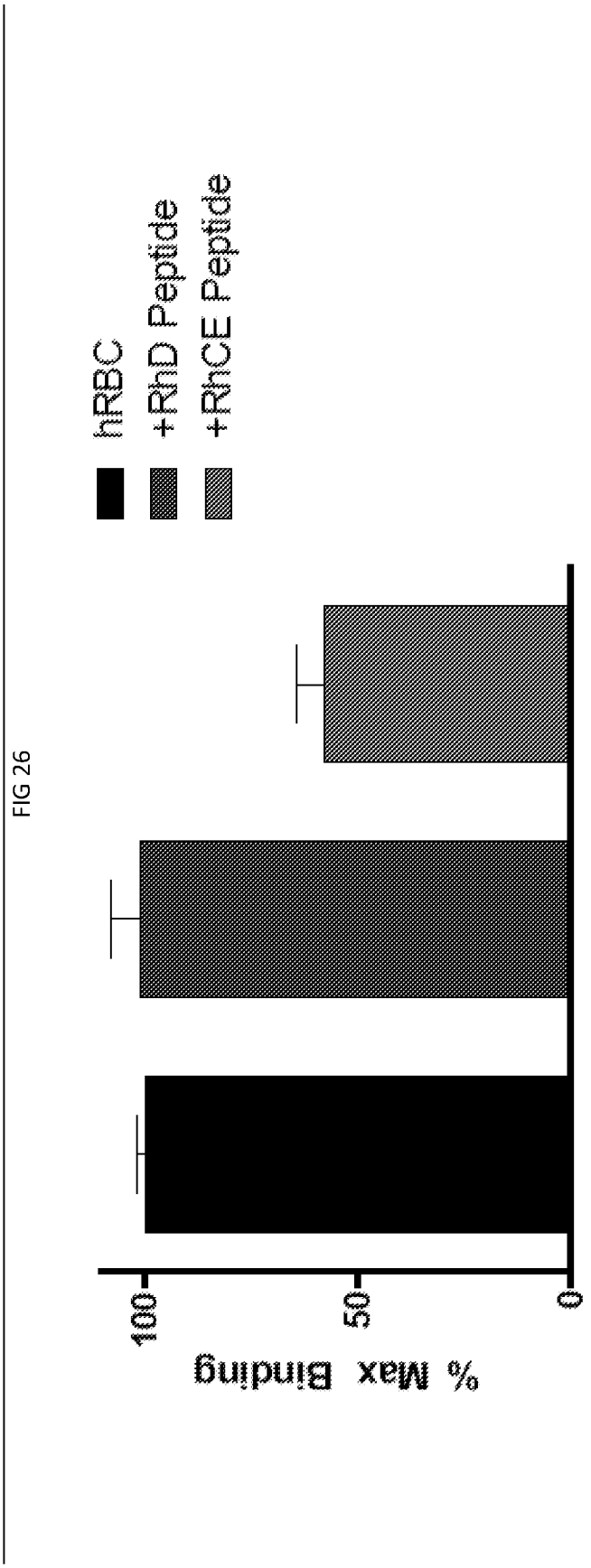
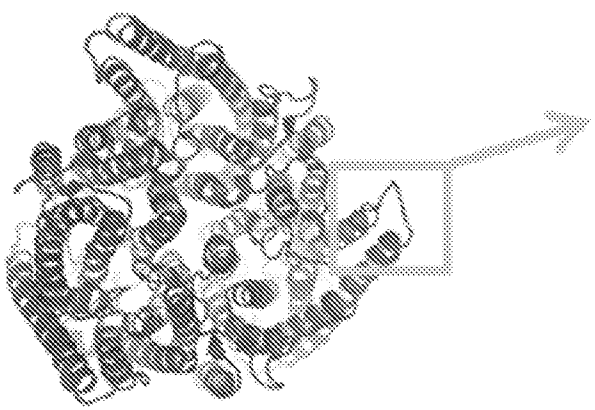


FIG 26

FIG 27



LVL**H**TV**WN**GNGM	Human RHCE	SEQ ID NO: 361
LVL**D**TV**GA**GNGM	Human RHD	SEQ ID NO: 362
QIV**TEPKS**SDLW	Mouse RHD	SEQ ID NO: 363
MAL**RVFWA**SSNM	Macaque RHD	SEQ ID NO: 364

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/64089

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C07K 16/00, 16/18, 16/34; G01N 33/80 (2019.01)
CPC - C07K 16/00, 16/18, 16/34; G01N 33/80

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENBANK ACCESSION AAC02646.1; Publication [online]. 11 February 1998 [retrieved 21 March 2019]. Retrieved from the Internet: <URL: https://www.ncbi.nlm.nih.gov/protein/AAC02646.1?report=genbank&log $=protalign&blast_rank=1&RID=929Z3S4Y015>; pp 1-2.	1
X	PASVOL et al. 'Inhibition of Malarial Parasite Invasion by Monoclonal Antibodies Against Glycophorin A Correlates with Reduction in Red Blood Cell Membrane Deformability' Blood, 1989, Vol. 74, No. 5, pp. 1836-1843; abstract; page 1836; second column, second paragraph; Figures 2B, 3; Table 2	4-5, 6/4-5
A	US 2016/0347830 A1 (LABORATOIRE FRANCAIS DU FRACTIONNEMENT ET DES BIOTECHNOLOGIES) 01 December 2016; abstract	2-3
A	US 2011/0033450 A1 (THULLIER et al.) 10 February 2011; paragraph [0060]	2-3
A	ANDRIS et al. 'Variable Region Gene Segment Utilization In Rhesus Monkey Hybridomas Producing Human Red Blood Cell-Specific Antibodies: Predominance Of The VH4 Family But Not VH4-21 (V4-34)' Molecular immunology, 1997, Vol. 34, No. 3, pp. 237-253	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 March 2019 (21.03.2019)	08 APR 2019
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/64089

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-30
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-5, 6/4-5; SEQ ID NOs.: 1, 19-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US18/64089

-***-Continued from Box No. III Observations where unity of invention is lacking: -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-6 and SEQ ID NOs: 1, 19 and 20 are directed toward antibodies and their corresponding VH and VL domain sequences, wherein said antibodies specifically bind to erythrocyte epitopes without causing significant adverse alteration of membrane deformability.

The antibodies will be searched to the extent they encompass a CDR encompassing SEQ ID NO: 1 (first exemplary VH CDR); a VH encompassing SEQ ID NO: 19 (first exemplary VH domain) and a VL encompassing SEQ ID NO: 20 (first exemplary VL domain). Applicant is invited to elect additional CDR sequence(s), and, where applicable, VH or VL sequence(s) encompsasing the CDR sequence(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), to be searched. Additional CDR sequence(s), and, where applicable, variable region sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-3 (each in-part) and 4-6 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (VH CDR); SEQ ID NO: 19 (VH domain) and SEQ ID NO: 20 (VL domain). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 2 (VH CDR2).

No technical features are shared between the antibody sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features of: an antibody or fragment thereof comprising at least one CDR of a defined sequence, wherein said antibody or fragment thereof specifically binds an erythrocyte; an antibody or fragment thereof comprising a VH and VL, each of a defined sequence, wherein said antibody or fragment thereof specifically binds an erythrocyte; and an antibody or fragment thereof which specifically binds an epitope on an erythrocyte without causing significant adverse alteration of membrane deformability; however, these shared technical features are previously disclosed by WO 2016/156940 A1 to Uniwersytet Jagiellonski (hereinafter 'Jagiellonski') in view of the publication entitled, "Inhibition of Malarial Parasite Invasion by Monoclonal Antibodies Against Glycophorin A Correlates with Reduction in Red Blood Cell Membrane Deformability," by Pasvol et al. (hereinafter 'Pasvol').

Jagiellonski discloses an antibody or fragment thereof comprising at least one CDR of a defined sequence (heavy chain (VH) and light chain CDRs of a defined sequence; page 14, lines 5-11; claim 1), wherein said antibody or fragment thereof specifically binds an erythrocyte (IgG antibody specific for erythrocyte B antigen; page 4; lines 25-30; page 8, lines 22-25); and an antibody or fragment thereof comprising a VH and VL (page 14, lines 1-11; claim 1), each of a defined sequence (claim 1), wherein said antibody or fragment thereof specifically binds an erythrocyte (IgG antibody specific for erythrocyte B antigen; page 4; lines 25-30; page 8, lines 22-25). Jagiellonski does not disclose an antibody or fragment thereof which specifically binds an epitope on an erythrocyte without causing significant adverse alteration of membrane deformability.

Pasvol discloses an antibody which specifically binds an epitope on an erythrocyte (antibodies directed against red blood cell epitopes; page 1839; second column, first paragraph; Figure 2B) without causing significant adverse alteration of membrane deformability (reduction of erythrocyte membrane deformability caused by antibody binding is correlated with reduced malarial parasite invasion; abstract; page 1836; second column, second paragraph; Figure 3). It would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the disclosure of Jagiellonski to provide wherein an antibody or fragment thereof which specifically binds an epitope on an erythrocyte without causing significant adverse alteration of membrane deformability, because the effectiveness of binding of erythrocyte-specific antibodies in reducing membrane deformability to lessen malarial parasite interactions with the erythrocyte membrane as disclosed by Pasvol would have motivated the skilled artisan to utilize antibodies such as those previously disclosed by Jagiellonski to in order to prevent adverse alterations of membrane deformability.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the combination of the Jagiellonski and Pasvol references, unity of invention is lacking.