Phage display cDNA library screens

Phage display cDNA screens were performed to identify human RBC binding proteins of *P. falciparum* as previously described. Phage cDNA libraries were constructed from *P. falciparum* 3D7 mRNA using T7 select system (Novagen) as described. Phage clones were selected through four rounds of biopanning using human RBCs as bait in the binding buffer containing PBS (Phosphate Buffered Saline), 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.4, and 3.0% BSA. The reaction mixture (700 µL) was centrifuged through 500 µL Silicone oil to separate RBCs at 12,000 rpm (Eppendorf 5415D). Phage particles bound to RBCs were washed three times with PBS, eluted with 3.0 M NaCl for 20 minutes, and amplified in BLT5403 *E. coli* for 3.0 hours at 37°C. Plaque assays were performed as described in the Novagen's T7Select Manual. Phage inserts were amplified from independent plaques by PCR, further purified using E.Z.N.A. Cycle-Pure Kit, and sequenced at Tufts University Genomics Core Facility.

To identify potential host RBC receptors for PfGARP, reverse phage display screens were performed using purified recombinant PfGARP. Using Novogen’s T7 select system, multiple cDNA libraries were constructed from human reticulocyte mRNA with either random primed or oligo (dT) primed cDNA synthesis protocols. The discarded de-identified reticulocyte-rich blood was obtained from hemochromatosis patients from the Tufts Blood Bank with approval under IRB # 9750. The number of independent clones were approximately 6x10⁶ pfu for random primed cDNA library, and 1.8x10⁷ pfu for oligo (dT) primed cDNA library. These libraries were screened using purified TRX-PfGARP-M and His-PfGARP-M fusion proteins as bait. Several screens of both cDNA libraries yielded 83 potential clones that were sequenced and analyzed. The ELISA plates were coated with purified PfGARP fusion proteins (10 µg/mL in PBS) overnight at 4°C. Wells were washed three times with PBS, blocked with blocking buffer (1% BSA in PBS), and washed five times with deionized water. Phage display cDNA libraries
were diluted in PBST (PBS plus 0.05% TWEEN 20) for the binding experiments. Plates were washed five times with PBST and bound phages were eluted and amplified as described above.

Expression and purification of PfGARP constructs

PfGARP coding sequences, PfGARP<sub>356-552</sub> (PfGARP-L) of 3D7 strain and PfGARP<sub>392-437</sub> (PfGARP-S) of FCR-3 strain, were amplified by PCR with EcoRI and HindIII adaptors and cloned in pET-32b vector (Novagen). The constructs added His x 6 and TRX (thioredoxin)-Tags in the fusion proteins expressed in NEB 5-alpha competent <i>E. coli</i>. The primers used to amplify and sequence the PfGARP-S and PfGARP-L proteins were T7 up-5<sup>'</sup>-GGAGCTGTCA GTATTCCAGTC-3<sup>'</sup> and T7 down-5<sup>'</sup>-AACCCCTCAAGACCGCTT-3<sup>'</sup>. Primers used for the colony PCR and sequencing of the inserts in pET-32b vector were 5<sup>'</sup>-CGAACGCCAGCATGGACA-3<sup>'</sup> (Forward, pETF) and 5<sup>'</sup>-TGCTAGTTATTTGCTACGCGG-3<sup>'</sup> (Reverse, pETR). PfGARP<sub>370-444</sub> (PfGARP-M) construct was designed using PSIPRED software, and chemically synthesized after codon optimization (GenScript USA). The cDNA construct in pUC57 vector contained PvuII and ApaI restriction sites linkers to allow insertion in pRE4 vector generating the pRE4-PfGARP-M construct. PfGARP-M was also cloned into pET32b to express TRX-PfGARP-M protein. Sub-clones of PfGARP-M, PfGARP<sub>370-416</sub> (PfGARP-M1), and PfGARP<sub>417-444</sub> (PfGARP-M2) were cloned into pGEX2T for expression of GST fusion proteins. The TRX-PfGARP-M<sub>1(370-416)</sub> and PfGARP-M<sub>2(417-444)</sub> clones were generated by transferring the inserts from pGEX2T to pET32a. The His-PfGARP-M was cloned into pLIC-His plasmid (ligation independent cloning vector) with His-tag at the N-terminus.<sup>2</sup>

Recombinant proteins were expressed in <i>E. coli</i> BL21 (DE3). The His-tagged PfGARP proteins were purified through Nickel (Ni) affinity chromatography using high density Ni agarose beads or Cobalt resin for small volume purification. For larger volume purification, fast protein liquid chromatography (FPLC) equipped with a Nickel column was used. Protein-bound beads were washed three times with washing buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM Imidazole), eluted with elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 400 mM Imidazole), and dialyzed against sterile PBS overnight at 4°C. Proteins were stored at -80°C with 0.1% glycerol. In some cases, proteins were dialyzed again against PBS after thawing. Protein concentration was determined using NanoDrop according to the molecular weight and extinction
coefficient of the protein or BCA Protein Assay Kit, and proteins were analyzed by 12% SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB).

**Enzyme treatment of human RBCs and binding assays**

Human blood was collected from healthy de-identified donors of both sexes in the ACD (Acid Citrate Dextrose) buffer with approval under IRB # 9750. Alternatively, human RBCs (AB+) from de-identified donors of both sexes were obtained as packed cells from the Red Cross (Dedham, MA). The GYPB null RBCs were obtained from ITxM Clinical Services, Rosemont IL 60018. Blood samples were washed three times in DPBS (Dulbecco's Phosphate Buffered Saline) without calcium and magnesium or RPMI 1640. Enzyme treatment of human RBCs was performed as described with minor modifications. RBCs were incubated with the following enzymes in PBS for one hour at 37°C on a rotator. Enzymes used were α-chymotrypsin from bovine pancreas (0.5- 1.0 mg/mL, Sigma), trypsin from bovine pancreas (0.5- 1.0 mg/mL, Sigma), and neuraminidase from *Clostridium perfringens* (5 units/mL, Sigma) in 100 mM sodium acetate, 2.0 mM CaCl₂, pH 5.0. Treated RBCs were washed with PBS and incubated with trypsin-chymotrypsin inhibitor (Sigma) in PBS for 20 minutes while rotating at room temperature. The RBCs were then washed three times with PBS and used for the binding assays.

The protocol for the RBC-binding assay of recombinant proteins was as described previously. Equal amounts (1.0 µM final concentration) of recombinant proteins and control protein were mixed with 1.0 mg/mL BSA (0.1%) in PBS or DPBS with calcium and magnesium, 20 µL of fresh packed RBCs (80 µL of 25% Hematocrit) were added. After incubation with gentle rotation for 2 hours at room temperature, cells were centrifuged on 500 µL Silicone oil at 12,000 rpm. In some experiments, 0.5 mM DTT (Dithiothreitol) was added to the binding buffer. The pellet was washed with PBS and bound proteins were eluted from RBCs by 1.5 M NaCl. The TRX-tagged proteins were detected by Western blotting using anti-TRX monoclonal antibody. Proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose membrane for Western blotting. Non-specific binding was blocked using 5% milk in PBST. Inputs were prepared by diluting the binding mixture in PBS before the addition of RBCs. Western blotting signal was detected using the goat anti-mouse IgG HRP polyclonal secondary antibody and ECL.
CHO-K1 erythrocyte-binding assay

Chinese Hamster Ovary (CHO-K1 cells) (Sigma) were cultured in Ham's F-12 modified medium supplemented with 10% HyClone fetal bovine serum, 1X L-Glutamine, and 1% Penicillin-Streptomycin. Cells were harvested with 0.05% trypsin-EDTA, and RBC binding assays were performed essentially as described previously. CHO-K1 cells (1.0 x10^6) were transfected with 4.0 µg pRE4 plasmid vector using Lipofectamine 2000 transfection reagent (Invitrogen). This vector ensures the expression of protein on the surface of cells by substituting part of the internal region of the herpes simplex virus glycoprotein D (HSV gD1) with recombinant PfGARP and EBA-175. The control plasmid pRE4-EBA-175, transfected into CHO-K1 cells, was used as an internal positive control since EBA-175 binds host glycophorin A expressed on the surface of human RBCs, whereas the pRE4 empty vector with GFP insert was used as a negative control. RBC binding assays using CHO-K1 cells transfected with pRE4-PfGARP-M, along with appropriate controls, were performed 24 hours following transfection. Each coverslip containing the CHO-K1 cells and 100 µL of RBCs at 10% hematocrit diluted in the CHO-K1 culture media was incubated at 37°C for 2 hours. After incubation, coverslips were transferred to new wells and incubated in PBS for 15 minutes to allow unbound RBCs to fall off. Coverslips were fixed in 1% glutaraldehyde in cold PBS. Rosettes were visualized, counted, and imaged at 40X magnification objective using a Nikon Eclipse TE200 microscope and SPOT RT3 camera/SPOT software.

Immunofluorescence assays without permeabilizing the cells were performed to confirm the transfection and extracellular expression of proteins. Cells were fixed by 3.7% formaldehyde and stained with mouse mAb ID3 and mAb DL6 against HSVgD1 epitopes cloned on either side of the vector. These specific antibodies were kindly provided by Drs. Gary H. Cohen and Roselyn J. Eisenberg at the University of Pennsylvania. Secondary antibody (goat anti-mouse IgG with Alexa Fluor 633 conjugate) was used at 1:1000 dilution. Images were captured using 60X objective on Nikon Eclipse TE2000-E inverted microscope and MetaMorph Microscopy Automation & Image Analysis Software.
Human erythrocyte aggregation assay

We designed an in vitro RBC binding assay with modifications from a previously published protocol\textsuperscript{10} to visualize the formed unfixed human RBC aggregates. Briefly, 2.0 \( \mu \text{L} \) of RBCs at 20\% hematocrit were added to a final volume of 500 \( \mu \text{L} \) containing synthetic peptides in PBS with rotation at room temperature for two hours. Relatively lower number of RBCs were used to better visualize formation of any rouleaux and aggregates under Nikon Eclipse TE2000-E inverted microscope using 10X objective. Only RBC clusters forming clear 3D structures were counted and considered as positive hits. Representative images were captured using the MetaMorph Software. Two synthetic peptides of PfGARP termed M2K4 and M2K5 originating from the PfGARP-M2 region (Fig. 10A) dissolved in PBS produced positive aggregates. The M1E4R and M1G4R peptides derived from PfGARP-M1 region served as negative controls. The sequence information of these peptides is shown in Table 1.

Blot overlay assay (Far Western blotting)

Untreated and enzyme-treated RBC ghosts were prepared from 100 \( \mu \text{L} \) of packed human RBCs using cold lysis buffer (5 mM sodium phosphate pH 8.0, 1.0 mM EDTA pH 8.0) containing 1X protease inhibitor cocktail\textsuperscript{11,12}. Ghosts were analyzed by 12\% gels for SDS-PAGE, transferred to nitrocellulose membranes, and stored in distilled water at 4\°C until used for experiments. Blot overlay assay was performed as described previously with slight modifications\textsuperscript{3}. Briefly, membranes were incubated with PBST or TBST (Tris-buffered saline with TWEEN 20) containing 20 mM Tris-HCl, pH 7.6, 136 mM NaCl, 1\% TWEEN 20 for 30 minutes followed by incubation with the blocking buffer (10\% milk, 2\% BSA in PBST/TBST) with rotation overnight. Test proteins (1.0 \( \mu \text{g/mL} \)) in PBST/TBST were added to the nitrocellulose membranes and incubated overnight rotating at 4\°C. Membranes were washed with the blocking buffer five times at 4\°C. Primary antibody (anti-TRX mAb) in the blocking buffer was added overnight at 4\°C, followed by incubation with goat anti-mouse (GAM) alkaline phosphatase (AP) secondary antibody for one hour at 4\°C. After multiple washings of the membranes, the signal from bound proteins was detected using SuperSignal West Pico Chemiluminescent substrate kit.
Pull-down assay

We adapted the pull-down protocol with slight modifications\textsuperscript{12-14}. Briefly, untreated and chymotrypsin-treated ghosts were incubated with the solubilizing buffer containing 1\% C\textsubscript{12}E\textsubscript{8} (Octaethylene glycol monododecyl ether (Sigma), 5 mM sodium phosphate, pH 8.0, 1.0 mM EDTA, and 1X protease inhibitor cocktail for 20 minutes on ice, and cell lysate was centrifuged at 18,000 RCF for 30 minutes at 4\textdegree C. Preclearance of solubilized ghosts was performed by incubating them with 100 µL of washed 50\% slurry of Cobalt beads in PBS for one hour at 4\textdegree C, followed by centrifugation at 18,000 RCF for 5 minutes at 4\textdegree C. TRX and TRX-PfGARP-M2 fusion proteins (~125 µg) were incubated with Cobalt beads in PBS for one hour rotating at 4\textdegree C. Protein-bound beads were washed and centrifuged at 500 RCF for 5 minutes two times with 0.1\% C\textsubscript{12}E\textsubscript{8} in PBS. The precleared soluble ghosts were incubated with TRX fusion proteins bound to beads overnight at 4\textdegree C in PBS. The beads were washed six times with 0.1\% C\textsubscript{12}E\textsubscript{8} in PBS and analyzed by 12\% gels for SDS-PAGE. One gel was used for CBB staining and the other gel for Western blotting to detect band 3 binding using a polyclonal antibody raised against the N-terminal cytoplasmic domain of human band 3\textsuperscript{15}.

Malaria parasite culture and invasion assay

\textit{P. falciparum} 3D7 parasites were cultured \textit{in vitro} in complete malaria media (CMM) containing RPMI-1640 supplemented with 0.5\% Albumax II, 25 mM HEPES, 50 mg/L Hypoxanthine, 50 mg/L Gentamicin) at 37\textdegree C incubator maintained with a gas mixture of 5\% CO\textsubscript{2}, 3\% oxygen (O\textsubscript{2}), and balanced by nitrogen (N\textsubscript{2}) as described previously\textsuperscript{16-18}. Media was changed every day and fresh human RBCs were added when needed. Blood smears were fixed with 100\% methanol for 30 seconds, stained by Giemsa, and parasitemia was quantified by microscopy. To harvest parasite culture supernatant, \textit{P. falciparum} 3D7 schizont-infected RBCs were isolated using LS magnetic columns (Miltenyi Biotec) according to standard protocols. Purified schizonts (~5 x 10\textsuperscript{5}) were incubated for 20 hours in CMM at 37\textdegree C without the addition of RBCs in a total volume of 200 µL. The upper supernatant layer was gently harvested from the schizonts, centrifuged for 5 minutes at 500 RCF, and supernatant was centrifuged again at 1,200 RCF for 10 minutes. The final collected culture supernatant was saved at -80\textdegree C in the presence of 1X protease inhibitor cocktail. CMM without schizonts was used as a negative control. Both supernatant groups were analyzed by SDS-PAGE and Western blotting using a mouse
monoclonal antibody mAb (GM7) against PfGARP-M. The details of GM7 monoclonal are described below.

The invasion assay was performed as described previously. Synchronized magnetically purified parasites were adjusted to 1% parasitemia, and invasion assay was performed in a 96-well plate (200 µL final volume of containing CMM and PBS) at 2.5% hematocrit. Soluble fusion proteins and antibodies in PBS were added to each well at maximum volume of 50 µL. Uninfected and infected wells with no proteins were included as controls. Plates were incubated for 24 hours, and RBCs were incubated with 2.0 µM Hoechst 33342 dye in RMPI at 37°C for one hour. The pellet was washed with PBS and fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS at 4°C rotating in the dark for 45 minutes. Stained and fixed RBCs were washed twice with PBS, re-suspended in PBS, and kept at 4°C in the dark until quantification of parasitemia by flow cytometry (Becton Dickinson LSR II) at Tufts core facility. The Indo blue 450/50 filter was used to count 100,000 events for each sample. Summit software version 4.3 was used for data analysis.

**Generation of a monoclonal antibody against PfGARP**

*Immunization of mice with PfGARP-M:* Six BALB/c female mice (8-12 weeks old) were immunized with PfGARP-M protein. Recombinant TRX-PfGARP-M protein was expressed in *E. coli* BL21 (DE3) and purified through Ni-beads affinity chromatography. Eluted protein was further purified by Mono Q ion exchange chromatography using AKTA-FPLC system (GE Healthcare). For the first immunization, 70 µg of TRX-PfGARP-M protein mixed in Freund’s complete adjuvant was injected into mice by IP (intraperitoneal). To avoid excessive immune response against TRX, recombinant His-PfGARP-M (without TRX tag) protein was used for the subsequent boost injections. The His-PfGARP-M was expressed in *E. coli* BL21 (DE3) and purified through Ni-beads and Mono-Q column chromatography as described above. Three boost injections were given at three weeks intervals with 50 µg of His-PfGARP-M mixed in Freund’s incomplete adjuvant by IP. Test bleeds were collected 10 days after each boost, and antibody titers were determined by ELISA using the His-PfGARP-M. The final (4th) boost was given three weeks after the third boost to the best-responding mouse by injecting His-PfGARP-M in PBS (30
μg) by IV and 30 μg by IP. Three days after the final boost, splenocytes were harvested from the immunized mouse and used for the hybridoma production19.

Characterization of GM7 monoclonal antibody: Hybridoma clone #7 was selected from 32 positive hybridomas for further characterization. Hybridoma #7 clone was further sub-cloned by limiting dilution and secreted an IgG3 monoclonal antibody termed GM7-1 (for simplicity, we call it GM7 monoclonal). Affinity purified GM7 monoclonal was stored at 1.84 mg/mL in the storage buffer (0.02 M Potassium phosphate pH 7.2 -7.4, 0.15 M Sodium chloride). Since the GM7 mAb is of IgG3 class, it tends to precipitate after long-term storage at 4°C. Therefore, warming up of GM7 solution to 37°C followed by brief centrifugation was necessary before use.

Development of an ELISA for PfGARP: Streptavidin-coated as well as non-coated ELISA plates were used to immobilize either His-PfGARP-M antigen or synthetic peptides of interest by incubation overnight at 4°C. Plates were washed with 1X PBST twice for five minutes, blocked with 3% BSA in 10 mL PBST, and incubated for 30 minutes at room temperature. Plate was then washed with PBST twice for five minutes. Initial dilution of human plasma was either 1/400 or 1/1000 for subsequent GM7 screens. Plasma samples were incubated for 90 minutes at room temperature, and the plates were washed with PBST twice for five minutes. The negative controls included PBS alone and human plasma from Babesia microti infected patients (ImmuneTrics Inc.). Anti-human horseradish peroxidase (HRP) diluted at 1/10,000 or anti-mouse HRP diluted at 1/5,000 were used as secondary antibodies. Plates were incubated for 60 minutes at room temperature and washed with PBST three times for five minutes. TMB (Tetramethylbenzidine, Sigma) liquid substrate system for ELISA was used for signal detection. The plates were incubated with TMB for five minutes to allow development of blue color, and reaction was quenched with the addition of 1.0 M HCl. The plates were incubated for five minutes and development of yellow color was quantified using VersaMax Microplate reader.

Localization of PfGARP by immunofluorescence microscopy (IF)

A thin blood smear of P. falciparum 3D7 culture was air dried, fixed, and permeabilized with ice-cold methanol for 45 minutes. After three washes with PBS, cells were incubated in the blocking buffer (3% BSA in PBS) for 30 minutes at room temperature. Slides were washed
briefly with 0.05% TWEEN 20 in PBS. GM7 monoclonal antibody was diluted in the blocking buffer and cells were incubated for one hour at room temperature. Cells were washed three times with PBS for 5 minutes each. Alexa Fluor 488 goat anti-mouse antibody was diluted in the blocking buffer, and cells were incubated for one hour at room temperature. Cells were washed with PBS three times for 5 minutes each. Coverslips were mounted using ProLong Diamond Antifade with DAPI and allowed to cure overnight before imaging. Cells were imaged on a Nikon eclipse TE2000-E fluorescence microscope with an exposure length of 300 milliseconds.

**Live parasite IF:** Magnetic cell-sorted mature parasites were washed three times in 3% BSA in PBS. The parasite pellet was incubated with 1: 2,000 dilution of GM7 monoclonal antibody and 1: 2,000 dilution of Hoechst 33342, and incubated at 4°C for 30 minutes. The secondary antibody Alexa 488 conjugated anti-mouse antibody was diluted 1:1,000 and parasites were incubated for 30 minutes at 4°C. Labeled parasites were layered on a 35 mm glass-bottom culture dish with a 14 mm microwell and imaged.

**Parasite IF under non-permeabilizing conditions:** Magnetic cell-sorted mature parasites were fixed with 4% paraformaldehyde and 0.00625% glutaraldehyde in PBS for 30 minutes at room temperature (RT). Parasites were washed 3 times with PBS and free aldehydes were quenched with 0.1M glycine for 10 minutes at RT followed by 3 additional washes with PBS. Fixed parasites were blocked with 3% BSA in PBS for 30 minutes at RT. Parasites were incubated with GM7 monoclonal antibody (1: 2,000 dilution) in BSA/PBS for 1 hour at RT, washed 3 times with PBS, and incubated with Alexa 488 conjugated anti-mouse antibody (1:1,000 dilution) in BSA/PBS for 1 hour at RT, followed by 3 washes in PBS. Stained parasites were mounted on a glass slide with ProLong Diamond Antifade Mountant with DAPI and imaged as described previously.

**Note:** Further details are included under “Additional Results”.
REFERENCES

Supplemental Figure S1. Human RBC aggregates induced by PfGARP synthetic peptides. (A and B) M2K5 peptide promotes RBC aggregation in a dose-dependent manner. Multiple and larger RBC aggregates were clearly detected at 50 μM and 70 μM peptide concentrations (pointed by white arrows). No aggregates were detected in the negative controls PBS, TRX, M1E4R and M1G4R peptides (shown in panel B). Magnification of RBC aggregates (white arrows) and rouleaux structures (red arrows) are shown at the bottom of panel A. RBC aggregates were also detected with M2K4 peptide (bottom of panel B) and fewer RBC aggregates were detected with TRX-PfGARP-M2 (panel B). M2K4 peptide appears to be less efficient than M2K5 peptide for RBC aggregation. As indicated in the text, higher concentration of TRX-PfGARP-M2 fusion protein could not be achieved under these conditions.

Supplemental Figure S2. Effect of PfGARP inhibition on *P. falciparum* invasion of human erythrocytes. Recombinant PfGARP-M, PfGARP-M2, and multiple concentrations of GM7 monoclonal antibody did not interfere with *P. falciparum* invasion in human RBCs. Parasitemia was measured by flow cytometry. One-way ANOVA (Analysis of variance) was performed between iRBC groups. No significant difference was detected. These experiments were repeated three times with similar results.

Supplemental Figure S3. RBC receptor identification by blot overlay assay. Immobilized proteins from RBC ghosts were evaluated for binding using TRX-PfGARP-M and TRX-GARP-M2 as detectors. Human RBC ghosts: NU, normal untreated, NC, normal chymotrypsin-treated, GU, Glycophorin B null untreated, GC, Glycophorin B null chymotrypsin-treated human RBCs. Panel 1: Ghosts were analyzed by CBB-stained 12% SDS-PAGE. Panel 2: Ghosts blotted on nitrocellulose membranes were visualized by Ponceau S stain. Panel 3: Blot overlay results after incubation with 1.0 μg/ml of TRX-PfGARP-M. Panel 4: TRX-PfGARP-M2. Panel 5: TRX using TBST. Panel 6: TRX-PfGARP-M2 using PBST. Anti-TRX monoclonal antibody was used for binding and signal detection. Panel 7: Western blotting using rabbit polyclonal anti-band 3
antibody against the N-terminal domain. Asterisks indicate the positions of full length band 3, 
~60 kDa chymotryptic fragment of band 3, and possibly 48 kDa degradation product of band 3.

**Supplemental Figure S4.** Quantitative ELISA to detect antibodies against PfGARP. (A) A summary of PfGARP-M peptides. PfGARP-M protein was divided into several segments based on its repeat content. Several peptides were synthesized to cover these segments. M1P1 peptide contains two repeats of GEHKE and one repeat of EEHKE. M1P2 contains one repeat of each EEHKK, EEHKS and KEHKS. M1P4 contains first four repeats of PfGARP-M1. M1E4R peptide contains four single repeats of EEHKE. M1G4R peptide contains four single repeats of GEHKE. M1P6 peptide contains six repeats (three EEHKE repeats and three GEHKE repeats) of PfGARP-M1. M2K4 and M2K5 peptides contain four and five repeats of PfGARP-M2, respectively. (B) Reactivity of GM7 monoclonal antibody to PfGARP-M peptides as shown in panel A. GM7 recognized M1E4R peptide much more efficiently than M1G4R indicating that the epitope is likely to be encoded by EEHKE repeats. Similarly, M1P1 peptide was more reactive than M1P2. ELISA results show that M1P6 peptide encodes the most reactive antigenic epitope recognized by GM7 indicating that the epitope is located within EEHKE-GEHKE repeats. (C) His-PfGARP-M coated on the ELISA plate was used to screen human plasma from malaria endemic region of Mali. 20 out of the 380 malaria samples revealed high plasma reactivity. (D) M1P6 peptide was coated on the ELISA plate to screen the same 380 samples. M1P6 peptide confirmed the immune response detected by PfGARP-M as shown in panel C. The background signal was reduced from the M1P6 coated plate.

**Supplemental Figure S5.** Secretion of PfGARP into culture supernatant. (A) Blood smear images over 48 hours tracking development of magnetically-purified schizonts to demonstrate intact infected-RBCs at each parasite development stage. (B) Western blot analysis of PfGARP in culture supernatant over 48 hours. PfGARP was detected starting at 12 hours and reached maximal expression at 28 hours. Reduced PfGARP levels were observed at 36 and 48 hours suggesting PfGARP binding to infected erythrocytes during development. (C) PfGARP was detected in infected erythrocyte ghosts of a ring-synchronized culture over 24 hours. Band 3 was used as a loading control. Blood smear images (bottom panels) show representative parasites at each stage.
Supplemental Fig. S1

A

1 µM M2K5

15 µM M2K5

30 µM M2K5

50 µM M2K5

70 µM M2K5

50 µM M2K5

B

PBS

50 µM M1E4R

50 µM M1G4R

1 µM TRX

1 µM TRX-PIGARP-M2

50 µM M2K4
Supplemental Fig. S3

Human RBC Ghosts

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kDa

135 100 60 48
Supplemental Fig. S4

A. PfGARP-M (75 AA)

B. GM7 monoclonal Antibody

C. His-Pf-GARP-M Protein (coated)

D. M1P6 Peptide (coated)
Supplemental Fig. S5

A.

0 hr

12 hr

24 hr

36 hr

48 hr

Schizont

Free merozoites and rings

Ring

Trophozoite

Schizont

B.

kDa

63

48

35

25

Uninfected

Culture Supernatant (WB: α PfGARP)

C.

0 hr

8 hr

24 hr

α Band 3

α PfGARP

Ring

Trophozoite

Schizont
ADDITIONAL RESULTS

Synthetic peptides from PfGARP-M2 induce RBC aggregation

Direct attachment of human RBCs forming rosette-like structures on the surface of PfGARP-M expressing CHO-K1 cells led us to hypothesize that PfGARP has the intrinsic ability to link RBCs to form similar clusters. This phenomenon is directly relevant to the pathology of malaria since the rosette formation has been correlated with occlusion of microvasculature in the brain. To test this hypothesis, a series of peptides derived from PfGARP-M sequence were synthesized (Table 1). Two peptides termed M2K4 and M2K5 were derived from the PfGARP-M2 region while others were designed to cover the repetitive sequences within PfGARP-M1 region (Table 1, Fig. S4A). The experimental approach used to test the RBC aggregation activity of synthetic peptides was slightly modified from a published assay. Human RBCs incubated with M2K4 and M2K5 peptides formed aggregates within two hours of incubation at room temperature (Fig. S1). The RBC aggregates formed in solution did not require any fixation suggesting a high-affinity interaction between the cells. M2K5 peptide, covering the entire PfGARP-M2 sequence, displayed the most optimal RBC aggregation-stimulating activity consistent with the binding of PfGARP-M2 fusion protein with RBCs (Fig. S1A). M2K4 peptide, lacking a distal repeat of PfGARP-M2 region, also formed RBC aggregates albeit the frequency of clusters observed was relatively less than M2K5 (Fig. S1B, bottom panel). M2K5 peptide caused aggregation of RBCs in a dose-dependent manner with an optimal concentration of 50 µM. Few RBC aggregates were formed at lower peptide concentrations of M2K5 peptide (1.0-30 µM) (Fig. S1A). Similarly, TRX-PfGARP-M2 fusion protein also formed fewer clusters at 1.0 µM concentration indicating the concentration-dependence of the RBC aggregation activity (Fig. S1B). Importantly, no RBC aggregates were formed by either M1E4R or M1G4R peptides derived from the PfGARP-M1 region (Fig. S1B). Collectively, these findings indicate that five repeats contained within the PfGARP-M2 region are endowed with the RBC aggregation activity under these conditions.
PfGARP-M and GM7 do not inhibit *P. falciparum* invasion in RBCs

Identification of PfGARP as a parasite ligand that binds to the surface of intact RBCs necessitated evaluation of its functional role in the RBC invasion process. First, we tested whether PfGARP plays a role in merozoite invasion by using purified PfGARP proteins as competitive inhibitors. In addition, we tested the inhibitory effect of GM7 mAb on RBC invasion by *P. falciparum* 3D7. Invasion assays were performed using normal human RBCs preincubated with either PfGARP-M or PfGARP-M2 fusion proteins. Similarly, RBCs were incubated with GM7 mAb at increasing concentration (0.1 to 0.46 mg/mL) for two hours at 37°C. Magnetically purified schizonts (1%) were added to 2.5% hematocrit, and infected RBCs were quantified after 24 hours under standard malaria culture conditions. Parasitemia increased from 1.0 to 3% in all treatment groups without any significant effect on RBC invasion as measured by flow cytometry (Fig. S2). Addition of TRX-PfGARP-M, TRX-PfGARP-M2, and GM7 mAb did not inhibit RBC invasion efficiency under these conditions as compared to TRX, PBS, and dematin mAb as negative controls (Fig. S2). These results suggest that PfGARP-M does not mediate RBC invasion by *P. falciparum* 3D7 under these conditions. Future quantification of this observation will require a more rigorous evaluation of the effects of PfGARP segments, GM7 mAb, and defined PfGARP peptides on parasite invasion and growth using multiple laboratory-adapted and field isolates of *P. falciparum*.

**RBC receptor identification by human reticulocyte phage display cDNA screens**

To identify putative chymotrypsin-sensitive RBC receptor(s) for PfGARP, we first attempted to use the reverse phage display cDNA library approach with PfGARP-M as bait. Two phage display cDNA libraries were constructed using either random or oligo (dT) primed human reticulocyte mRNA isolated from a hemochromatosis patient. No specific interactions or potential RBC receptors were identified from these screens. Most of the 83 clones sequenced encoded peptides of multiple globin chains. Therefore, a major limitation of the reticulocyte-derived phage cDNA libraries approach is the hyper abundance of globin clones in such libraries. Although our conventional solution-based approach using immobilized PfGARP fusion proteins was not successful for the identification of host receptors, the Phage Display human reticulocyte cDNA libraries we have generated could be optimized for screening novel ligands using plaque-
based phage peptides immobilized on the nitrocellulose filters. In the future, we hope that the human reticulocyte phage display cDNA libraries may be useful for broad applications in the field of hematology.

**Human RBC Glycophorin B is not a host receptor for PfGARP**

Our unsuccessful attempts to identify the chymotrypsin-sensitive receptor on human RBCs by reverse phage display cDNA library screens prompted us to investigate the potential role of glycophorin B and band 3, two well-known RBC receptors that are sensitive to cleavage by chymotrypsin. Chymotrypsin is known to cleave the extracellular region of glycophorin B, a type I transmembrane protein. First, we measured the binding of TRX-PfGARP-M to glycophorin B-null human RBCs that lack the antigens carried by the GPB receptor (S-s-U-). Unlike band 3 null erythrocytes, glycophorin B-null erythrocytes are structurally stable and therefore suitable for the biochemical binding studies. Western blotting demonstrated that TRX-PfGARP-M bound to both normal and glycophorin B null S-s-U- human RBCs (Fig. 5C). In fact, binding of TRX-PfGARP-M appears to be relatively more efficient with glycophorin B null S-s-U- RBCs as compared to normal RBCs (Fig. 5C). To investigate the effect of chymotrypsin treatment on glycophorin B null RBCs, we compared the binding of TRX-PfGARP-M to glycophorin B null RBCs and chymotrypsin-treated GYPB-null RBCs (Fig. 5D). We found a significant enhancement of TRX-PfGARP-M binding to GYPB-null S-s-U- RBCs that were treated with chymotrypsin as compared to untreated GYPB-null S-s-U- RBCs (Fig. 5D). These observations suggest that the surface accessibility of the putative RBC receptor(s) for PfGARP could be influenced by the orientation and conformational stability of these receptor(s) in the absence of glycophorin B.

**Quantification of PfGARP immune response in malaria endemic areas**

Since PfGARP shows exclusive expression in *P. falciparum*, an expectation is that it may contribute to the pathogen’s virulence by modulating the adhesive properties of the parasite that are unique to *P. falciparum*. If this model turns out to be correct, then PfGARP must be exposed to the immune system while functioning in the extracellular environment. To test this model, we first developed a quantitative ELISA to detect antibodies against PfGARP. The GM7 monoclonal antibody developed against PfGARP recognized only the M1 repeats but not the M2
repeats (Fig. 3A, B). In fact, all 32 hybridoma clones reacted against PfGARP-M1 but not PfGARP-M2 (data not shown). To further map the GM7 epitope within PfGARP-M1, we synthesized a series of peptides to determine whether multiple distinct epitopes are recognized by the 32 hybridomas. If so, then these hybridomas could also be used to develop a Sandwich type ELISA that will allow quantification of both PfGARP antigen and the corresponding antibodies. PfGARP-M is composed of characteristic repeat structures (Fig. S4). PfGARP-M1 consists of nine repeats of 5-amino acids represented by the consensus xEHKx. Moreover, PfGARP-M1 can be further divided into two groups; the first six repeats consist of highly conserved EEHKE and GEHKE repeats followed by three repeats consisting of somewhat diverse EEHKK-EEHKS-KEHKS sequence motifs (Fig. S4A). Since PfGARP-M2 consists of five repeats of highly basic 5-amino acids defined by the consensus KxKKx, we elected to synthesize two peptides covering the entire PfGARP-M2. The M2K4 peptide contains four repeats whereas the M2K5 peptide includes five repeats. Both peptides contained an SGSG linker to provide flexibility for conjugation to biotin. The composition and location of each synthetic peptide is shown in (Fig. S4A), and PfGARP-M1 and PfGARP-M2 repeats are highlighted in red and green, respectively.

To identify the specific repeats within PfGARP-M that are recognized by GM7 mAb, we first evaluated peptide M1P1 containing repeats 4-6 and M1P2 peptide containing repeats 7-9. ELISA showed that GM7 recognized M1P1 but bound very weakly to M1P2 (Fig. S4B) indicating that the GM7 epitope is located within M1P1 repeats. Although we considered the M1P2 peptide essentially as negative, compared to M1P1 peptide, due to low-level reactivity by GM7, this peptide could be a potential target for other hybridomas. Therefore, we screened the remaining 31 hybridomas for reactivity against M1P2. Again, no reactivity was detected with the M1P2 peptide (data not shown). Moreover, as expected, no reactivity for GM7 was detected with either M2K4 or M2K5 peptides (Fig. S4B). Together, these results demonstrate that GM7 mAb and the other 31 hybridomas recognize an epitope located within repeats 1-6 of PfGARP-M1. Since repeats 1-6 are assembled by multiple EEHKE and GEHKE repeats, we evaluated whether one or both repeats contain the GM7 epitope. Two peptides containing four repeats of either EEHKE (M1E4R) or GEHKE (M1G4R) were synthesized and tested by ELISA. GM7
recognized M1E4R peptide but not M1G4R peptide (Fig. S4B) indicating that the EEHKE repeat constitutes the most immunogenic site for antibody response against PfGARP.

Guided by the results of peptide-scanning experiments, we designed an M1P6 peptide containing the first 6 repeats of PfGARP-M1 (Fig. S4A). Since the repeating pattern of EEHKE and GEHKE repeats in M1P6 breaks down after four repeats, we also designed another M1P4 peptide containing only the first four repeats of PfGARP-M1 (Fig. S4A). A comparison of M1P6 and M1P4 peptides by ELISA demonstrated that M1P6 peptide produced the most robust response with GM7 mAb (Fig. S4B). Based on these results, it was concluded that M1P6 peptide containing a defined configuration of three EEHKE and three GEHKE repeats encodes an optimal epitope recognized by GM7 mAb and all other hybridomas tested. Therefore, M1P6 peptide was selected for the development of an ELISA-based screen to test the human plasma samples originating from malaria endemic areas of Africa.

To obtain the proof-of-principle evidence for clinical relevance of GM7 epitope present in the PfGARP-M, and particularly in M1P6 peptide, we acquired 380 plasma samples from subjects living in rural areas of Kambila, Mali, where transmission of P. falciparum is seasonal and intense\(^8,9\). Plasma samples came from subjects representing both sexes as well as young children exposed to malaria. These samples were originally obtained from the National Institutes of Health (NIH) as part of a screen to search for potential biomarkers of malaria infection in the endemic areas. We first screened the plasma samples using purified His-PfGARP-M protein immobilized to an ELISA plate. This screen was designed to capture all positive hits targeting the epitopes located within PfGARP-M. This screen identified several hits from the 380 samples with high seropositive response, and data from the representative samples are shown (Fig. S4C). To further validate this screen, we tested the same plasma samples for antibodies against PfGARP using immobilized M1P6 peptide as antigen. Consistent with the results from the His-PfGARP-M screen (Fig. S4C), the M1P6 immobilized ELISA showed high antibody reactivity in the same plasma samples (Fig. S4D). In fact, background signal was substantially reduced in the M1P6 coated ELISA screen (Fig. S4D). In summary, the peptide-based mapping of the reactive epitope recognized by GM7 monoclonal antibody led to the development of M1P6 peptide-based
ELISA, which is likely to allow quantification of immune response against PfGARP in subjects exposed to malaria infection in the endemic areas.

REFERENCES