

In Vitro Genetic Analysis of an Erythrocyte Determinant of Malaria Infection

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Invasion of erythrocytes by *Plasmodium falciparum* is an obligatory step in the life cycle of the parasite. A major challenge is the unambiguous identification and characterization of host receptors. Because erythrocytes lack nuclei, direct genetic analyses have been limited. In this work, we combined an in vitro erythrocyte culture system, which supports *P. falciparum* invasion and growth, with lentiviral transduction to knock down gene expression. We genetically demonstrate, in an isogenic background, that glycophorin A is required for efficient strain-specific parasite invasion. We establish the feasibility of in vitro systematic functional analysis of essential erythrocyte determinants of malaria and erythrocyte biology.

The human malaria parasite, *Plasmodium falciparum*, causes severe clinical disease and mortality during the erythrocytic stage of the life cycle, marked by invasion and uncontrolled proliferation in mature enucleated human erythrocytes, for which it has a unique tropism. Many critical molecular interactions occur between the parasite and the host during the processes of erythrocyte invasion and intracellular establishment and transformation of the erythrocyte.

The identification of essential host determinants of malaria is a prerequisite for the development of improved host-targeted

interventions. Biochemical studies suggest that the major sialoglycoprotein of the erythrocyte membrane, glycophorin A (GPA), is a receptor for the parasite ligand EBA-175, which is being developed as a vaccine candidate [1]; however, assessment of the functional impact on parasite invasion has relied on the use of extremely rare natural mutants [2–4]. Few studies have addressed the erythrocyte contribution to host-parasite interactions, largely because the absence of a nucleus in the terminally differentiated erythrocyte precludes direct genetic manipulation.

Human hematopoietic stem cells (HSCs) have been targeted with lentiviral vectors in the development of gene therapy for inherited erythrocyte disorders; however, differentiation to mature erythrocytes has required implantation into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice [5]. Recent advances in ex vivo erythrocyte culture, such as facilitated growth on a murine stromal layer, have resulted in the generation and the large-scale expansion (up to 10⁶ cells) of synchronous, terminally differentiated erythrocytes with highly efficient enucleation [6]. On the basis of these recent advances, efforts have been made to generate human erythrocytes from CD34⁺ HSCs in vitro that support *Plasmodium* species culture [7, 8].

In this work, we have combined in vitro culture of human erythrocytes with lentiviral transduction to knock down expression of GPA. Our results demonstrate the importance of this erythrocyte receptor for strain-specific parasite invasion and the feasibility of our in vitro genetic approach for the study of erythrocyte biology.

Methods. CD34⁺ HSC precursor cells isolated from bone marrow of a single donor were purchased commercially (Lonza). The in vitro culture protocol is essentially the same as that described elsewhere [6], with minor modifications. Briefly, HSCs were incubated in Iscove modified Dulbecco medium (IMDM; Biochrom) supplemented with 10% fetal bovine serum and hydrocortisone (1 μmol/L; Sigma), interleukin 3 (IL-3; 5 ng/mL; R&D Systems), stem cell factor (SCF; 100 ng/mL; Stem Cell Technologies), and erythropoietin (3 U/mL; Amgen) for 8 d. Cells were incubated with SCF and erythropoietin for 3 d. On day 11, cells were co-incubated on an MS-5 murine stromal cell monolayer with erythropoietin. On day 15, cells were incubated in complete IMDM without cytokines until mature erythrocytes were observed on day 18.

Viral particles containing pLKO plasmids (Addgene plasmid 10878; D. Root) that express small hairpin RNA (shRNA) against GPA (Open Biosystems) or scrambled shRNA controls (Addgene

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plasmid 1864; D. Sabatini) were produced in 293T cells. Approximately 1×10^7 transduction units were used to transduce day 4 CD34⁺ cells in the presence of polybrene (8 $\mu\text{g}/\text{mL}$) for 6 h, followed by culture in IMDM with hydrocortisone, IL-3, SCF, and erythropoietin and selection with 1 $\mu\text{g}/\text{mL}$ puromycin (Sigma) 24 h after transduction. Transduced HSCs were incubated ex vivo to mature erythrocytes. The level of protein knockdown was measured by flow cytometry with the use of ectodomain-reactive antibodies specific to GPA (with no cross-reactivity to glycophorin B; Open Biosystems, StemCell Technologies). Data were acquired with a BD FACS Calibur and analyzed with FlowJo software (version 8.8.6; Tree Star). The knockdown percentage was calculated as follows: $100 - \text{MFI}_{\text{GPA KD}}/\text{MFI}_{\text{cRBC}} \times 100$, where $\text{MFI}_{\text{GPA KD}}$ is the mean fluorescence intensity of GPA knockdown erythrocytes and MFI_{cRBC} is the mean fluorescence intensity of cultured erythrocytes.

Invasion assays were performed, as described elsewhere [9], by mixing enzyme-treated, parasitized donor erythrocytes with equal numbers of cultured erythrocytes, shRNA-expressing erythrocytes, or enzyme-treated negative control erythrocytes. Sorbitol-synchronized, ring-stage parasitized donor cells were treated with α -2-3,6,8-*Vibrio cholera* neuraminidase (66.7 mU/mL; Calbiochem), trypsin (1 mg/mL; Sigma), and chymotrypsin (1 mg/mL; Worthington) to prevent reinvasion. Parasites were plated in triplicate at a final parasitemia of 1%, at 2% hematocrit in complete Roswell Park Memorial Institute medium. Following reinvasion, the parasitemia was assessed by microscopic analysis. A Miller reticle was used to count 4500 erythrocytes for each sample. The parasite multiplication rate was determined as the ratio of the final parasitemia to the initial parasitemia over 1 cycle of invasion. The selectivity index was calculated as the ratio of the observed number of multiply infected cells to the expected number of multiply infected erythrocytes, as described elsewhere [10].

Results. A requirement for the functional analysis of human erythrocyte determinants of malaria by reverse genetics in vitro is a robust method for generating erythrocytes that are susceptible to *P. falciparum* invasion. To this end, we reproducibly generated synchronous, terminally differentiated erythrocytes with highly efficient enucleation and expansion, by means of co-culture on a stromal cell line [6] (Figure 1A). Importantly, these cultured erythrocytes from bone marrow-derived HSCs contain >90% hemoglobin A and are morphologically and hematologically indistinguishable from mature erythrocytes isolated from peripheral blood, with normal surface expression of the mature erythrocyte surface markers GPA, glycophorin C, and Duffy antigen receptor for chemokines (DARC) (Figure 1B).

Cultured erythrocytes incubated with *P. falciparum* parasites were fully susceptible to invasion, growth to the trophozoite and schizont stages, and subsequent reinvasion (Figure 1C),

which allowed for the generation and study of the complete asexual life cycle in cultured erythrocytes. Invasion by *P. falciparum* parasites into cultured erythrocytes occurred at higher levels than those of invasion into erythrocytes from normal donors (Figure 1D). The multiplicity of infection was also higher in the cultured erythrocytes, with a considerable proportion of cells supporting >4 parasites (Figure 1E). These findings support observations that young erythrocytes show increased susceptibility to *P. falciparum* invasion [11]. Although this study is focused on enucleated erythrocytes, we observed that nucleated orthochromatic erythroblasts are susceptible to invasion by *P. falciparum*, as reported elsewhere [8]. We also noted formation of sexual-stage gametocytes in short-term cultures, which indicates that cultured erythrocytes support this life cycle stage (data not shown).

To directly assess the function of GPA in malaria, we adopted a strategy of shRNA-based knockdown of gene expression in HSCs, followed by terminal differentiation to erythrocytes in vitro (Figure 2A). We used lentiviral transduction for stable gene delivery and sustained gene expression during subsequent in vitro erythrocyte culture. Bone marrow-derived CD34⁺ HSCs were transduced with pLKO lentivirus expressing either GPA-specific shRNA or a scrambled shRNA control. Positive selection of cultures with puromycin yielded pure populations of transduced cells.

To confirm stable, targeted knockdown of gene expression, we used flow cytometry to measure GPA erythrocyte surface expression on day 18 of ex vivo culture. In 8 independent experiments, the mean level of GPA expression in GPA knockdown erythrocytes was knocked down by 79% (standard deviation, 9%) compared with that in cultured erythrocyte controls (Figure 2B). Importantly, transduction and gene knockdown did not affect erythroid maturation, as assessed by the levels of erythrocyte surface markers glycophorin C and DARC (Figure 2C) and by the enucleation percentage (Figure 2D). Surface expression of the Wright b antigen, which requires interaction between Band 3 and GPA, decreased in knockdown cells (Figure 2E). In contrast, CD47 and Kell levels did not decrease, whereas Band 3 levels were elevated.

To determine the impact of decreased GPA expression on *P. falciparum* parasite invasion, we conducted invasion assays with W2Mef and 3D7 parasite strains, which utilize different erythrocyte receptors for invasion. The former strain is highly reliant on sialylated receptors, such as GPA, whereas the latter uses predominantly sialic acid-independent receptors for invasion. The *P. falciparum* ligand for GPA is EBA-175. Genetic evidence from parasite knockouts suggests that both W2Mef and 3D7 strains use this ligand for erythrocyte invasion [9]. Invasion efficiency into GPA knockdown erythrocytes was compared with that into erythrocytes transduced with scrambled pLKO. Invasion assays of the W2Mef parasite line showed significantly

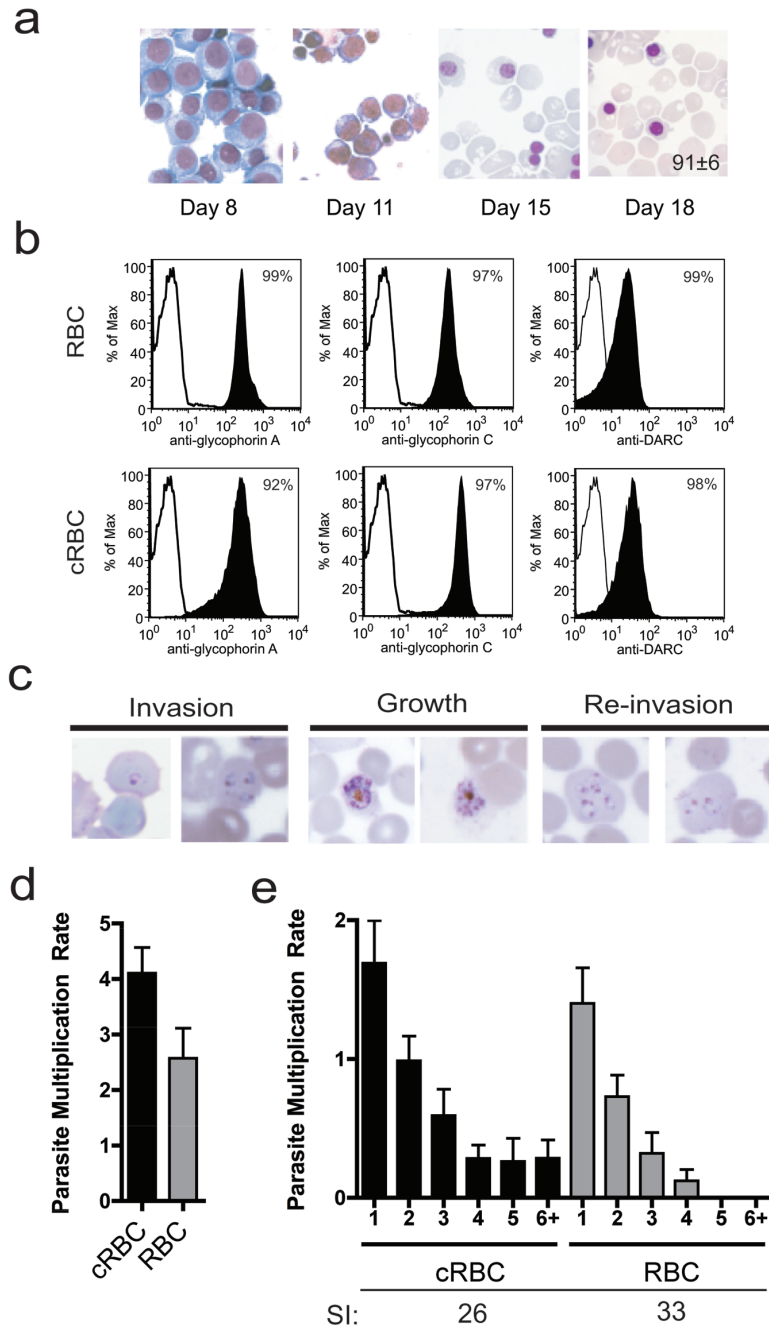


Figure 1. Susceptibility of cultured erythrocytes to invasion and growth of *Plasmodium falciparum*. *a*, Cytopsin analysis of cells taken on days 8, 11, 15, and 18. Spots were stained with the May-Grünwald Giemsa staining technique. Day 8 cells correspond to basophilic erythroblasts, day 11 cells correspond to orthochromatic erythroblasts and are co-cultured on the MS-5 stromal layer, day 15 cells correspond to reticulocytes, and day 18 cells correspond to mature erythrocytes. For day 18 cells, the enucleation percentage is shown as the mean (\pm standard deviation). *b*, Expression of maturation markers (glycophorin A, glycophorin C, and Duffy antigen receptor for chemokines [DARC]) on erythrocytes (red blood cells) from normal donors (RBC) and day 18 cultured erythrocytes (cRBC), as measured by flow cytometry. Negative controls for all experiments were isotype control (fluorescein isothiocyanate-conjugated secondary antibody) stained erythrocytes. The percentage of positive staining of erythrocyte gate is indicated. *c*, Invasion, growth, and reinvasion of *P. falciparum* parasites into day 18 cultured erythrocytes, as monitored by microscopic analysis of thin smears with Giemsa staining. *d*, Invasion into cultured erythrocytes and erythrocytes from normal donors, shown as the mean parasite multiplication rate from 2 experiments. Assays were performed in duplicate and triplicate. Error bars represent the standard deviation of the mean. *e*, Percentage of singly, doubly, and multiply (3, 4, 5, and 6 or more parasites per cell) infected erythrocytes in 2 invasion assays, shown as the mean parasite multiplication rate. Assays were performed in duplicate and triplicate. Error bars represent the standard deviation of the mean. The selectivity index (SI) is shown.

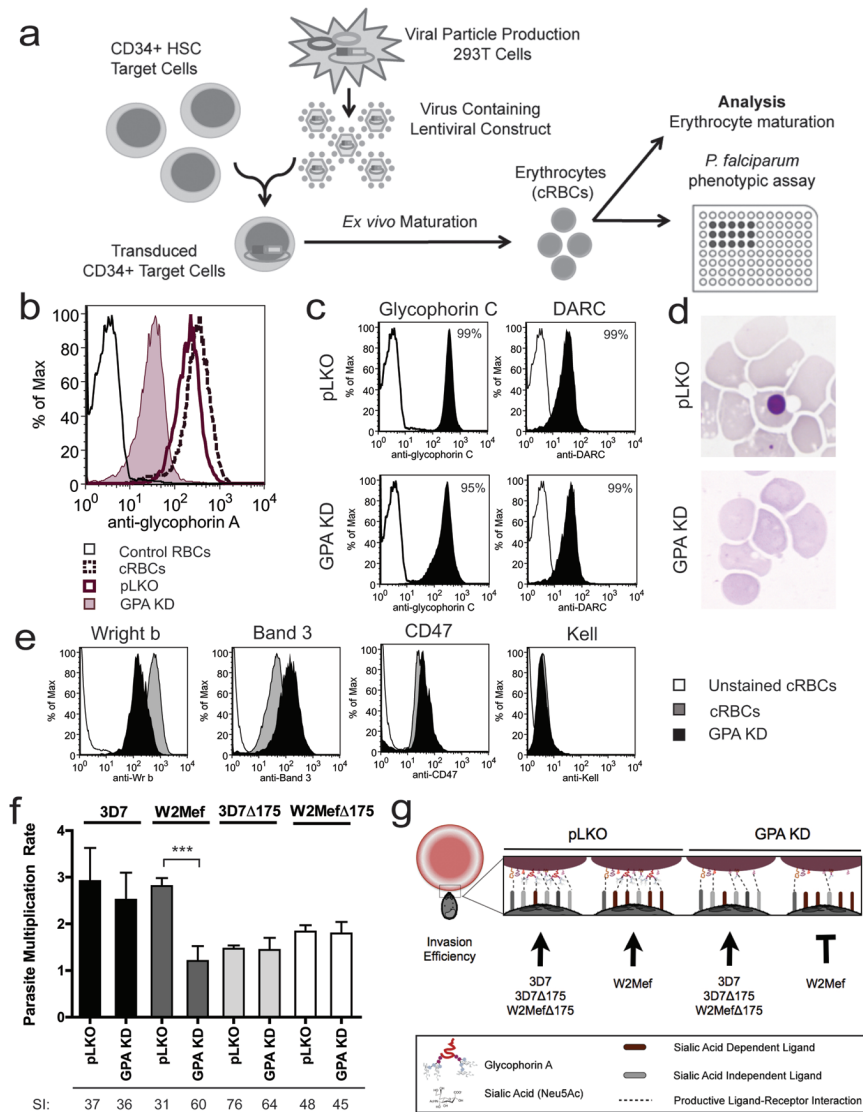


Figure 2. Stable knockdown of glycophorin A (GPA) on the erythrocyte surface resulting in no effect on erythrocyte maturation, but dramatic impact on invasion by *Plasmodium falciparum* parasites reliant on EBA-175. *a*, Schematic illustrating the method for reverse genetic analysis of human erythrocytes. Lentiviral particles are produced in 293T cells through co-transfection of small hairpin RNA (shRNA)-expressing plasmids (pLKO), envelope, and packaging plasmids. CD34⁺ hematopoietic stem cells (HSCs) are transduced and puromycin-selected cells are matured into erythrocytes ex vivo, at which point phenotypic analyses are conducted. *b*, Expression of GPA in cultured erythrocyte untransduced control cells (cRBCs), pLKO scramble-transduced control cells (pLKO), GPA knockdown cells (GPA KD), and isotype control stained erythrocytes (control RBCs). *c*, Expression of maturation markers (glycophorin C and Duffy antigen receptor for chemokines [DARC]) on transduced cells—pLKO scrambles shRNA-expressing cells (pLKO) and GPA shRNA-expressing cells (GPA KD). Negative controls were isotype control stained erythrocytes. The percentage of positive staining of erythrocyte gate is indicated. *d*, Erythrocyte morphology on day 18, as assessed by cytospin and May-Grunwald Giemsa staining. *e*, Expression levels of Band 3-GPA complex antigens (Wright b, Band 3, and CD47) and other surface proteins (Kell) on transduced cells (GPA KD) compared with those on cultured erythrocyte controls (cRBCs). Negative controls were unstained cultured erythrocytes. *f*, Parasite multiplication rate and invasion efficiency of the 3D7 and W2Mef parasite strains and EBA-175 mutants into GPA knockdown erythrocytes (GPA KD) and invasion efficiency into pLKO-transduced controls (pLKO), shown as the mean invasion percentage for 4 GPA knockdown transduction, performed in triplicate. W2Mef showed a significant difference in invasion percentage between GPA knockdown cells and pLKO control cells ($P < .001$) by the Student *t* test (2-tailed; significance level, $\alpha = 0.05$). Error bars represent the standard deviation of the mean. The selectivity index (SI) is shown. *g*, Model depicting erythrocyte invasion by both sialic acid-independent (3D7) and sialic acid-dependent (W2Mef) *P. falciparum* strains. Arrows denote efficient invasion into erythrocytes; the blocked bar denotes inhibited invasion.

impaired invasion efficiency into GPA knockdown erythrocytes (invasion efficiency of 42.8% compared with pLKO control) and an increased selectivity index (a measure of the distribution of multiply infected erythrocytes [10]) (Figure 2F). In contrast, the invasion efficiency of the 3D7 parasite line was not significantly impeded in GPA knockdown erythrocytes, indicating that this parasite strain predominantly uses other erythrocyte receptors for invasion (Figures 2F and 2G). To specifically determine the impact of EBA-175, the invasion efficiency of W2Mef Δ 175 and 3D7 Δ 175 mutants (knockouts of EBA-175) into GPA knockdown erythrocytes was assessed. Both W2Mef Δ 175 and 3D7 Δ 175 demonstrated reduced invasion efficiency into cultured erythrocytes compared with those of their wild-type parents (Figure 2F), as observed elsewhere [12]. In contrast to W2Mef, W2Mef Δ 175 showed no reduction in invasion efficiency in GPA knockdown erythrocytes relative to pLKO, suggesting that W2Mef Δ 175 parasites do not contain other invasion ligands that utilize GPA for efficient invasion. No difference in invasion efficiency was observed for 3D7 Δ 175 parasites.

Discussion. Here, we present a novel approach for the functional analysis of host-pathogen interactions in malaria. Few erythrocyte determinants of malaria have been identified through manipulation of erythrocyte ghosts [13] and immunodepletion of defined erythrocyte proteins [14]. Although such methods are ideally suited to the study of cytoplasmic proteins, they cannot be applied to the study of transmembrane proteins such as erythrocyte surface receptors. The system developed in this work combines in vitro culture of erythrocytes with lentiviral transduction to knock down gene expression. We provide direct genetic evidence that GPA is a major receptor for invasion by *P. falciparum* strains that rely on the parasite ligand EBA-175 for efficient erythrocyte invasion.

The utilization of alternative erythrocyte receptors by different *P. falciparum* strains for invasion may impede development of single specific invasion ligands, such as EBA-175, as vaccine candidates. The important role of GPA proposed for *P. falciparum* invasion is based on several lines of evidence with the use of rare natural GPA-null erythrocytes, blocking anti-GPA Fab fragments, and enzymatic treatment of erythrocytes to remove surface receptors [2–4, 9, 15]. By generating erythrocytes in which GPA gene expression has been depleted with shRNA, we are able to directly assess the role of this erythrocyte receptor in the absence of other confounding factors. The use of HSCs from a single donor for the generation of knockdowns produces isogenic lines, thus eliminating the influence of donor blood group differences or other co-inherited erythrocyte polymorphisms. In this work, we observed that the W2Mef parasite strain displays reduced invasion efficiency into erythrocytes depleted of GPA. However, this reduced invasion efficiency was not observed with the 3D7 parasite strain or with genetic mutants in which EBA-175 is disrupted, which indicates that the

different *P. falciparum* strains vary dramatically in their GPA dependence, despite expression of EBA-175 (Figure 2G). Interestingly, an incomplete depletion of GPA resulted in reduced erythrocyte invasion efficiency, which suggests that there may be a threshold of receptor levels necessary for efficient invasion. This could be further addressed by titrating GPA levels by use of a drug-inducible knockdown system.

Erythrocyte proteins exist in complexes, and depletion of one may affect the surface expression of other complex members. In our system, GPA knockdown resulted in no substantial change in the surface expression of many erythrocyte proteins, including glycophorin B (data not shown), with an expected decrease in the surface expression of Wright b antigen. Interestingly, increased Band 3 expression was observed, suggesting an interaction with GPA that can be elucidated with further mechanistic studies. Depletion of erythrocyte proteins may affect trafficking and surface expression of other erythrocyte receptors and could affect maturation and membrane deformability. Both surface expression of maturation markers and standard hematological and biophysical parameters should be considered for each independent knockdown.

The reverse genetics approach we have developed can be extended to identify the precise contribution of many erythrocyte proteins involved in the processes of parasite invasion and growth. However, genes that are required during erythropoiesis pose a challenge, as knockdown will be deleterious prior to erythrocyte differentiation. In addition, some erythrocyte mutations may lead to unviable reductions in erythrocyte stability or alterations in morphology, whereas in some cases, an incomplete level of knockdown may be insufficient to reveal a phenotype. Vectors for inducible expression of shRNA and transgenes or that use lineage- and stage-specific promoters, such as β -globin, may allow appropriate maturation and transgene expression in erythrocytes. Importantly, the method we employed produces large numbers of synchronous, enucleated, and terminally differentiated human erythrocytes that are susceptible to *P. falciparum* infection and that are suitable for phenotypic analyses.

In conclusion, this study demonstrates the first (to our knowledge) genetic knockdown of a protein involved in *P. falciparum* invasion that has been achieved entirely in vitro in mature erythrocytes. The identification of essential determinants for malaria will provide candidates for development of host-targeted therapies. In vitro genetic analysis of erythrocyte proteins will advance research in the diverse fields of hematology, erythrocyte biophysics, human genetics, and malaria.

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