Inherited Glutathione Reductase Deficiency and \textit{Plasmodium falciparum} Malaria—A Case Study

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\textbf{Introduction}

The tripeptide glutathione (\textit{N}-\textit{gamma}-glutamylcysteinylyglycine) is present in millimolar concentrations in the malaria parasite \textit{Plasmodium falciparum} as well as in the host red blood cell (RBC) \cite{1–5}. Reduced glutathione (GSH) plays an essential role in antioxidant defense in both parasitic and host cell \cite{1–5}. Parasite GSH supports cell growth by providing electrons for deoxyribonucleotide synthesis and takes part in detoxifying heme, a product of hemoglobin digestion \cite{6}. Furthermore, GSH is the coenzyme of the glyoxalase system, which detoxifies methylglyoxal \cite{7}, and of glutathione-S-transferase (GST)-based reactions. GSTs contribute to the detoxification of exogenous and endogenous cytotoxic metabolites. In \textit{Plasmodium}, GST is involved in peroxide detoxification and the binding of parasitotoxic heme as well as in the development of drug resistance \cite{8}. The flavoenzyme glutathione reductase (GR, EC 1.8.1.7) reduces oxidized glutathione (GSSG) to back to GSH \cite{3,9}. Due to its central position in redox control, \textit{P. falciparum} GR (PfGR) is ranked number one in the list of proposed antimalarial drug targets for \textit{Plasmodium} (http://tdtargets.org/), and a wide range of respective drug development approaches is currently being followed \cite{9–11}. In addition, the inhibition of RBC GR has been proposed as an approach to reduce the risk of multidrug resistance in malaria parasites \cite{9}.

In the GR-catalyzed reaction, reducing equivalents are provided by NADPH. NADPH is generated in the first half of the hexose monophosphate shunt by glucose-6-phosphate dehydrogenase (G6PD). Therefore, G6PD (producer of NADPH) as well as GR (utilizer of NADPH) are equally essential to maintain GSH homeostasis in the parasite-host unit \cite{2,4}. Mutations affecting either G6PD or GR might thus induce similar metabolic and functional consequences in the RBC. G6PD deficiency occurs in numerous genotypes, some of which are polymorphic and particularly frequent in areas where malaria is or was endemic \cite{12,13}, affecting approximately 330 million people worldwide \cite{14}. Decreased GR activity due to low saturation with FAD is also common in certain malaria-endemic regions \cite{16}. By contrast, hereditary GR deficiency is rare \cite{17}, and only recently a full biochemical and molecular characterization of a GR mutation...
leading to complete GR deficiency has been performed [18]. In this patient, RBCs and leukocytes did not contain any GR activity, and the GR protein could not be detected by Western blotting. DNA sequencing revealed a 2242-bp deletion, starting at nucleotide +658 in intron 11 and ending at nucleotide 639 in the 3′ untranslated region of exon 13 of the GR gene, which is located on chromosome 8. As a result, translated GR missed the complete dimerization domain, resulting in an inactive enzyme [18].

In view of (a) the potentially similar metabolic effects of G6PD and GR deficiency, (b) the well documented protection from severe falciparum malaria afforded by G6PD deficiency [14,19] and (c) the fact that P. falciparum GR and human GR represent most promising antimalarial drug targets, we studied invasion and growth of several P. falciparum strains in GR-deficient RBCs as well as the stage-dependent pathological alterations induced by parasite growth in these erythrocytes. We directly compared these changes to GR-sufficient control cells as well as to analogous data obtained with malaria-infected G6PD-deficient RBCs and to senescent RBCs [13,20,21]. Analogies with RBCs from patients with sickle-cell trait, β-thalassemia [22], and pyruvate kinase deficiency [23] are discussed.

Results

Unless otherwise indicated, all experiments reported below were performed with RBCs from the index patient.

Invasion and multiplication of P. falciparum grown in GR-deficient RBCs

Twenty-four hours after inoculation of GR-deficient RBCs with malarial parasites (strains 3D7 or K1, experiment 1, see Materials and Methods), ring stages of Plasmodium were detectable in all wells. As determined by Giemsa staining and by having an experienced technician count infected cells under the light microscope, the parasitemia for the 3D7 strain was 5.9±0.5% in the GR-deficient cells and 4.0±0.4% in the controls. This indicated that Plasmodium is able to invade GR-deficient RBCs as efficiently as normal RBCs. Subsequently, parasites were grown in the respective RBC cultures for four complete 48-h cycles, showing a mean multiplication rate of 4.9±0.3 per RBC cycle for the GR-deficient RBCs, as well as 4.3 (0% blood) and 6.5 (A+ blood) for the controls (mean ±SEM of the different individuals included and two to three parallel determinations per sample).

Also, in the second experiment (strain 3D7, see Methods), all RBC samples (index patient and three controls) were efficiently invaded by the parasites. Forty-eight hours later about 4% infected RBCs (IRBCs) were again determined in all 4 cultures. After splitting and another 48-h cycle, a very similar parasitemia (given in the following for 3D7) was determined in all samples (9.8±0.3% in the GR-deficient cells, 9.3±0.4% in the controls). Also the percentages of ring stages (36±2% in the patient and 37±3% in the controls) and trophozoite/schizont stages (64±1% in the patient, 62±3% in the controls) did not differ significantly. The morphology of the parasites as determined by light microscopy after Giemsa staining was unchanged throughout the experiments (data not shown). In a separate set of experiments performed in the other participating laboratory in Turin with the Polo Alto parasite strain, similar data were obtained, showing that the invasion rate of GR-deficient RBCs was as high as that of control RBCs (30% parasitemia at 19 h after inoculation with 6.4% schizonts), and regular development into trophozoites was observed on day 2 (30% parasitemia).

GR activity, total glutathione content and drug sensitivity of P. falciparum grown in GR-deficient RBCs

Malarial parasites grown in GR-deficient RBCs were tested for total glutathione content, GR activity, and their sensitivity towards redox-active antimarial as well as inducers of oxidative and nitrosative stress. Data were collected in two independent experiments (including venipuncture and shipment of the blood samples) with different controls and two different parasite strains (K1 and 3D7). Before determining the above parameters, parasites were grown for 4–5 cycles (corresponding to 8–10 days) in the respective RBCs. Control cells had the same blood group (O+) as the patient’s.

In both experiments the parasites displayed a glutathione content of about 70 nmol/mg protein, which did not differ between parasites grown in GR-deficient and normal host cells (Table 1). However, in the parasites grown in GR-deficient cells, GR activity was found to be significantly reduced to 61% (experiment 1, see Table 1) of control values. A comparable value (66% of control activity) was determined in experiment 2.

The IC50 values for CQ, artemisinin, and methylene blue were very similar between parasites grown in GR-deficient RBCs and in controls. GR-deficient RBCs were significantly less sensitive towards the superoxide generating agent paraquat and of sodium nitroprusside (Table 1). This was verified in both experiments and for both parasite strains K1 and 3D7. However, the IC50 values of the superoxide generating agent paraquat and of sodium nitroprusside were found to be significantly lower in parasites grown in GR-deficient RBCs than in controls (Table 1).

Membrane binding of hemichromes, complement C3 fragment, autologous IgG, and phagocytosis in ring stage-infected GR-deficient RBCs

Membrane-bound hemichromes, indicators of oxidative membrane damage and inducers of RBC membrane modifications producing enhanced phagocytosis [20–22], were measured in ring
stage-infected RBCs of the index patient, individual 2 (brother), and individual 3 (sister), all homozygous for the GR deficiency, and were compared to ring stage-infected normal RBCs. As shown in Fig. 1A, parasite growth significantly increased membrane-bound hemichromes in all three GR-deficient individuals.

Membrane binding of complement fragments C3 and C3b2 and autologous IgG, two powerful opsonins known to be instrumental in inducing phagocytosis, were assayed in ring-infected RBCs in the index patient as well as in individuals 2 and 3. Oposide complement C3 fragments C3b/C3b2 were measured as C3c, a stable C3 derivative, making use of anti-C3c antibodies, as detailed in the Materials and Methods section. As shown in Figure 1B–C, membrane deposition of complement fragment C3c was remarkably increased in all three GR-deficient individuals, while binding of IgG was strongly enhanced in the index patient but unchanged in the other two individuals. In general, changes observed in the index patient were more pronounced compared to the other two GR-deficient individuals. Phagocytosis was assayed with the human monocytic cell line THP-1 (Figure 2A) and with human mononuclear cells (Figure 2B). Both phagocytic cell types displayed increased phagocytosis of ring-infected cells from all three GR-deficient individuals. Again the effect was more pronounced in the index patient. Co-cultivated, non-infected normal or deficient RBCs were not phagocytosed in significant amounts (Figure 2A–B). Trophozoites were phagocytosed more intensely than ring stages, with minor differences between GR-deficient and control RBCs (Figure 2B).

The data on membrane-bound hemichromes, complement C3c fragment, IgG, and phagocytosis were obtained from single samples from the GR-deficient individuals and 3–4 normal controls. Except for hemichromes where 3–4 repeats were constantly performed and formal statistical treatment was possible, the paucity of sample material did not allow us to perform a sufficient number of repeats in the GR-deficient samples. Therefore representative values are shown in Figures 1 and 2. The supplementary figure S1 compares results shown in Figures 1 and 2 with an additional series of normal control rings obtained using 24–36 h old normal blood from 7 healthy Italian donors. In general, SDs were distinctly lower in the Italian control series, increasing the robustness of comparisons.

**Discussion**

**RBC mutations leading to enhanced ring phagocytosis**

A relatively small number of mutations affecting hemoglobins and RBC enzymes are present in large numbers in human populations. It is accepted that these widespread polymorphisms afford protection against *falciparum* malaria [24–26]. A group of these conditions including sickle cell anemia, β-thalassemia, possibly hemoglobins C [27,28] and E [29], as well as G6PD deficiency [14,20] and PK deficiency [23] appear to provide antimalarial protection based on a common mechanism [20,22]. The aberrant RBCs are characterized by an increased production of reactive oxygen species (ROS) (hemoglobin mutants), or decreased antioxidant defense (G6PD deficiency, PK deficiency [23]). Non-infected, mutant RBCs display slight but significantly increased membrane deposition of hemichromes accompanied by low-grade but distinct enhancement of phagocytic uptake [20,22,23]. Parasites growing in any of the aforementioned mutated cells induce typical modifications in the host RBCs which start manifesting at ring stage. Normal RBCs harboring ring stages show little band 3 aggregation, poor opsonization, and slightly enhanced phagocytic recognition and uptake [30]. By contrast, rings growing in mutant RBCs sequentially display (1)

![Figure 1. Membrane-bound hemichromes, complement C3c fragment, and autologous IgG in GR-deficient and GR-sufficient RBCs.](image)

- **HEMICROMES**
  - Panel A: Membrane-bound hemichromes (black bars) and complement C3c (open bars) in ring-infected and non-infected GR-deficient RBCs from the index patient, brother (individual 2) and sister (individual 3) of index patient, and from normal GR-sufficient controls. Hemichromes are expressed as pmoles/µg membrane protein. Mean values of index patient, individuals 2 and 3, and normal controls (mean ± SD, n = 3) are shown. Hemichromes were significantly higher (p < 0.02) in the ring-infected RBCs of the index patient and individuals 2 and 3 compared to ring-infected RBCs of normal controls. Complement C3c fragment and autologous IgG are expressed as Mean Fluorescence Intensity. Representative data of GR-deficient individuals and mean values of normal controls (mean ± SD, n = 2–3). For experimental details see Materials and Methods.

- **BOUND IgG**
  - Panel C: Mean Fluorescence Intensity of bound IgG in ring-infected and non-infected GR-deficient RBCs from the index patient, brother (individual 2) and sister (individual 3) of index patient, and from normal GR-sufficient controls. Black bars indicate ring-infected RBCs; open bars, non-infected RBCs. Hemichromes are expressed as pmoles/µg membrane protein. Mean values of index patient, individuals 2 and 3, and normal controls (mean ± SD, n = 2–4) are shown. Hemichromes were significantly higher (p < 0.02) in the ring-infected RBCs of the index patient and individuals 2 and 3 compared to ring-infected RBCs of normal controls. Complement C3c fragment and autologous IgG are expressed as Mean Fluorescence Intensity. Representative data of GR-deficient individuals and mean values of normal controls (mean ± SD, n = 2–3). For experimental details see Materials and Methods.

- **BOUND C3c**
  - Panel B: Mean Fluorescence Intensity of bound C3c in ring-infected and non-infected GR-deficient RBCs from the index patient, brother (individual 2) and sister (individual 3) of index patient, and from normal GR-sufficient controls. Black bars indicate ring-infected RBCs; open bars, non-infected RBCs. Hemichromes are expressed as pmoles/µg membrane protein. Mean values of index patient, individuals 2 and 3, and normal controls (mean ± SD, n = 2–3) are shown. Hemichromes were significantly higher (p < 0.02) in the ring-infected RBCs of the index patient and individuals 2 and 3 compared to ring-infected RBCs of normal controls. Complement C3c fragment and autologous IgG are expressed as Mean Fluorescence Intensity. Representative data of GR-deficient individuals and mean values of normal controls (mean ± SD, n = 2–3). For experimental details see Materials and Methods.
Advantages of enhanced ring phagocytosis for the malaria patient

Enhanced and preferential phagocytosis of ring-parasitized mutant RBCs may be advantageous to the host in two ways: (i) the reduction of parasitic growth and parasite density, observed for example in patients with HbAS and beta-thalassemia trait [24,25], and (ii) the rapid digestion of phagocytosed rings by monocytes and the frequent repetition of this process without loss of efficiency [31]. By contrast, phagocytosis of hemoglobin-containing mature parasites inhibits the ability of monocytes to repeat the phagocytic process [31], enhances their production of inflammatory cytokines [32], and impairs their ability to kill ingested pathogens [33], to express MHC class II and other membrane antigens upon interferon-gamma stimulation [34], and to correctly present antigens [35]. Another way enhanced ring phagocytosis may be advantageous to the host is by lowering the number of trophozoites and schizonts adhering to endothelia in specific organs and provoking there severe clinical conditions such as cerebral malaria, placental malaria, and possibly dyserythropoietic-based anemia [36,37]. Other protection mechanisms probably concur with this based on enhanced ring-phagocytosis of mutant RBCs underscored here. For example, decreased adhesion to endothelia has been suggested to be involved in anti-malaria defense in sickle-cell trait [38,39] and Hb AC/CC [40].

Enhanced ring phagocytosis associated with GR deficiency

Data presented here show that malaria-infected GR-deficient RBCs behave very similarly to infected G6PD-deficient RBCs
Endemic GR deficiency caused by undersaturation with FAD

Complete absence of RBC GR is very rare [12,17,18]. This defect evidently did not arise in parallel to malaria expansion in humans to provide protection. However, impairment of GR activity is widespread and geographically coincident with past or present occurrence of malaria. Since GR is a flavoprotein critically dependent on FAD [43], flavin-deficient RBCs would contain decreased GR activity. In fact, riboflavin deficiency is widespread in underdeveloped countries due to low dietary intake [44,45]. Several studies have shown that a reduced riboflavin status and non-genetic low GR activity are associated with lower Plasmodium parasitemia in humans [46] and in animal [47] models and are frequently found in malaria-endemic areas [48]. Furthermore, high prevalence of familial flavin-deficient RBCs not due to dietary riboflavin deficiency was detected in parts of Italy where malaria used to be prevalent, namely the Maremma region in Tuscany, the delta region of the river Po, and coastal areas in Sardinia [48–50]. Interestingly, in Sardinia [50] micro-regional accumulation in villages formerly exposed to malaria was observed for carriers of the putative malaria-protective low-activity GR and FMN-dependent pyridoxine phosphate oxidase [50]. Accumulation in the same villages was previously observed for carriers of β-thalassemia and G6PD deficiency [51]. Finally, low GR activity was found to be frequently associated with other malaria-protective mutations such as sickle-cell anemia and β-thalassemia [52–55].

Conclusions

In conclusion, rare RBC mutations such as PK deficiency and GR deficiency resist malaria infection and may provide protection by the same paradigm applicable to widespread mutations such as those affecting hemoglobin, G6PD, or membrane proteins. Thus the pharmacologic inhibition of parasite and/or host cell GR might lead to metabolic consequences proven by nature to be effective against the parasite. Indeed the inhibition of RBC GR has been proposed as a feasible antimalarial approach likely to reduce the risk of resistance development [9]. Normal RBCs pretreated with high doses of the cytostatic agent carmustine (BCNU) have no detectable GR activity. These RBCs do not serve as host cells of Plasmodium falciparum in vitro unless the medium contains high levels of glutathione. Thus, in contrast to genetically GR-deficient RBCs, RBCs rendered GR-deficient by BCNU appear to be unable to maintain sufficient GSH levels, possibly because the high BCNU doses also affect the GSH synthesizing enzymes [56]. Nevertheless erythrocytes pretreated with lower doses of nitroso-ureas in vivo or in vitro are probably suitable for corroborating and extending the findings of this present study [57].

When testing GR-inhibitors as antiplasmodial agents it should be taken into account that the effects on parasite growth and multiplication in vitro might be less pronounced than in vivo – as observed in genetic GR deficiency. Thus additional tests such as parallel application of oxidant stressors and/or determination of membrane alterations and phagocytosis should be performed for assessing the antimalarial effects of GR inhibitors. Furthermore their hemolytic potential needs to be evaluated carefully.

Materials and Methods

Patients

The GR-deficient patient – the index patient – who kindly agreed to donate blood for this study was a 54-year old woman whose clinical history has been described before [17,18]. She was the index patient in a family with GR deficiency who suffered a hemolytic crisis after eating fava beans. Two siblings – brother and sister of the index patient, indicated as individual 2 and individual 3, respectively – were also homozygous for GR deficiency [17]. In all three individuals GR activity in RBCs and leukocytes was undetectable and could not be stimulated by either riboflavin ingestion in vivo or FAD addition in vitro [18].

All family members included in this study are fully aware of their unique enzyme deficiency and have given their written consent for the research carried out. The research has been explained to them both in written and oral form. The research was performed as part of the diagnostic work on the patients’ blood samples, which is fully approved by the Sanquin Ethical Medical Committee.
heme, hemichromes, IgG and complement C3c fragment, assay of phagocytosis with stage-separated infected and control RBCs), full blood from the GR-deficient individuals and three normal controls was anticoagulated with CPD, shipped on crushed ice, and utilized within 24 h of venipuncture. The further processing of the blood samples is described below.

**Cultivation of *P. falciparum* and preparation of parasite extracts**

In the Giessen lab CQ sensitive (3D7-Netherlands) and resistant (K1-Southeast Asia) strains of *P. falciparum* were grown in continuous culture as described [38] with slight modifications. Parasites were maintained at 1–10% parasitemia and 3.3% hematocrit in RPMI 1640 culture medium supplemented with A + RBCs (normal culture conditions) or 0 + RBCs (for the GR-deficient patient and a control sample), 4% A + human serum, 0.2% lipid-rich bovine serum albumin (Albumax), 9 mM glucose, 0.2 mM hypoxanthine, 2.1 mM L-glutamine, and 22 μg/ml gentamicin. All incubations were carried out at 37°C, 3% O2, 3% CO2 and 94% N2. Synchronization of parasites in culture to ring stages was carried out by treatment with 5% (v/v) sorbitol [59]. The morphology of the parasites as well as the multiplication rate were determined by light microscopy after Giemsa staining. Before determining either the glutathione content or the GR activity in *P. falciparum*, parasites were grown over 5 cycles (10 days) in their respective RBCs. Trophozoite stage parasites were then isolated by suspending the RBCs in a 20-fold volume of buffer containing 7 mM KH2PO4, 1 mM NaH2PO4, 11 mM NaHCO3, 58 mM KCl, 56 mM NaCl, 1 mM MgCl2, 14 mM glucose, and 0.02% saponin for 10 min at 37°C. The pellets were washed two times in the same saponin buffer for RBC lysis and then isolated by suspending the RBCs in a 20-fold volume of buffer containing 7 mM KH2PO4, 1 mM NaH2PO4, 11 mM NaHCO3, 58 mM KCl, 56 mM NaCl, 1 mM MgCl2, 14 mM glucose, and 0.02% saponin for 10 min at 37°C. The pellets were washed two times in the same saponin buffer for RBC lysis and one time in PBS (centrifugation for all steps 1,500 g, 5 min, room temperature). The free parasites were finally diluted in PBS and disrupted by freezing and thawing three times in the presence of protease inhibitors (40 μl/ml Complete (Roche) and 1 mg/ml Pefabloc (Roche)). After centrifugation (ultracentrifuge 100,000 g, 30 min, 4°C), the protein content of the supernatant was determined by the Bradford method and the extract was used for the various analyses.

**Invasion and multiplication of *P. falciparum* grown in GR-deficient RBCs**

To determine if *P. falciparum* can successfully invade and multiply in GR-deficient RBCs, EDTA full blood was taken from patients and controls in the Netherlands and shipped at 4°C to the Giessen lab. About 24 h after venipuncture, plasma and buffy coat of the blood samples were removed by centrifugation (1,500 g, 3 min, 4°C) and aspiration followed by washing three times in a 10-fold volume of RPMI 1640 medium (1,500 g, 3 min, 4°C). For some of the invasion studies control samples freshly taken in Germany were processed and studied in parallel.

**EXPERIMENT 1:** Ten μl of RBCs from the index patient (0 + blood) and two German controls (A + and 0 + blood) each were added to 300 μl of complete cell culture medium in 48-well plates. Two μl of parasitized RBCs (3D7, mainly trophozoite stage; final parasitemia 0.7%) were added to the RBC cultures. The K1 strain was inoculated for drug sensitivity tests.

**EXPERIMENT 2:** In a separate experiment, RBCs from the index patient (0 + blood) were shipped together with 3 controls (0 +, A +, A + blood). After washing, 0.5 ml of RBCs were added to 15 ml of cell culture medium and inoculated with 1 ml of parasitized RBCs (3D7, synchronized to ring stages; this resulted in the same final parasitemia of 0.7% as in experiment 1).

**Determination of total glutathione and PfGR activity in parasite extracts**

For the determination of total glutathione content, 40 μl of parasite extract was deproteinized by adding 2 vol of 5% (w/vol) sulfosalicylic acid; the samples were mixed and centrifuged, and the supernatant was used for analyses. The glutathione content was measured by the GR-coupled 5,5’-dithio-bis-2-nitrobenzoic acid, DTNB-GSH-recycling assay [60]. A standard curve was prepared using appropriate concentrations of GSH and sulfosalicylic acid. *P. falciparum* PfGR activity in the parasite extracts was assayed at 25°C with 100 μM NADPH and 1 mM GSSG in 47 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, pH 6.9. The consumption of NADPH was followed spectrophotometrically at 340 nm [61]. Specific activities (μmol/min/mg of protein) were calculated using the enzyme activities at 25°C and the protein concentrations of the parasite extracts.

**Drug effects on *P. falciparum***

An isotopic drug sensitivity assay using the semi-automated microdilution technique [62] was employed to investigate the effects of CQ, artemisinin, methylene blue, paraquat and sodium nitroprusside (SNP) on parasites grown in GR-deficient RBCs. The method is based on the incorporation of radioactive 3H-hypoxanthine – which is taken up by the parasite as a precursor of purine deoxynucleotides for DNA and RNA synthesis – and was modified according to Fivelman [63]. In 96-well microtiter plates (Nunc™), a two-fold serial dilution of the starting concentration of each drug to be tested was carried out. Parasites were incubated at a parasitemia of 0.125% (>70% ring forms) and 1.25% hematocrit in hypoxanthine-free medium. After 48 h, 0.5 μCi [3H]-hypoxanthine was added into each well and the plates were incubated for another 24 h. The cells from each well were harvested on a glass fiber filter (Perkin-Elmer, Rodgau-Jugelsheim, Germany), washed, and dried. Their radioactivity in counts per minute was considered to be proportional to the respective growth of *P. falciparum* in the well. IC50 values (drug concentrations that produce 50% reduction in the uptake of 3H-hypoxanthine) were calculated as described in [63].

For statistical analyses of the data on multiplication rates, biochemical parameters and IC50 values (given as mean values ± SEM), the unpaired t-test as well as the Mann-Whitney U Test were employed.

**Stage-dependent separation and opsonization of parasites**

*P. falciparum* parasites (Pallo Alto strain, *Mycoplasma*-free) were cultivated in normal and GR-deficient RBCs at 2% hematocrit and synchronized as described [30]. Briefly, schizont stage infected normal RBCs (parasitemia >95%) were mixed for invasion with washed GR-deficient or normal RBCs and kept in growth medium (RPMI 1640), containing 25 mM Hepes, 30 mM glucose, 2 mM glutamine, 0.02 mM adenine, 24 mM NaHCO3, 32 mg/l gentamicin, and 10% A + complemented human plasma (time 0). After 19 h incubation in a humidified CO2/air incubator, the ring-enriched fraction was separated on and collected from a discontinuous 40/80/90% Percoll gradient, containing mannitol (6% wt/vol). The bottom Percoll fraction contained non-infected RBCs and approximately 15–20% rings, morphologically identical to those of the ring-enriched fraction. After 41 h incubation the trophozoite-enriched fraction was separated onto and collected from a discontinuous 10/40/80% Percoll gradient. Rings (after 19 h incubation) as well as trophozoites (after 41 h incubation) were enriched to approx. 80–85% and approx. 90%, respectively.
by the above procedure. Parasitemia and parasite morphology were assessed by light microscopy after Diff-Quik® Fix staining (Medion Diagnostics GmbH, Duedingen, Switzerland). Non-infected control RBCs of each donor were incubated and treated in a similar way without schizont inoculation at time 0. Infected and non-infected RBCs were washed, reequilibrated for 1 h in PBS-G, and opsonized with freshly drawn serum of a healthy AB/Rh- donor in a ratio: RBCs/PBS containing 2 mM glucose (PBS-G)/ serum 1/1/2 (vol/vol). Non-opsonized RBCs were incubated in parallel substituting serum with PBS-G. After 30 min at 37°C cells (ring-enriched, trophozoite-enriched or non-infected RBCs) were washed 3 times with PBS-G, adjusted to 50% hematocrit and centrifuged at 200 × g for 5 min. The cell pellet was resuspended in 50 μl PBS-G containing 3 mM EDTA and 0.05% (vol/vol) Triton X100, and used to quantify hemoglobin. Heme from both ghost aliquots (hemoglobin + insoluble hemes) was solubilized for 1 h at room temperature in 1 ml of PBS containing 3 mM EDTA and 0.05% (vol/vol) Triton X100 to quantify the total heme content (hemoglobin + insoluble hemes). A second aliquot of 10 μl ghosts was solubilized for 1 h at room temperature in 1 ml of PBS containing 5 mM EDTA and 0.05% (vol/vol) Triton X100 and used to quantify hemoglobin. Heme from both ghost aliquots was quantified by measuring the heme-dependent luminol-enhanced luminescence as described [65]. Each luminescence measurement (2 μl aliquots) was repeated 3–4 times. Inter-measurement variability never exceeded 2–3%. Results obtained from cell samples that contained approx. 15–20% rings were extrapolated to 100% rings using the calculation suggested by Cappadoro et al. [20]. The hemicrine content was calculated from the difference between total heme and hemoglobin heme. Hemicrine heme was the major component of total heme in control rings (88% of total heme), index patient (96%), individual 2 (99%) and individual 3 (87%), while membrane-bound hemoglobin was a minor fraction of total heme.

Quantification of membrane-bound total heme and hemichromes in ghosts prepared from stage-separated infected RBCs

Heme bound to the cytoplasmic face of the membrane as hemoglobin and insoluble hemicrines was analyzed in the hypotonic ghosts [64] prepared under non-reducing conditions in the presence of Complete® protease inhibitor cocktail from the bottom Percoll fraction of the unseparated, infected GR-deficient and normal RBCs [22]. This fraction contained approximately 15–20% rings. Every ghost preparation was divided into two aliquots. A first aliquot of 10 μl ghosts was solubilized for 3 h at 4°C in 1 ml of 0.1 N NaOH containing 3 mM EDTA and 0.05% (vol/vol) Triton X100 to quantify the total heme content (hemoglobin + insoluble hemes). A second aliquot of 10 μl ghosts was solubilized for 1 h at room temperature in 1 ml of PBS containing 5 mM EDTA and 0.05% (vol/vol) Triton X100 and used to quantify hemoglobin. Heme from both ghost aliquots was quantified by measuring the heme-dependent luminol-enhanced luminescence as described [65]. Each luminescence measurement (2 μl aliquots) was repeated 3–4 times. Inter-measurement variability never exceeded 2–3%. Results obtained from cell samples that contained approx. 15–20% rings were extrapolated to 100% rings using the calculation suggested by Cappadoro et al. [20]. The hemicrine content was calculated from the difference between total heme and hemoglobin heme. Hemicrine heme was the major component of total heme in control rings (88% of total heme), index patient (96%), individual 2 (99%) and individual 3 (87%), while membrane-bound hemoglobin was a minor fraction of total heme.

Phagocytosis of stage-separated infected and control RBCs by THP-1 cells and human mononuclear cells

The phagocytosis assay was performed utilizing the human monocytic cell line THP-1 or human mononuclear cells.

1. THP-1 cells were maintained at 0.2 × 10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The phagocytic ability of THP-1 cells was stimulated by supplementing IFN-gamma (50 U/ml) and TNF (250 U/ml) to the cell suspension 3 h before the start of phagocytosis. Shortly before phagocytosis, cells were washed three times with RPMI 1640 medium and resuspended in Macrophage-SFM medium supplemented with 10% heat-inactivated FBS to obtain 150,000 cells/300 μl medium. Ring- or trophozoite-infected and non-infected normal or GR-deficient RBCs were fluorescent-labeled for the phagocytosis assay during 10 min incubation at room temperature with 0.5 μM carboxyfluorescein diacetate, succinnimidyl ester, (CFDA-SE, Sigma, Milano, Italy) in PBS-G at 0.05% hematocrit. Labeling was stopped by adding 2 ml of heat-inactivated FBS for 5 min. RBCs were then washed three times with PBS-G and co-incubated with THP-1 cells at a THP-1:RBC ratio of 1:100 in round-bottom polystyrene tubes at 37°C for 2.5 h (CO₂ 5%). At the end of the incubation, non-ingested RBCs were lysed with 3.5 ml of ice-cold distilled water for 2 min. Physiologic osmolarity was restored by adding 10-times concentrated PBS. Cells were finally washed with cold PBS +1% FBS and analyzed with a BD FACS Calibur Flow Cytometer using Cell Quest software (BD Biosciences). Events were displayed on green fluorescence (FL1) versus forward scatter (FSC) dot plots or FL1 versus events number histogram. THP-1 live cells were gated on light scatter characteristics, and a total of at least 30,000 events in gating were collected for each sample. Data analysis was done with WinMDI software.

Supporting Information

Figure S1 Membrane-bound hemichromes, phagocytosis, complement C3c fragment, and autologous IgG in/of GR-deficient and GR-sufficient RBCs - comparison with healthy Italian donors. Data from Figure 1 and Figure 2 of the manuscript were compared to ring-infected and non-infected GR-sufficient control RBCs prepared from 24–36 h old blood (kept at +4°C) from 7
healthy Italian donors. Mean values of normal controls (mean±SD, n = 7).

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References

1. Beutler E (2001a) Composition of the erythrocyte. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, eds. Williams Hematology, 6th edition. New York: McGraw-Hill. pp 299–293.

2. Beutler E (2001b) Energy metabolism and maintenance of erythrocytes. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, eds. Williams Hematology, 6th edition. New York: McGraw-Hill. pp 319–332.

3. Becker K, Rahlfs S, Nickel G, Schirmer RH (2003) Glutathione-function and metabolism in the malarial parasite Plasmodium falciparum. Biol Chem 384: 551–566.

4. Becker K, Tilley L, Vernerstrom J, Roberts F, Rogerson S, Ginsburg H (2004) Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. Parasitology 128: 163–189.

5. Becker K, Koncarevic S, Hunt NH (2005) Oxidative stress and antioxidant defense in malarial parasites. In: Sherman IW, ed. Molecular Approaches to Malaria. Herndon: American Society of Microbiology Press. pp 365–383.

6. Atamna H, Ginsburg H (1995) Heme degradation in the presence of glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells. J Biol Chem 270: 24876–24883.

7. Akoachere M, Iffezel R, Rahlis S, Deponte M, Mannervik B, et al. (2005) Characterization of the glycosylases of the malarial parasite Plasmodium falciparum and comparison with their human counterparts. Biol Chem 386: 41–45.

8. Hiller N, Fritz-Wolf K, Deponte M, Wendt D, Zimmermann H, et al. (2006) Plasmodium falciparum glutathione S-transferase: structural and mechanistic studies on ligand binding and enzyme inhibition. Protein Sci 15: 281–289.

9. Krauth-Siegel R, Bauer H, Schirmer RH (2005) Dithiol proteins as guardians of the intracellular redox milieu in parasites: old and new drug targets in trypanosomes and malaria-causing plasmodia. Angew Chem Int Ed Engl 44: 690–715.

10. Buchholz K, Mwingela Mala B, Heiner Schirmer R, Becker K (2007) Structure-Based Drug Development Against Malaria. Frontiers in Drug Design & Discovery 3: 225–255.

11. Rahili S, Becker K (2006) Interference with redox-active enzymes as a basis for the design of antimalarial drugs. Mini Rev Med Chem 6: 163–76.

12. Beutler E (2001c) Glucose-6-phosphate dehydrogenase deficiency and other red cell enzyme abnormalities. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, eds. Williams Hematology, 6th edition. New York: McGraw-Hill. pp 527–545.

13. Luzzato L, Mehta A, Vulchanova T (2001) Glucose-6-phosphate dehydrogenase deficiency. In: Scrivener CR, Beaudet RAL, Sly WS, Valle D, eds. The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill. pp 4517–4533.

14. Greene L (1993) G6PD deficiency as protection against falciparum malaria: an epidemiologic critique of population and experimental studies. Yearb Phys Anthropol 36: 26.

15. Nkhoti ET, Poole C, Vammapattig V, Hall SA, Beutler E (2009) The global prevalence of glucose-6-phosphate dehydrogenase deficiency: A systematic review and meta-analysis. Blood Cells Mol Dis, (pub ahead of print, Feb. 2009).

16. Beutler E (1996) G6PD deficiency: population genetics and clinical manifestations. Blood 10: 45–52.

17. Loos H, Roos D, Wening R, Hoovenruij J (1976) Familial deficiency of glutathione reductase in human blood cells. Blood 48: 53–62.

18. Kamebeck N, van Zwieteren R, de Boer M, Morren G, Vull H, et al. (2007) Malarial basis of glutathione reductase deficiency in human blood cells. Blood 109: 3560–3566.

19. Rinvede C, Hill A (1998) Glucose-6-phosphate dehydrogenase deficiency and malaria. J Mol Med 76: 581–588.

20. Cappadoro MG, Giribaldi E, O’Brien F, Turrini F, Mannu D, et al. (2000) Enhanced phagocytosis of ring-parasitized mutant erythrocytes: a common mechanism that may explain protection against falciparum malaria in sickle trait and beta-thalassemia trait. Blood 100: 3364–3371.

21. Aki K, Min-Oo G, Serghides L, Crockett M, Kirby-Allen M, et al. (2008) Pyruvate kinase deficiency and malaria. N Engl J Med 358: 1085–1010.

22. Williams T (2006) Human red blood cell polymorphisms and malaria. Curr Opin Microbiol 9: 388–394.

23. Roberts DIJ, Harris T, Williams T (2004) The influence of inherited traits on malaria infection. In: Bellamy R, ed. Susceptibility to Infectious Diseases: the Importance of Host Genetics. Cambridge: Cambridge University Press. pp 139–184.

24. Min-Oo G, Gros P (2005) Erythrocyte variants and the nature of their malaria protective effect. Cell Microbiol 7: 753–763.

25. Arai T, Fairhurst R, Brittain N, Wellem T, Dvorak J (2005) Hemoglobin C modulates the surface topography of Plasmodium falciparum-infected erythrocytes. J Struct Biol 150: 163–169.

26. Tokumasu F, Fairhurst R, Osga G, Brittain N, Hwang J, et al. (2005) Band 3 modifications in Plasmodium falciparum-infected AA and CC erythrocytes assayed by autocorrelation analysis using quantum dots. J Cell Sci 118: 1091–1098.

27. Chotivanich K, Udomsangphet R, Pattanapanyasat K, Chierakul W, Simpson J, et al. (2002) Hemoglobin E: a balanced polymorphism protective against high parasitemias and thus severe P. falciparum malaria. Blood 100: 1172–1176.

28. Turini F, Ginsburg H, Busolinom F, Pescarmoma GP, Serra MV, Ares P (1992) Phagocytosis of Plasmodium falciparum-infected human red blood cells by human monocytes: involvement of immune and nonimmune determinants and dependence on parasite developmental stage. Blood 80: 801–808.

29. Schwarzer E, Turini F, Ullers D, Giribaldi G, Ginsburg H, et al. (1992) Impairment of macrophage functions after ingestion of Plasmodium falciparum-infected erythrocytes or isolated malarial pigment. J Exp Med 176: 1033–1041.

30. Hanesch T, Egan T, Groschus M (2007) Haemoglobin from methionin pigment as drug target, diagnostic tool, and immune modulator. Lancet Infect Dis 7: 675–685.

31. Fiers P, Rappelli P, Mirkarimi S, Ginsburg H, Cappuccinelli P, et al. (1993) Reduced microbical and anti-tumour activities of human monocytes after ingestion of Plasmodium falciparum-infected red blood cells. Parasite Immunol 15: 647–655.

32. Schwarzer E, Alessio M, Ullers D, Ares P (1998) Phagocytosis of the malarial pigment, hemozoin, impairs expression of major histocompatibility complex class II antigen, CD54, and CD11c in human monocytes. Infect Immun 66: 1601–1606.

33. Serraz T, Magee S, Bro L, De Baetselier P (1999) Hemozoin is a key factor in the induction of malaria-associated immunosuppression. Parasite Immunol 21: 545–554.

34. Skorokhod O, Alessio M, Mordmeliller B, Ares P, Schwarzer E (2004) Hemoglobin (malarial pigment) inhibits differentiatation and maturation of human monocyte-derived dendritic cells: a peroxisome proliferator-activated receptor-gamma-mediated effect. J Immunol 173: 4066–4074.

35. Chakarova S, Hughes K, Craig A (2008) Host response to cytoadherence in Plasmodium falciparum-infected human red blood cells. J Immunol 180: 1613–1621.

36. Aki K, Min-Oo G, Serghides L, Crockett M, Kirby-Allen M, et al. (2008) Pyruvate kinase deficiency and malaria. N Engl J Med 358: 1085–1010.

37. Aki K, Min-Oo G, Serghides L, Crockett M, Kirby-Allen M, et al. (2008) Pyruvate kinase deficiency and malaria. N Engl J Med 358: 1085–1010.

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Conceived and designed the experiments: VG ES RHS PA KB. Performed the experiments: VG ES RVz. Analyzed the data: VG ES RHS KB. Contributed reagents/materials/analysis tools: ES RHS DRz PA KB. Wrote the paper: VG ES RHS DRz PA KB.
47. Kaikai P, Thurnham D (1983) The influence of riboflavin deficiency on Plasmodium berghei infection in rats. Trans R Soc Trop Med Hyg 77: 680–686.
48. Anderson B, Giuberti M, Perry G, Sahimi G, Casadio I, et al. (1993) Low red blood cell glutathione reductase and pyridoxine phosphate oxidase activities not related to dietary riboflavin: selection by malaria? Am J Clin Nutr 57: 666–672.
49. Anderson B, Scattoni M, Perry G, Galvan P, Giuberti M, et al. (1994) Is the flavin-deficient red blood cell common in Maremma, Italy, an important defense against malaria in this area? Am J Hum Genet 55: 975–980.
50. Anderson B, Corda L, Perry G, Platas D, Giuberti M, et al. (1994) Is the flavin-deficient red blood cell common in Maremma, Italy, an important defense against malaria in this area? Am J Hum Genet 55: 975–980.
51. Siniscalco M, Bernini L, Filippi G, Latte B, Meera-Khan P, et al. (1966) Population genetics of haemoglobin variants, thalassaemia and glucose-6-phosphate dehydrogenase deficiency, with particular reference to the malaria hypothesis. Bull World Health Organ 34: 379–393.
52. El-Hazmi M, Warsy A (1985) Glutathione reductase deficiency in association with sickle cell and thalassaemia genes in Saudi populations. Hum Hered 35: 326–332.
53. Anderson B, Perry G, Clements J, Studds C, Fashola R, et al. (1989) Genetic and other influences on red-cell flavin enzymes, pyridoxine phosphate oxidase and glutathione reductase in families with beta-thalassaemia. Eur J Haematol 42: 334–360.
54. Clements J, Anderson B, Perry G (1981) Low red cell activity of pyridoxine (pyridoxamine) phosphate oxidase and glutathione reductase associated with thalassaemia. Biomedicine 34: 119–123.
55. Varmuza R, Manikad V, Phelps D, Jenkins L, Suskind R (1983) Depressed erythrocyte glutathione reductase activity in sickle cell disease. Am J Clin Nutr 38: 884–887.
56. Zhang YA, Konig I, Schirmer RH (1988) Glutathione reductase-deficient erythrocytes as host cells of malarial parasites. Biochem Pharmacol 37: 861–865.
57. Frischer H, Ahmad T (1977) Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU [1,3-bis(chloroethyl)-1-nitrosourea]. J Lab Clin Med 89: 1000–1001.
58. Trager W, Jensen J (1976) Human malaria parasites in continuous culture. Science 193: 673–675.
59. Lambros C, Vanderberg J (1979) Synchronization of Plasmodium falciparum erythrocytic stages in culture. J Parasitol 65: 418–420.
60. Becker K, Gui M, Traeder A, Kirsten C, Schirmer RH (1994) Redox processes in malaria and other parasitic diseases. Determination of intracellular glutathione. Histochemistry 102: 389–395.
61. Nordhoff A, Bächeler U, Werner D, Schirmer RH (1993) Folding of the four domains and dimerization are impaired by the Gly446->Glu exchange in human glutathione reductase. Implications for the design of antiparasitic drugs. Biochemistry 32: 4060–4066.
62. Desjardins R, Canfield C, Haynes J, Chulay J (1979) Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother 16: 4097–4102.
63. Fivelman Q, Adagu I, Warhurst D (2004) Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of Plasmodium falciparum. Antimicrob Agents Chemother 48: 4097–4102.
64. Steck T, Weinstein R, Straus J, Wallach D (1970) Inside-out red cell membrane vesicles: preparation and purification. Science 160: 253–257.
65. Schwarzer E, Turini F, Arese P (1994) A luminescence method for the quantitative determination of phagocytosis of erythrocytes, of malaria-parasitized erythrocytes and of malarial pigment. Br J Haematol 88: 740–745.
66. Lutz HU (2004) Innate immune and non-immune mediators of erythrocyte clearance. Cell Med Biol 50: 107–116.
67. Skorokhod O, Schwarzer E, Cerotto M, Arese P (2007) Malarial pigment haemozoin, IFN-gamma, TNF-alpha, IL-1beta and LPS do not stimulate expression of inducible nitric oxide synthase and production of nitric oxide in immuno-purified human monocytes. Malar J 6: 73.

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