Accelerated Clearance of *Plasmodium*-infected Erythrocytes in Sickle Cell Trait and Annexin-A7 Deficiency

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Key Words

Annexin-A7 • Ca²⁺-permeability • Calpain • Phagocytosis • Phospholipid asymmetry • *Plasmodium falciparum* • Prostaglandin E₂

Abstract

The course of malaria does not only depend on the virulence of the parasite *Plasmodium* but also on properties of host erythrocytes. Here, we show that infection of erythrocytes from human sickle cell trait (HbA/S) carriers with ring stages of *P. falciparum* led to significantly enhanced PGE₂ formation, Ca²⁺ permeability, annexin-A7 degradation, phosphatidylserine (PS) exposure at the cell surface, and clearance by macrophages. *P. berghei*-infected erythrocytes from annexin-A7-deficient (*annexin-A7*^{-/-}) mice were more rapidly cleared than infected wildtype cells. Accordingly, *P. berghei*-infected *annexin-A7*^{-/-} mice developed less parasitemia than

wildtype mice. The cyclooxygenase inhibitor aspirin decreased erythrocyte PS exposure in infected *annexin-A7*^{-/-} mice and abolished the differences of parasitemia and survival between the genotypes. Conversely, the PGE₂-agonist sulprostone decreased parasitemia and increased survival of wild type mice. In conclusion, PS exposure on erythrocytes results in accelerated clearance of *Plasmodium* ring stage-infected HbA/S or *annexin-A7*^{-/-} erythrocytes and thus confers partial protection against malaria *in vivo*.

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Introduction

Malaria causes 300-500 million clinical cases and results in 1-3 million deaths per year [1]. In its blood cycle, the malaria parasite *P. falciparum* invades host erythrocytes. To survive in the new environment, the

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1015-8987/09/0246-0415\$26.00/0

Accessible online at:
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parasite has to induce new permeability pathways in the host erythrocyte membrane. These new pathways provide the parasites with essential nutrients, dispose of metabolic waste products, modify the electrolyte composition of the host cell, and decrease the colloid osmotic pressure of the erythrocyte. By doing so, the new pathways prevent premature hemolysis of the host cell [2-4]. The infection-stimulated pathways include a Ca^{2+} -permeable cation permeability [5]. Sustained Ca^{2+} entry triggers suicidal death of erythrocytes [6]. We thus investigated whether interaction of *P. falciparum* with human erythrocytes results in premature suicidal death of the host erythrocyte and whether such a premature death plays a role in the course of malaria.

Viable cells retain phosphatidylserine (PS) in the inner leaflet of the cell membrane, while apoptotic cells are unable to maintain the membrane asymmetry, and expose PS at their surface [7]. Phospholipids scrambling of the cell membrane with subsequent PS exposure has been shown to be an “eat me” signal involved in the rapid engulfment of apoptotic nucleated cells by phagocytes [8] which seems to be conserved from worm [9, 10] to man [11, 12]. PS exposure may accelerate the clearance of circulating injured erythrocytes [7, 13-15]. Whether PS exposure, however, is a primary “eat me” signal in the clearance of senescent erythrocyte is still under debate [16]. Established “eat me” signals of senescent erythrocytes include surface deposition of immunoglobulins and complement to modified band 3 and a decline in sialic acid in the glycocalyx [16].

P. falciparum ages the host erythrocyte during its intracellular development, i.e., it induces a dramatic, time-compressed enhancement of normal erythrocyte senescence: infection fosters binding of hemichromes to its specific, high-affinity binding site on the cytoplasmic domain of band 3, clustering of band 3, and deposition of complement C3 fragments and anti band 3 immunoglobulins [17]. In addition, infection alters the amounts of membrane cholesterol and phospholipids, declines the amount of sialic acid at the erythrocyte surface [18]. Moreover, many (but not all [19, 20]) studies have observed break-down of the phospholipid asymmetry in *P. falciparum*-infected human erythrocytes [21-28] and *Plasmodium knowlesi*-infected rhesus monkey erythrocytes [29]. In contrast to senescent erythrocytes, PS exposure has been demonstrated to participate in the removal of parasitized erythrocytes [30, 31].

The alterations of the host erythrocytes and the subsequent clearance by monocytes increase with intraerythrocytic parasite development [31]. Unlike normal

erythrocytes (HbA/A), parasitized erythrocytes with hemoglobinopathies such as sickle cell trait (HbA/S) show features of senescent erythrocytes already at early ring stages of *P. falciparum* [17] which accelerates their clearance from circulating blood. This clearance of early stages is thought to underlie the partial malaria resistance of sickle cell trait carriers [32, 33]. In contrast, erythrocytes parasitized with late *Plasmodium* stages (trophozoite/schizont) partially evade the innate immune system by tissue sequestration (although parasitized erythrocyte sequestered in the deep vasculature are not totally protected from immunity and certainly, most of the merozoites which emerge from schizonts into the circulating blood are phagocytosed or prevented from reinvasion). In addition, trophozoites digest hemoglobin and detoxify the free heme to hemozoin which consists of crystallized Fe (III) protoporphyrin IX dimers. Engulfment of hemozoin by phagocytes reportedly alters the function of macrophages, monocytes and dendritic cells thus unfavorably influencing the clinical course of malaria [34-38]. Moreover, hemozoin-fed monocytes increase matrix metalloproteinase-9 activity fostering extravasation of phagocytic cells and parasitized erythrocytes into brain tissues [39].

To investigate the signalling leading to PS exposure on *P. falciparum*-infected erythrocytes and to test whether PS exposure modulates the infection process and contributes to the partial malaria resistance observed in sickle cell trait, we infected erythrocytes from four European sickle cell trait donors, who have never had malaria infection before, and several age- and sex-matched control individuals with *P. falciparum*. As a result, we identified proteolytic cleavage of annexin-A7 as a key event in *P. falciparum*-infected sickle cell trait erythrocytes. In addition, we assessed the functional significance of annexin-A7 cleavage by using the *P. berghei* model of rodent malaria in annexin-A7-deficient mice.

Materials and Methods

Haematological parameters

Erythrocyte number, packed cell volume (haematocrit), mean corpuscular volume, mean corpuscular haemoglobin, and haemoglobin concentration were determined using a MDM 905 electronic particle counter (Medical Diagnostics Marx, Butzbach, Germany). Reticulocyte count was determined by Reti-COUNT reagent (Becton Dickinson, Erembodegem-Aalst, Belgium) in flow cytometry according to the manufacturer's instructions.

In vivo and in vitro malaria infection

For mouse infection *P. berghei* ANKA-parasitized murine RBCs (2×10^6) as counted by flow cytometry upon staining with the DNA/RNA-specific dye syto 16 were injected intraperitoneally into sex- and age-matched (2–6 months old male and female) wildtype (*annexin-A7^{+/+}*) and annexin-A7 knockout (*annexin-A7^{-/-}*) mice (obtained from the Institute for Biochemistry I, University of Cologne, Germany), and parasitaemia was determined daily by syto 16 staining. For Fig. 5A and for the clearance studies in Fig. 4D–G, asynchronous infected wildtype and *annexin-A7^{-/-}* erythrocytes were injected intravenously (10^8 to 2×10^8 parasitized cells). In some infection experiments aspirin (100 mg / kg body weight / d) was administered permanently by addition to the drinking water starting one day before infection. This aspirin treatment was well tolerated by non-infected *annexin-A7^{+/+}* and *annexin-A7^{-/-}* mice. In further experiments a single dose of the prostaglandin E_2 (PGE₂) agonist sulprostone (2.5 µg / kg body weight) was administered intra-peritoneally on day 22 p.i.. Animal experiments were performed according to the German Animal Protection Law and approved by the local authorities. For *in vitro* infection of human erythrocytes the *P. falciparum* strains BINH and FCR-3 were grown *in vitro* in human HbA/A and HbA/S erythrocytes as described [40]. The sickle cell trait genotype was confirmed by sequencing. Donors gave informed consent, and procedures were performed according to the declaration of Helsinki with local ethical committee approval. Human erythrocytes were stored up to 6 weeks in SAG-mannitol after removal of leukocytes. Parasites were cultured in serum-free Albumax II (0.5 %)-supplemented RPMI 1640 medium as described earlier [40]. Ring stage synchronization of infected human and murine erythrocytes (see Fig. 4A) was accomplished by hemolyzing trophozoite-infected cells in isosmotic sorbitol solution (in mM: 290 sorbitol, 5 glucose, 5 HEPES/NaOH, pH 7.4) for 20 min and 10 min at 21°C, respectively (mouse erythrocytes express an endogenous sorbitol permeability, which hemolyzes also non-infected and ring stage-infected cells when incubated for longer times in isosmotic sorbitol solution [40]). *P. berghei* grows asynchronously in mice. Asynchronous parasites were used in Fig. 1A,B, 2A,D,E,G, 3A, 4C–H, and 5, ring stage-synchronized parasites in the experiments in Fig. 1C, 2B,C,H 3B–D and 4A,B. In all experiments parasitaemias of infected *annexin-A7^{-/-}* and wildtype erythrocytes or those of HbA/A and HbA/S erythrocytes were adjusted to equivalent values.

Annexin-V binding and forward scatter

P. berghei-infected mouse erythrocytes were drawn, ring stage-synchronized and incubated for 0 or 24 h at 37°C in Albumax II-containing RPMI 1640 medium (Fig. 4A,B). The increase in syto 16 fluorescence (see dot blots in Fig. 4A) indicates intraerythrocytic development of the parasites under these conditions. In further experiments (Fig. 4C,H) asynchronous *P. berghei*-infected mouse erythrocytes were grown for 0.5 h and 8 h at 37°C in NaCl Ringer (in mM: 125 NaCl, 5 KCl, 1 MgSO₄, 32 HEPES/NaOH, 5 glucose, 1 CaCl₂; pH 7.4) containing 0 or 0.5 µM of annexin-V (MBI, Nagoya, Japan) and 0 or 10 µM diclofenac, respectively. Post-incubated infected mouse erythrocytes, cultures of *P. falciparum*-infected human HbA/

A and HbA/S erythrocytes, or infected human HbA/A and HbA/S erythrocytes post-incubated for 8 h and 24 h at 37°C in (glucose-containing) NaCl Ringer containing 0 or 10 µM diclofenac and 0 or 25 µM calpain inhibitor I (Calbiochem, Darmstadt, Germany) and calpain inhibitor II (Sigma, Taufkirchen, Germany), respectively, were washed in NaCl Ringer, loaded (for 20 min at 21°C) in annexin binding buffer (NaCl Ringer solution additionally containing 4 mM of CaCl₂ to promote annexin-V binding as a measure of PS exposure) with fluorescence-labelled annexin-V-568 (1:50 dilution; Roche Diagnostics, Mannheim, Germany) or annexin-V-APC (1:20 dilution; BD Biosciences, Heidelberg, Germany) and with the DNA/RNA-specific dye syto 16 (30 nM; Molecular Probes, Göttingen, Germany), and analyzed by flow cytometry (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). The syto 16- and annexin-V-568-specific fluorescence were analyzed in fluorescence channels FL-1 (530 nm emission) and FL-2 (585 nm emission) at 488 nm excitation, respectively. Annexin-V-APC-specific fluorescence was analyzed in fluorescence channel FL-4 (635 nm excitation and 661 nm emission). In addition, forward scatter was used as a measure of cell size. To assess PS exposure of non-infected human erythrocytes, cells were stained with annexin-V-Fluos (1:20 dilution; Roche Diagnostics) and analyzed in FL-1. In dot blots non-infected cells, erythrocytes infected with early stages (ring stages) and cells infected with late stages (trophozoites, schizonts) were differentiated by their low (background), intermediate, and high syto 16 fluorescence, respectively (see Fig. 1A and 4A). In our experiments, the percentage of annexin-V binding (i.e., PS-exposing cells) in non-infected erythrocytes ranged between almost 0 and 20 % (compare Figs 1B, 2E, 2H, 4B, and 4C). The malaria infection, reportedly, damages also co-cultured uninfected bystander erythrocytes probably by parasite-derived oxidative stress. Alterations of bystander cells comprise accelerated aging [31] and PS exposure (own unpublished observations). The degree of these alterations depends highly on parasite stage (synchronized rings versus unsynchronized cultures), parasitemia, incubation time, and incubation medium (complete medium versus NaCl Ringer) which differed between the experiments (see Figure Legends and preceding paragraph).

In vivo clearance and infectivity of PS-exposing erythrocytes

Erythrocytes from non-infected wild type mice were washed (NaCl-Ringer), left untreated or were treated with ionomycin (10 µM in NaCl Ringer for 1 h) to induce PS exposure, washed in NaCl Ringer, subsequently coated for 20 min at 37°C with annexin-V (0 and 0.5 µM in annexin binding buffer, Nagoya, Japan), centrifuged, and the pellet was labelled with 5-carboxyfluoresceine diacetate succinimidylester (5 µM; CFSE; Molecular Probes, Eugene, Oregon, USA). After washing twice and re-suspending in NaCl Ringer (50 % hematocrit), cell suspensions were re-injected intravenously into wild type mice, and clearance of CFSE-positive cells from peripheral blood and sequestration of these cells in the spleen (upon extraction by gently crushing the spleen in ice cold PBS and straining the suspension through a 40 µM nylon cell strainer; Becton Dickinson; Heidelberg, Germany) were assessed by flow

cytometry. Erythrocytes from *P. berghei*-infected wild type or annexin-A7^{-/-} mice (~50 % parasitemia) were washed (NaCl Ringer), subsequently left uncoated or were coated with annexin-V (MBI, Nagoya, Japan 0.5 µM in annexin binding buffer, 10 % hematocrit) for 30 min at 37°C, centrifuged. For infectivity experiments cells were directly washed twice, re-suspended in NaCl Ringer (50 % hematocrit) and then re-injected intravenously into wild type mice. For clearance experiments, the pellet was labelled for 20 min at 37°C with syto 16 (1 µM in annexin binding buffer, 5 % hematocrit; Molecular Probes, Göttingen, Germany), washed, and controlled for syto 16 labelling by flow cytometry prior to injection. The clearance was defined by the decrease of syto 16 positive cells from peripheral blood. Ring stage-parasitized cells were defined by their intermediate syto 16 fluorescence staining. Sequestration of these cells in the spleen (upon extraction) were assessed by flow cytometry. The infectivity was defined by the parasitaemia determined 24 h after injection by syto 16 staining.

Isolation of monocytes

Peripheral blood mononuclear cells were isolated from heparinized venous blood of healthy adult donors by Ficoll-Paque plus density gradient centrifugation (Amersham Biosciences, Freiburg, Germany). Monocytes were positively isolated using CD14 Microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Monocytes (1 x 10⁵) were resuspended in RPMI 1640 (Gibco, Karlsruhe, Germany) supplemented with penicillin G (200 U/ml), gentamicin (20 ng/ml), L-glutamine (0.3 mg/ml) and 10 % fetal calf serum (FCS).

Erythrophagocytosis assay

The parasitaemia of ring stage-synchronized cultures of *P. falciparum*-infected human HbA/A and HbA/S erythrocytes was adjusted to 5 % parasitaemia, and cells were incubated in the presence or absence of annexin-V (0.5 µM for 30 min), washed and labelled with syto 16 (1 µM). In further experiments non-infected human erythrocytes were treated with ionomycin (0 or 1 µM), incubated with annexin-V (0 or 0.5 µM) and stained with CFSE (1 µM). The labelled erythrocytes were washed in 10 % FCS-containing RPMI 1640 medium and co-incubated with monocytes (100 : 1 RBC to monocyte ratio) for 5 h at 37°C and 5 % CO₂. Non-internalized erythrocytes were lysed osmotically in ice-cold 20 % phosphate buffered saline (PBS) for 1 min. After resuspending and rinsing the monocytes in PBS, the erythrocyte ingestion by monocytes was determined by flow cytometry. Syto 16 positive monocytes and extra-erythrocytic parasite-containing vacuoles (which were also pelleted together with the monocytes) were distinguished by forward scatter.

⁴⁵Ca²⁺ flux

For determination of ⁴⁵Ca²⁺ entry into non-infected or *P. falciparum*- or *P. berghei*-infected ring stage-synchronized human erythrocytes (~10 % parasitemia), cells were suspended in EGTA/KCl/NaCl solution containing (in mM): 80 KCl, 70 NaCl, 10 HEPES, 5 glucose, 0.2 MgCl₂, 0.1 EGTA, pH 7.4. High K⁺ concentrations were used to counteract infection-induced shrinkage of HbA/S and annexin-A7^{-/-} erythrocytes.

Cells were washed in KCl/NaCl solution containing (in mM): 80 KCl, 70 NaCl, 10 HEPES, 5 glucose, 0.2 MgCl₂, pH 7.4, and then pre-incubated for 10 min at 37°C in KCl/NaCl solution supplemented with 10 mM inosine (to replenish the erythrocytic nucleoside pool which diminishes by ATP release) and 1 mM Na⁺-orthovanadate (to inhibit the Ca²⁺-ATPase). Then, cells were incubated in the same solution (final hematocrit ~ 5-10 %) supplemented with the radioactive tracer (~1 µCi / ml ⁴⁵Ca²⁺) and 0.2 mM CaCl₂. After the indicated time points, 100 µl aliquots were delivered into KCl/NaCl solution and centrifuged at 4000 g for 10 s. The cells were washed a second time using the same solution, lysed and deproteinized by addition of trichloroacetic acid (6 %) and finally centrifuged. The radioactivity of the supernatant was measured using a β-scintillation counter (Wallac 1406, Freiburg, Germany). Since uptake of non-infected cells was negligible uptake of the infected samples was normalized to 100 % parasitaemia. For non-infected controls mock-cultured and freshly drawn erythrocytes were used yielding identical results.

Prostaglandin E₂ (PGE₂) measurements

Several stress stimuli induce erythrocyte PGE₂ formation [6, 42]. For determination of erythrocyte PGE₂ formation, non-infected and ring stage-synchronized infected human and non-synchronized or ring stage-synchronized infected mouse erythrocytes were washed and adjusted to a haematocrit of 20 %. After incubation for 24 h at 37°C in NaCl Ringer, cells were pelleted and the supernatant was stored at -80°C. PGE₂ concentrations in the supernatant were determined using the Correlate-EIATM Prostaglandin E₂ Enzyme Immunoassay Kit (Assay Designs, Inc., Ann Arbor, MI, USA) and normalized to 100 % parasitaemia. In some experiments, the 24 h-incubation was performed in the presence of the PLA₂ inhibitors palmitoyl trifluoromethyl ketone (PACOCF₃; 4 µM), arachidonyl trifluoromethyl ketone (AACOCF₃; 8 µM) or bromoenol lactone (Br-enol-L; 8 µM) or in the presence of arachidonic acid (AA, 1 µM).

Human erythrocyte membrane preparation and Western blot

Non-infected human erythrocytes were washed in NaCl Ringer and centrifuged at 2000 rpm for 5 min. 200 µl of the pellet was haemolysed by hypotonic shock in 20 mM HEPES (pH 7.4) containing a cocktail of protease inhibitors (Roche Diagnostics). Ghost membranes were pelleted (15,000 g for 20 min at 4°C) and lysed in lysis buffer containing (in mM): 125 NaCl, 25 HEPES/NaOH (pH 7.3), 10 EDTA, 10 Na-pyrophosphate, 10 NaF and 0.1 % SDS, 0.5 % deoxycholic acid, 1 % triton-X, 0.4 % β-mercaptoethanol.

P. falciparum-infected erythrocytes (2 % hematocrit, 30 % parasitemia) were washed in NaCl Ringer solution and then lysed for 20 min in an isosmotic sorbitol solution (290 mM sorbitol, 10 mM HEPES, pH 7.4) containing a cocktail of protease inhibitors. Non-hemolysed cells were spun down (10 min, 2000 rpm). Ghost membranes in the supernatant were pelleted (15,000 g for 20 min at 4°C) and lysed as described above.

Lysates were separated by 12 % SDS-PAGE for annexin-A7 and 10 % for calpain (50 µg protein per lane) and transferred

electrophoretically from gel to Protan BA83 nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Protein transfer was controlled by Ponceau red staining. After blocking the non-specific sites with 5 % nonfat milk, the blots were probed overnight at 4°C, either with a monoclonal anti-human annexin-A7 antibody [43, 44] or with a polyclonal rabbit anti-human μ -calpain (domain IV) antibody (affinity isolated antibody C5611 from Sigma, Taufkirchen, Germany) diluted at 1:500. After washing, the blots were incubated for 1 h at 21°C either with a secondary sheep anti-mouse antibody (GE Healthcare, Chalfont Buckinghamshire, UK) or with a secondary goat anti-rabbit antibody (Cell Signaling Technology, Danvers, USA) conjugated with horseradish peroxidase at a 1:1000 dilution. Antibody binding was detected with the enhanced chemoluminescence (ECL) kit from Amersham.

Statistical analysis

Data are presented as arithmetic means $\pm$ SEM; statistical analysis was made by two-tailed unpaired t-test or ANOVA test, where appropriate. $p \leq 0.05$ was considered statistically significant.

Results

PS-exposing erythrocytes are rapidly cleared

Blood parameters did not differ between the sickle cell trait carriers (HbA/S) and the healthy control persons (HbA/A) apart from higher reticulocyte counts in sickle cell trait carriers (2.4 ± 0.3 vs. 1.5 ± 0.2 %). Moreover, *in vitro* growth of *P. falciparum* was not significantly different in HbA/S and HbA/A erythrocytes. *P. falciparum* grew within 48 h from 1 % parasitemia to 7.4 ± 0.4 % in HbA/A cells and to 6.7 ± 0.4 % in HbA/S cells (means $\pm$ SEM; $n = 30 - 32$). Staining of the DNA with syto 16 in flow cytometry was used to detect early stage-parasitized, late stage-parasitized erythrocytes and non-infected bystander cells in asynchronous parasite cultures. Double staining with fluorescent annexin-V was applied to assess the phospholipid asymmetry of the erythrocyte membrane in a parasite stage-dependent manner. Infection with early (ring)- and late (trophozoites/schizonts)-stages of *P. falciparum* resulted in PS exposure, which was significantly higher ($p \leq 0.05$) in HbA/S cells than in HbA/A cells (Fig. 1A,B).

To test for PS-mediated clearance of ring stage-parasitized human HbA/S cells were coincubated with human monocytes. In some samples the PS signal of the ring stage-parasitized cells was masked by coating the erythrocyte surface with the PS-binding protein annexin-V. As a result, ring stage-parasitized human HbA/S cells were more rapidly phagocytosed by human monocytes

Fig. 1. Phosphatidylserine (PS) exposure and *in vitro* phagocytosis of *P. falciparum*-infected human HbA/A and HbA/S erythrocytes. A,B. Co-cultured non-infected, and asynchronous infected HbA/A and HbA/S cells were retrieved from the parasite culture and double-stained with fluorescent annexin-V and the DNA/RNA-specific dye syto 16. PS exposure as measured by fluorescent annexin-V-binding in flow cytometry (dot plot in A and means $\pm$ SEM; $n = 25$ in B) was analyzed differentially for non-infected human erythrocytes, early (i.e., ring) and late (i.e., trophozoites and schizonts) stage-infected cells as defined by their background, intermediate and high syto 16 fluorescence staining, respectively (mean parasitemia was adjusted to the same values in the HbA/A and HbA/S cultures). C. *P. falciparum*-infected HbA/A (open bars) or HbA/S cells (closed bars) were ring stage-synchronized, left uncoated (1st and 2nd bar) or coated with annexin-V (0.5 μ M for 30 min; 3rd and 4th bar), stained with syto 16 and co-incubated (5 h) with human monocytes. Shown is the mean percentage ($\pm$ SEM; $n = 10 - 39$) of syto 16 positive monocytes. *, **, and *** indicate significant ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively) difference, ANOVA.

than ring stage parasitized HbA/A cells (Fig. 1C). Moreover, neutralisation of PS on ring stage-infected HbA/S erythrocytes with annexin-V inhibited internalisation by monocytes (Fig. 1C). Non-infected HbA/S and HbA/A erythrocytes displayed almost no PS on the surface (Fig. 1A,B). These observations provide evidence that erythrocytes from sickle cell trait carriers expose higher amounts of PS upon *in vitro* infection with

Fig. 2. *P. falciparum*-infected human HbA/S erythrocytes exhibited a higher prostaglandin E₂ (PGE₂) formation, larger Ca²⁺-permeability and lower cell volume than infected HbA/A cells that leads to calpain activation and degradation of annexin-A7. A. Mean PGE₂ release (± SEM; n = 11 - 18) from non-infected (left) and infected (right) HbA/A (open bars) and HbA/S (closed bars) erythrocytes. Non-infected erythrocytes and erythrocytes from asynchronous parasite cultures were incubated for 24 h in NaCl Ringer solution containing 5 mM D-glucose and the PGE₂ release into the medium was determined by immunoassay. PGE₂ values of the infected cells were normalized to 100 % parasitaemia. B. Time-dependent uptake (means ± SEM) of ⁴⁵Ca²⁺ by non-infected (circles) and ring stage-synchronized *P. falciparum*-infected (triangles) HbA/A (open symbols) and HbA/S erythrocytes (closed symbols). Depicted are individual blood samples determined in quadruplicate. C. Mean ⁴⁵Ca²⁺ uptake (30 min values; box plots, n = 12) of ring stage-infected erythrocytes HbA/A (open box) and HbA/S erythrocytes (closed box). D. Co-cultured non-infected and infected HbA/A (open bars) and HbA/S (closed bars) cells were retrieved from the asynchronous parasite culture stained with DNA/RNA-specific dye syto 16 and analyzed by forward scatter in flow cytometry as a measure of cell volume. Depicted is the mean forward scatter (± SEM; n = 25) of non-infected, early stage-infected and late stage-infected HbA/A and HbA/S erythrocytes as defined by the syto 16 fluorescence. E. The cyclooxygenase inhibitor diclofenac decreased the PS exposure of early stage-infected HbA/S erythrocytes. Shown is the mean percentage (± SEM, n = 11) of PS-exposing cells retrieved from asynchronous parasite cultures and post-incubated for 8 h in (glucose-containing) NaCl Ringer in the absence (- diclofenac; black bars) or presence (+ diclofenac; grey bars) of diclofenac (10 µM). PS exposure was analyzed differentially for non-infected, early stage-infected and late stage-infected HbA/S erythrocytes by fluorescent annexin-V and syto 16 double staining. F,G. Immunoblots showing µ-calpain-specific (left) and annexin-A7 (AnxA7) (right)-specific immunoreactive proteins of non-infected (F) and *P. falciparum*-infected (G) HbA/A (1st lane) and HbA/S (2nd lane) erythrocytes. Infection stimulates (G, left blot, 2nd lane) the proteolysis of the latent calpain form p80 into its active form p78 and p76 and the degradation of annexin-A7 (AnxA7) only in HbA/S cells (G, right blot, 2nd lane). The numbers indicate the molecular mass (in kDa). In the calpain blot of non-infected cells (F, left blot), an additional very weak band of about 70 kDa appeared. The nature of this band is not defined. H. Inhibition of µ-calpain decreased the PS exposure (recorded and analyzed as in E) of infected HbA/S erythrocytes. Shown is the mean percentage (± SEM, n = 6) of PS-exposing cells retrieved from synchronized parasite cultures and post-incubated for 24 h in (glucose-containing) NaCl Ringer in the absence (- calpain inhibitor; black bars) or presence (+ calpain inhibitor; grey bars) of calpain inhibitors I and II (25 µM, each). ** indicates significant (p≤0.01) difference as assessed by two-tailed t-test in C or by ANOVA in A, D, E and H.

P. falciparum than erythrocytes from individuals carrying HbA/A. The data further demonstrate that PS exposure facilitates removal of *P. falciparum*-infected human erythrocytes.

P. falciparum induces a high Ca²⁺ permeability in HbA/S erythrocytes

To address mechanisms that mediate PS exposure on erythrocytes after infection with *P. falciparum* we

Fig. 3. *P. berghei* infection induced higher PGE₂ formation, larger Ca²⁺-permeability and lower cell volume in *annexin-A7*^{-/-} than in wild type erythrocytes. A. Mean PGE₂ formation (± SEM; n = 10 - 12) by erythrocytes drawn from non-infected (left) and *P. berghei*-infected (right) wild type (open bars) and *annexin-A7*^{-/-} (*AnxA7*^{-/-}) (closed bars) mice. Non-infected and non-synchronized infected erythrocytes were incubated (24 h) in Ringer and the PGE₂ release into the medium was determined. For infected mouse samples PGE₂ values were normalized to 100% parasitaemia. B. Inhibition of phospholipase A₂ (PLA₂) or supplementation with the PLA₂ product arachidonic acid (AA) did not blunt the difference in infection-stimulated PGE₂ formation between erythrocytes from wild type (open bars) and *annexin-A7*^{-/-} (closed bars) mice. Shown is the mean PGE₂ production (± SEM; n = 3) by ring stage-synchronized infected wild type and *annexin-A7*^{-/-} erythrocytes incubated for 24 h in the absence or presence of the PLA₂ inhibitors palmitoyl trifluoromethyl ketone (PACOCF₃; 4 μM), arachidonyl trifluoromethyl ketone (AACOCF₃; 8 μM) and bromoenol lactone (Br-enol-L; 8 μM) or of AA (1 μM). ** and *** indicate significant (p ≤ 0.01 and p ≤ 0.001, respectively) difference, ANOVA. In the inset, the data on PGE₂ formation of infected wild type (open bars) and *annexin-A7*^{-/-} erythrocytes (closed bars) are normalized to their respective controls in order to point out the inhibitory and amplifying effect of PLA₂ inhibition and substrate addition, respectively. *, ** and *** indicate significant (p ≤ 0.05, p ≤ 0.01 and p ≤ 0.001, respectively) difference from 1.0 (i.e., from the control), one-sample two-tailed t-test. C. Mean time-dependent uptake of ⁴⁵Ca²⁺ (± SEM, n = 3 - 5) by non-infected (circles) and ring stage-synchronized infected erythrocytes (triangles) from wild type (open symbols) and *annexin-A7*^{-/-} mice (closed symbols). D. Mean forward scatter (± SEM, n = 25) as a measure of cell volume of non-infected (i.e., syto 16 background staining), early stage-infected (intermediate syto 16-staining) and late-stage-infected erythrocytes from *annexin-A7*^{-/-} (closed bars) and wild type (open bars) mice incubated for 24 h in NaCl Ringer after blood withdrawal. * and ** indicate significant (p ≤ 0.05 and p ≤ 0.01, respectively) difference, ANOVA.

determined the release of PGE₂ and the influx of Ca²⁺, events that have previously been shown to be critically involved in PS exposure on erythrocytes upon application of stress [6, 42]. In particular, PGE₂ activates a nonselective cation channel in dying erythrocytes followed by increase of erythrocyte Ca²⁺ concentration, Ca²⁺-stimulated erythrocyte shrinkage and phospholipid scrambling with PS exposure [42].

P. falciparum infection increased PGE₂ formation (Fig. 2A), an effect significantly (p ≤ 0.01) stronger in HbA/S than in HbA/A cells (Fig. 2A). The enhanced PGE₂ formation in HbA/S cells was paralleled by enhanced ⁴⁵Ca²⁺ fluxes, which were larger in ring stage-parasitized HbA/S cells than in noninfected HbA/S cells or ring stage-infected HbA/A erythrocytes (Fig. 2B,C). In accordance with an elevated Ca²⁺ permeability, the cell size was significantly smaller in ring stage-parasitized

HbA/S cells than in ring stage-parasitized HbA/A cells (Fig. 2D). The cyclooxygenase inhibitor diclofenac decreased PS exposure of ring stage-infected HbA/S erythrocytes (Fig. 2E) indicating that the pronounced PS exposure of ring stage-infected HbA/S erythrocytes resulted from elevated PGE₂-stimulated Ca²⁺ permeability. Increased cytosolic free Ca²⁺ concentrations in infected HbA/S cells might result in activation of μ-calpain, a neutral endopeptidase in human erythrocytes. Calpain digests cytoskeleton elements such as annexins [45]. Therefore, we tested by immunoblotting for calpain activation and subsequent degradation of annexin-A7, the annexin isoform expressed in human erythrocytes [43, 44]. The immunoblots probed against μ-calpain (Fig. 2F, G, left) showed in infected HbA/A cells or in non-infected HbA/A and HbA/S cells only the inactive p80 protein (in the non-infected cells also a very faint yet undefined band

at 70 kDa was apparent, see legend to Fig. 2F). In infected HbA/S (Fig. 2G, left), in contrast, the protein band smeared towards lower molecular weights suggestive of the additional appearance of the active p78 and p76 calpain forms. This proposed μ -calpain activation

of infected HbA/S cells was paralleled by degradation of annexin-A7 (Fig. 2G, right), while annexin-A7 remained intact in infected HbA/A or in non-infected HbA/A and HbA/S cells (Fig. 2F, G, right). To test whether the proposed μ -calpain activation might be linked to the

elevated PS exposure of ring stage-infected HbA/S erythrocytes, non-infected and ring stage-synchronized HbA/S cells were incubated for 24 h in the presence or absence of calpain inhibitors I and II (25 μ M each) and PS exposure was analyzed. As shown in Fig. 2H, calpain inhibitors almost abolished the infection-stimulated PS exposure further hinting to a role of μ -calpain in infection-stimulated PS exposure and removal of HbA/S cells.

Annexin A7-deficient murine erythrocytes mimic the phenotype of human HbA/S cells

To define a putative role of annexin-A7 degradation for PS exposure and clearance of *Plasmodium*-infected erythrocytes we employed erythrocytes from mice lacking annexin-A7 (*annexin-A7*^{-/-}) [44]. Erythrocyte number ($8.93 \pm 0.30 \cdot 10^6/\mu$ l) and hematocrit (42.3 ± 1.2 %) were slightly but significantly ($p \leq 0.05$, two-tailed t-test) lower in *annexin-A7*^{-/-} mice ($n = 14$) than in wild type mice ($9.92 \pm 0.19 \cdot 10^6/\mu$ l; 46.4 ± 1.1 %; $n = 12$). In contrast, the percentage of reticulocytes was significantly ($p \leq 0.05$, two-tailed t-test) larger in *annexin-A7*^{-/-} mice (6.2 ± 0.6 %) than in wildtype mice (4.6 ± 0.3 %), pointing to a decreased lifespan and an enhanced turnover of *annexin-A7*^{-/-} erythrocytes *in vivo*.

Next, we infected *annexin-A7*^{-/-} and wild type mice with *P. berghei* and determined PGE₂ formation, ⁴⁵Ca²⁺ uptake, cell volume, and PS exposure. Similar to the findings in HbA/S erythrocytes, *P. berghei* infected *annexin-A7*^{-/-} erythrocytes formed significantly more PGE₂ than infected wild type erythrocytes (Fig. 3A). Inhibition of arachidonic acid formation by three different PLA₂ inhibitors (palmitoyl trifluoromethyl ketone, arachidonyl trifluoromethyl ketone and bromoenol lactone) decreased PGE₂ formation while arachidonic acid addition increased PGE₂ formation (Fig. 3B, inset) without abrogating the difference between genotypes (Fig. 3B). The observation suggests that annexin-A7 interferes with PGE₂ formation downstream of arachidonic acid. ⁴⁵Ca²⁺ uptake into *P. berghei* ring stage-infected mouse erythrocytes of *annexin-A7*^{-/-} mice was increased compared to non-infected cells (Fig. 3C) or ring-parasitized wildtype erythrocytes (Fig. 3C). Accordingly, cell size was significantly smaller in ring stage-infected *annexin-A7*^{-/-} erythrocytes as compared to wildtype erythrocytes (Fig. 3D). Most importantly, PS exposure in ring stage-parasitized *annexin-A7*^{-/-} cells was significantly stronger than in non-infected *annexin-A7*^{-/-} cells, late-stage-infected *annexin-A7*^{-/-} cells or ring-infected wildtype erythrocytes (Fig. 4A,B). Similar to the PS exposure on ring stage-parasitized HbA/S erythrocytes, diclofenac

prevented PS exposure in ring stage-infected *annexin-A7*^{-/-} erythrocytes (Fig. 4C) providing a functional link between infection-induced PGE₂ formation and PS exposure also for the *annexin-A7*^{-/-} mouse model.

PGE₂-induced PS exposure contributes to the partial resistance of annexin-A7^{-/-} mice against P. berghei ANKA malaria

Next, we addressed the biological significance of an increased exposure of PS after *Plasmodium* infection. Ring-parasitized *annexin-A7*^{-/-} erythrocytes that were injected into wildtype mice, were more rapidly cleared *in vivo* from the peripheral blood (Fig. 4D-F, 1st and 2nd column) and sequestered in the spleen (Fig. 4G) than ring-parasitized wildtype cells, very similar to the faster *in vitro* phagocytosis of ring-parasitized HbA/S cells. Masking PS at the surface of ring-parasitized erythrocytes with annexin-V protein delayed the *in vivo* clearance and abolished the difference between *annexin-A7*^{-/-} and wildtype erythrocytes (Fig. 4F,G, 3rd and 4th bar) demonstrating that an increased PS exposure facilitates uptake and removal of *P. berghei*-infected erythrocytes. Control experiments confirmed that preincubation of ring stage-infected erythrocytes with unlabelled annexin-V protein prior to incubation with fluorescent annexin-V decreased the number of unmasked (i.e. fluorescence-binding) PS molecules of *annexin-A7*^{-/-} erythrocytes to the value of wildtype cells (Fig. 4H).

If the rapid clearance of ring stage-infected *annexin-A7*^{-/-} erythrocytes via PS exposure is involved in host-parasite interactions, the levels of PS exposure after infection may affect the development of parasitaemia. To address this issue, we first infected wildtype mice i.v. with identical numbers of annexin-V-coated and uncoated *Plasmodium berghei*-infected cells retrieved from parasitized *annexin-A7*^{-/-} or wildtype mice and determined the initial parasitaemia 24 h thereafter. As shown in Fig. 5A, the injection of parasitized *annexin-A7*^{-/-} erythrocytes resulted in lower initial infection rates than infection of parasitized wildtype cells. Masking of PS by annexin-V on infected, parasitized erythrocytes prior to injection abolished this difference suggesting that PS exposure is critical for the attenuated parasitemia following injection of infected *annexin-A7*^{-/-} erythrocytes. To further define the functional significance of the accelerated clearance of ring parasitized *annexin-A7*^{-/-} erythrocytes for the course of malaria we infected *annexin-A7*^{-/-} and wildtype mice i.p. with *P. berghei* and determined parasitaemia and mouse survival. The results disclose a partial malaria resistance of *annexin-A7*^{-/-} mice.

Fig. 5. *Annexin-A7* deficiency caused a mild course of malaria in mice infected with the lethal *P. berghei* ANKA strain. A. Mean normalized infectivity ($\pm$ SEM; $n = 6$) of freshly drawn *P. berghei*-infected *annexin-A7*^{-/-} (*Anx*A7^{-/-}) (closed bars) and wild type (open bars) erythrocytes not coated (1st and 2nd bar) or coated with annexin-V (0.5 μ M, 30 min, 3rd and 4th bar) prior to i.v. injection into wild type mice. The infectivity was defined by the parasitaemia of the mice 24 h after injection and normalized for each experimental trial to the parasitaemia of those mice infected with uncoated parasitized wild type cells. B,C. Time dependence of mean parasitaemia ($\pm$ SEM) in wild type (open circles) and *annexin-A7*^{-/-} (closed triangles) mice infected intraperitoneally at time 0 d with 2×10^6 *P. berghei*-infected wild type erythrocytes. The mice were either kept without ($n = 13 - 17$, B) or with oral administration of aspirin (100 mg/kg body weight d; $n = 3 - 5$, C). D,E. Kaplan-Meier cumulative survival plots of the *P. berghei*-infected wild type and *annexin-A7*^{-/-} mice shown in (B,C). Survival was either studied under control conditions (D) or during permanent treatment with aspirin (E). F. Increase in parasitemia (means $\pm$ SEM; $n = 6 - 23$) of *annexin-A7*^{-/-} (closed bars) and wild type (open bars) mice in the absence or presence of aspirin treatment as calculated from (B, C) by linear regression between day 8 and day 20 p.i.. G. Increase in parasitemia (means $\pm$ SEM; $n = 10-13$) as calculated between day 22 and day 24 p.i. in untreated wild type mice (open bar) or in wild type mice receiving a single dose of sulprostone (2.5 μ g/kg body weight, i.p.; closed bar) at day 22 p.i.. * in (A) indicates significant ($p \leq 0.05$) difference from 1.0, one-sample two-tailed t-test; *, and *** indicate significant ($p \leq 0.05$ and $p \leq 0.001$, respectively) difference, ANOVA, in (F) and (G).

Parasitaemia rapidly developed in infected wildtype mice, but was significantly delayed in *annexin-A7*^{-/-} mice (Fig. 5B,F). The blunted increase in parasitaemia was paralleled by enhanced survival of *annexin-A7*^{-/-} mice after the third week of infection (Fig. 5D). In contrast, the early death within the first two weeks of infection did not differ between both genotypes (Fig. 5B,D). This early death was associated with severe neurological symptoms, which were unrelated to parasitaemia (data not shown) suggesting that these mice died from cerebral malaria [46].

To further support the role of PS exposure for

the host defence, we prevented PGE₂ formation by inhibition of cyclooxygenase with aspirin, which should reverse the protection of *annexin-A7*^{-/-} mice. Treatment of the animals with aspirin during infection restored the sensitivity of the *annexin-A7*^{-/-} mice to *P. berghei* infection and completely abrogated the differences in parasitaemia (Fig. 5C,F) and death rate between *annexin-A7*^{-/-} and wildtype mice, (Fig. 5E). *Vice versa*, application of the PGE₂ agonist sulprostone significantly blunted the increase in parasitaemia in wildtype mice (Fig. 5G), thereby providing further experimental evidence for the proposed clearance mechanism.

Discussion

The present data disclose similar properties of malaria-infected *annexin-A7*^{-/-} and HbA/S erythrocytes suggesting that degradation of annexin-A7 might be involved in the observed phenotype of infected HbA/S cells. Moreover, the data indicate that infection of erythrocytes with *P. falciparum* and *P. berghei* results in a PGE₂-mediated stimulation of a Ca²⁺ permeability resulting in exposure of PS on the surface of HbA/S and *annexin-A7*^{-/-} erythrocytes at an early stage of infection, respectively.

PS fosters internalization of these erythrocytes by macrophages *in vitro* and *in vivo*, an event that is prevented by neutralisation of surface PS on infected erythrocytes by coating the membrane with annexin-V protein. The accelerated clearance of PS-exposing infected *annexin-A7*^{-/-} erythrocytes confers relative resistance of *annexin-A7*^{-/-} mice to *P. berghei* infections. Accordingly, rapid removal of PS-exposing HbA/S erythrocytes by phagocytosis may similarly confer partial malaria resistance of sickle cell trait carriers. This is in contrast to normal (HbA/A) erythrocytes, where PS exposure-mediated removal of infected cells is only apparent at late stages of infection [31].

In addition to increased clearance of ring-parasitized cells, trophozoite-infected HbA/S erythrocytes display an altered expression of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), the parasite's major cytoadherence ligand and virulence factor expressed on knob-like protrusions at the surface of the host erythrocyte. The altered PfEMP-1 expression of trophozoite-parasitized HbA/S erythrocytes, in turn, results on the one hand in a decreased cytoadherence of the infected erythrocytes to monocytes via CD36, but on the other hand also in a decreased adherence to vascular endothelium including cerebral microvessels (via CD36 or ICAM-1) [47]. Thus, PS exposure-triggered accelerated clearance of early stage-infected erythrocytes in concert with reduced microvascular sequestration and blunted inflammatory response may contribute to the partial malaria resistance of sickle cell trait carriers.

⁴⁵Ca²⁺ uptake measurements pointed to elevated Ca²⁺ permeability specifically in ring stage-infected human HbA/S erythrocytes and in murine *annexin-A7*^{-/-} erythrocytes. The elevated Ca²⁺ permeability was paralleled by lower cell volume (which is most probably due to the activation of the Gardos K⁺ channel and efflux of KCl), increased PS exposure, and accelerated phagocytosis of the infected HbA/S erythrocytes.

The present observations reveal that enhanced PS exposure in HbA/S erythrocytes or *annexin-A7*^{-/-} erythrocytes resulted from elevated PGE₂ formation in those cells. Accordingly, the cyclooxygenase inhibitor diclofenac blunted the differences in PS exposure between ring stage-infected HbA/S or *annexin-A7*^{-/-} erythrocytes and infected control erythrocytes *in vitro*. Moreover, the cyclooxygenase inhibitor aspirin administered to malaria-infected mice abolished the benefit resulting from annexin-A7 deficiency *in vivo*. Finally, the PGE₂ receptor agonist sulprostone applied to infected wildtype mice mimicked annexin-A7 deficiency indicating that an increased PGE₂ formation of ring stage-infected HbA/S and *annexin-A7*^{-/-} erythrocytes underlies the increased PS exposure of these cells upon infection.

Deficiency in *annexin-A7* or presence of the HbS allele alone did not result in PGE₂ formation in non-infected cells. However, infection-induced PGE₂ formation was significantly higher in *annexin-A7*^{-/-} erythrocytes than in wildtype erythrocytes. This suggests that *annexin-A7* negatively regulates PGE₂ formation. Along those lines annexin proteins [48-50] have been demonstrated to associate with and inhibit PLA₂ [51, 52]. The present study suggests that removal/decrease of the inhibiting signal by annexin-A7 knock-out alone is not sufficient to stimulate PGE₂ formation. It is thus tempting to speculate that the Ca²⁺-stimulated and calpain-mediated annexin-A7 degradation acts as a positive feed back by amplifying the signal through further formation of PGE₂. The present experimental data also provide a mechanistic explanation for the clinical observation that children with enhanced PGE₂ plasma concentrations have a less severe clinical course of malaria [53]. PGE₂ formation in these patients may promote PS exposure and, thus, accelerate clearance of the parasite-harboring erythrocytes.

A wide variety of stimulators of eryptosis have been identified [54-76]. To the extent that those stimulators accelerate the suicidal death of infected erythrocytes, they may counteract the development of parasitemia and thus favourably influence the course of malaria.

In conclusion, our observations demonstrate that infection of human HbA/S erythrocytes with ring stages of the malaria parasite *P. falciparum* results in PGE₂ formation, Ca²⁺-entry, calpain activation, annexin-A7 degradation and PS exposure. Enhanced PS exposure in turn fosters phagocytosis of infected HbA/S erythrocytes by macrophages. The *annexin-A7*-deficient mouse model indicates that lack of annexin-A7 accelerates the clearance of infected cells from peripheral blood and

decreases the infectivity, delays the evolution of parasitaemia and confers partial protection against a lethal course of *P. berghei* malaria in a PGE₂- and PS exposure-dependent manner. Therefore, PGE₂- and PS exposure-stimulated clearance of infected HbA/S erythrocytes may contribute to the partial protection of sickle trait carriers from severe malaria.

Acknowledgements

We thank Hagai Ginsburg for helpful discussion and suggestions. This study was supported by the Deutsche Forschungsgemeinschaft (DFG, Gu335/16-1, La315/11-1/2 and Hu781/4-3), and by the DFG International Graduate School 1302.

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