Parasitology Research (2019) 118:377–382
https://doi.org/10.1007/s00436-018-6167-1

IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - SHORT COMMUNICATION

Eryptosis of non-parasitized erythrocytes is related to anemia in *Plasmodium berghei* low parasitema malaria of Wistar rats

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Received: 1 September 2018 / Accepted: 23 November 2018 / Published online: 1 December 2018

Abstract

It is known that premature elimination of non-parasitized RBCs (nRBCs) plays an important role in the pathogenesis of malarial anemia, in which suicidal death process (eryptosis) of nRBCs has been suggested to be involved. To check this possibility, we investigate eryptosis during infection of *P. berghei* ANKA in Wistar rats, a malaria experimental model that, similar to human malaria, the infection courses with low parasitemia and acute anemia. As expected, *P. berghei* ANKA infection was marked by low parasite burdens that reached a mean peak of 3% between days six and nine post-infection and solved spontaneously. A significant reduction of the hemoglobin levels (~30%) was also observed on days subsequent to the peak of parasitemia, persisting until day 16 post-infection. In eryptosis assays, it was observed a significant increase in the levels of PS-exposing nRBC, which coincided with the reduction of hemoglobin levels and was positively related to anemia. In addition to PS externalization, eryptosis of nRBC induced by *P. berghei* infection was characterized by cytoplasm calcium influx, but not caspases activity. These results confirm our previous studies evidencing a pro-eryptotic effect of malaria infection on nRBCs and show that a caspase-independent eryptotic process is implicated in anemia induced by *P. berghei* ANKA infection in Wistar rats.

Keywords Eryptosis · *P. berghei* · Malaria · Anemia

Introduction

Malaria, a mosquito-borne disease caused by *Plasmodium* parasites, remains a great problem of public health in 91 countries of tropical and subtropical areas of the globe (WHO 2017). Although it is a treatable disease, malaria can rapidly progress to severe and potentially lethal forms in the absence of prompt and appropriate treatment, notably during *P. falciparum* infections (Varo et al. 2018). As a result, malaria has been responsible for about one half million deaths annually, to which severe anemia has significant contribution (WHO 2017).

Anemia is a common feature of malaria and, despite its pathophysiology has not been fully elucidated; it is believed that premature elimination of non-parasitized red blood cells (nRBCs) may play an important role in its genesis (Jakeman et al. 1999; Collins et al. 2003). In this sense, different cellular and immunological mechanisms, such as hyperactivation of the phagocytic system, sensitization of the erythrocyte membrane by antibodies and complement, reduction of cell deformability as well as down-expression of complement regulatory proteins have been described as potential participants in the elimination of nRBC (Dondorp et al. 1999; Waitumbi et al. 2000; Stoute et al. 2003; Helegbe et al. 2007). In addition, our group has suggested that suicidal erythrocyte death, named eryptosis, can be implicated in the pathogenesis of malarial anemia by leading nRBC to precocious elimination through phagocytosis (Totino et al. 2016).

Enhanced levels of eryptosis, which shares many classical apoptotic hallmarks with nucleated cells, including phosphatidylserine (PS) externalization, intracellular calcium (Ca^{2+}) influx and activation of specialized proteases (i.e., caspases and calpains), have been reported in different clinical disorders in which anemia is a common feature (Jemaà et al. 2017). In agreement with this fact, our studies on lethal infection of *P. yoelii* 17XL in BALB/c mice showed, for the first time, an increase in eryptotic

Section Editor: Kevin S.W. Tan

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nRBC levels during the acute anemic phase, indicating a role of eryptosis in malarial anemia pathogenesis (Totino et al. 2010, 2013). Considering that the high parasite load typical of the majority of malaria models in mice could interfere in the investigation of nRBC commitment in anemia development—since anemia of these malaria models, such as *P. yoelii* 17XL infection, is closely related to intravascular hemolysis of the high pRBC rates (Lamikanra et al. 2007)—we attempted to study eryptosis of nRBC in *P. berghei* ANKA infecting Wistar rats model. This non-lethal experimental model, although poorly explored, develops acute anemia with low parasitemia levels similarly to the one recorded during human malaria (Evans et al. 2006).

Materials and methods

Experimental infection

Female Wistar rats aged 13–15 weeks were intraperitoneally inoculated with 1×10^6 erythrocytes infected with *P. berghei* ANKA expressing green fluorescent protein (GFP), obtained from passage BALB/c mice previously infected with cryopreserved parasites. Infected rats were monitored for 23–26 days with evaluation of parasitemia, anemia and eryptosis in tail blood samples. When indicated, non-infected, age matched rats were used as control. All animals were provided by the Institute of Science and Technology in Biomodels/Fiocruz and the experiments were performed as approved by the Ethics Committee on the Use of Animals of the Oswaldo Cruz Institute/Fiocruz, Rio de Janeiro, RJ, Brazil (CEUA/IOC 014/2015).

Evaluation of parasitemia

The parasitemia was monitored by flow cytometry through detection of RBC presenting GFP fluorescence (i.e., pRBC). Briefly, tail blood samples were 1:1000 diluted in phosphate-buffered saline (PBS) and, then, GFP fluorescence was identified in a FACSVerse flow cytometer (Becton Dickinson), using 488-nm blue laser and 527/32 bandpass filter. Parasitemia was defined as the percentage of GFP-positive RBCs within the total RBC population, after counting a minimum of 20,000 events. Blood samples from non-infected rats were used as negative control.

Determination of anemia

Anemia degree was examined by measuring hemoglobin concentration in approximately 10 μL of tail blood using a portable hemoglobinometer (HemoCue Hb 301), as indicated by manufacturer. Alternatively, anemia was determined by counting the number of RBC per mm^3 of blood. Briefly, 2 μL of blood were suspended in 0.5 mL heparinized PBS, 1:10 diluted in the same buffer and, then, the number of RBC was determined in a hemocytometer.

Eryptosis assays

Eryptosis of nRBCs during the course of infection was monitored by ex vivo examination of phosphatidylserine exposure at the cell surface of GFP-negative RBCs using annexin V staining. For this propose, rat tail blood samples were washed twice with PBS (350 *g*, 10 min) and RBCs were suspended in annexin-binding buffer (BD Pharmingen) at a density of 1×10^6 cells/mL. Five microliters of allophycocyanin (APC)-conjugated annexin V (BD Pharmingen) were added to 100 μL of RBC suspension and these cells were incubated for 15 min at room temperature. Finally, RBCs were diluted five times in annexin-binding buffer and analyzed using a FACSVerse flow cytometer (Becton Dickinson). GFP-negative RBCs (i.e., nRBC) were identified using 488-nm blue laser and 527/32 bandpass filter, while 633-nm red laser and 660/10 bandpass filter were used to detect annexin V (APC)-positive RBCs.

Additionally, Ca^{2+} influx and caspase activation were studied at the acute anemia stage of infection by using X-rhod-1 (Invitrogen) and Red-VAD-FMK (Calbiochem) staining, respectively. RBCs (1×10^5 cells) were suspended in 200 μL Ringer solution (in mM: 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, and 1 $CaCl_2$; pH 7.4) containing X-rhod-1 (1 μM) or Red-VAD-FMK (2 μL) and, then, incubated at 37° C for 30 min or 1 h, respectively. After incubation, RBCs stained with X-rhod-1 were washed and resuspended in Ringer solution, while a wash buffer provided by manufacturer was used for Red-VAD-FMK staining. Finally, cells were analyzed in a FACSVerse flow cytometer (Becton Dickinson) and X-rhod-1 and Red-VAD-FMK fluorescence were detected using 488-nm blue laser and 586/42 nm bandpass filter.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA, USA), as indicated in figure legends. A p value of < 0.05 was considered statistically significant.

Results and discussion

To evaluate the relationship between eryptosis and malarial anemia, we investigated the experimental infection of *P. berghei* ANKA in Wistar rats, which is a model of non-

lethal acute malarial anemia established by Evans and colleagues (Evans et al. 2006), to analyze immunological mechanisms contributing to precocious elimination of nRBC. These authors have shown that Wistar model is marked by a low burden parasitemia peaking at 3.4% that, while notably differing from the majority of rodent malaria models, resemble the human disease (Lamikanra et al. 2007). Indeed, a similar profile of *P. berghei* ANKA infection coursing with low parasitemia was also observed by us, in general reaching a mean peak of 3% between days six and nine post-infection and resolving spontaneously by day 19 (Fig. 1). On the other hand, an anemia degree corresponding to a reduction of hemoglobin concentration less intense than the 60% reported by the Evans' group (Evans et al. 2006) was noted. In our experiments, *P. berghei* infection led to a significant reduction of around 30% in the hemoglobin levels on days subsequent to the peak of parasitemia, persisting until day 16 post-infection, as compared with non-infected control animals (Fig. 1). Such a disparity in the anemia magnitude could be explained by the well-known variability of genetic background between different breeding colonies of outbred rats (Hedrich 2000), since host genetic diversity has, indeed, been shown to represent a determinant factor of severe malarial anemia susceptibility (Okeyo et al. 2013; Munde et al. 2017). These results showing a more moderate anemia are, however, in agreement with two more recent works in which 20–30% reduction of hemoglobin levels in *P. berghei* ANKA-infected Wistar rats was also detected (Gómez et al. 2011; Safeukui et al. 2015).

Since *P. berghei* ANKA induced an expressive anemia in Wistar rats, we examined the frequency of eryptotic nRBC during the course of infection by assaying externalized PS. As shown in Fig. 2, *P. berghei* ANKA infection was followed by a significant increase in the levels of PS-exposing nRBC on days 12 and 15 post-infection (Fig. 2c), which coincided with the reduction of hemoglobin concentrations (Fig. 2b). This rise in PS-exposing nRBC levels has been described during anemia of *P. yoelii* 17XNL non-lethal infection in Swiss mice (Fernandez-Arias et al. 2016), as well as in severely anemic children with falciparum malaria (Fendel et al. 2010). Similarly, our previous study on *P. yoelii* 17XL infection, which develops lethal anemia in BALB/c mice as a result of hyperparasitemia, also evidenced an eryptotic effect of malaria on nRBC, but failed to detect an association between eryptosis and anemia (Totino et al. 2010, 2013). Now, using Wistar model, it was remarkable that PS-exposing nRBC levels were inversely related to hemoglobin concentrations on day 15 post-infection (Fig. 2d), bringing additional evidence of eryptosis involvement in the pathogenesis of malarial anemia (Totino et al. 2016) as it has also been described for anemia associated with other clinical conditions in which excessive rates of eryptotic RBCs occur, including diabetes, renal insufficiency, sickle-cell anemia, chronic lead exposure, sepsis, and mycoplasmosis (Lang and Lang 2015).

In order to better characterize the eryptotic process of nRBC occurring in *P. berghei* ANKA-infected Wistar rats, Ca^{2+} influx and caspase activity were studied at the anemic phase of infection (day 12 post-infection). As evidenced by X-rhod-1 staining, *P. berghei* infection significantly increased the levels of nRBC with elevated cytosolic Ca^{2+} activity at the time that anemia was marked by a drop of 77% in the number of peripheral RBC, when compared to non-infected control rats (Fig. 3a). In contrast, although an increase in the levels of nRBC presenting caspase activity was detected in anemic infected rats by using Red-VAD-FMK staining, this increase was not significantly different from that observed in non-infected control animals (Fig. 3b), suggesting that

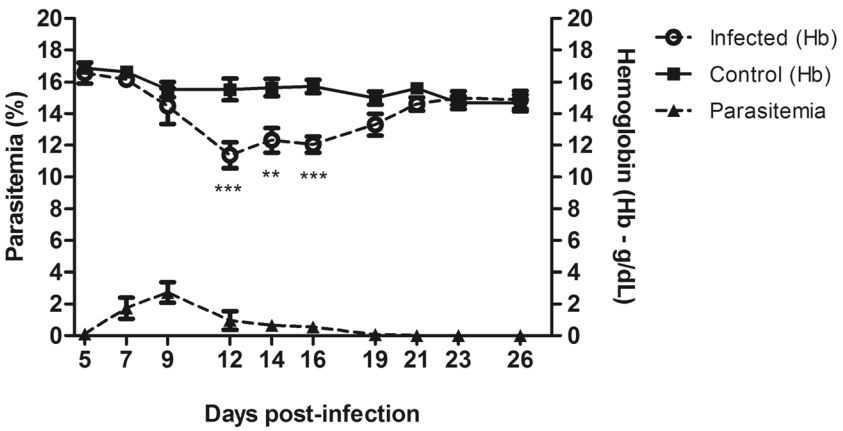

Fig. 1 Course of parasitemia and anemia in *P. berghei*-infected Wistar rats. Rats were infected with 1×10^6 red blood cells parasitized by GFP-expressing parasites and, then, parasitemia and blood hemoglobin (Hb) concentration were evaluated over 26 days post-infection by using flow cytometry and hemoglobinometer, respectively. In parallel, non-infected animals were used as control. Data are shown as mean ± standard error (SEM) and represent one of two independent experiments performed with 5 to 8 animals in each group (control or infected). Statistical difference of Hb concentration was tested comparing control and infected animals by two-way ANOVA and Bonferroni posttests. **: $p < 0.01$; ***: $p < 0.001$

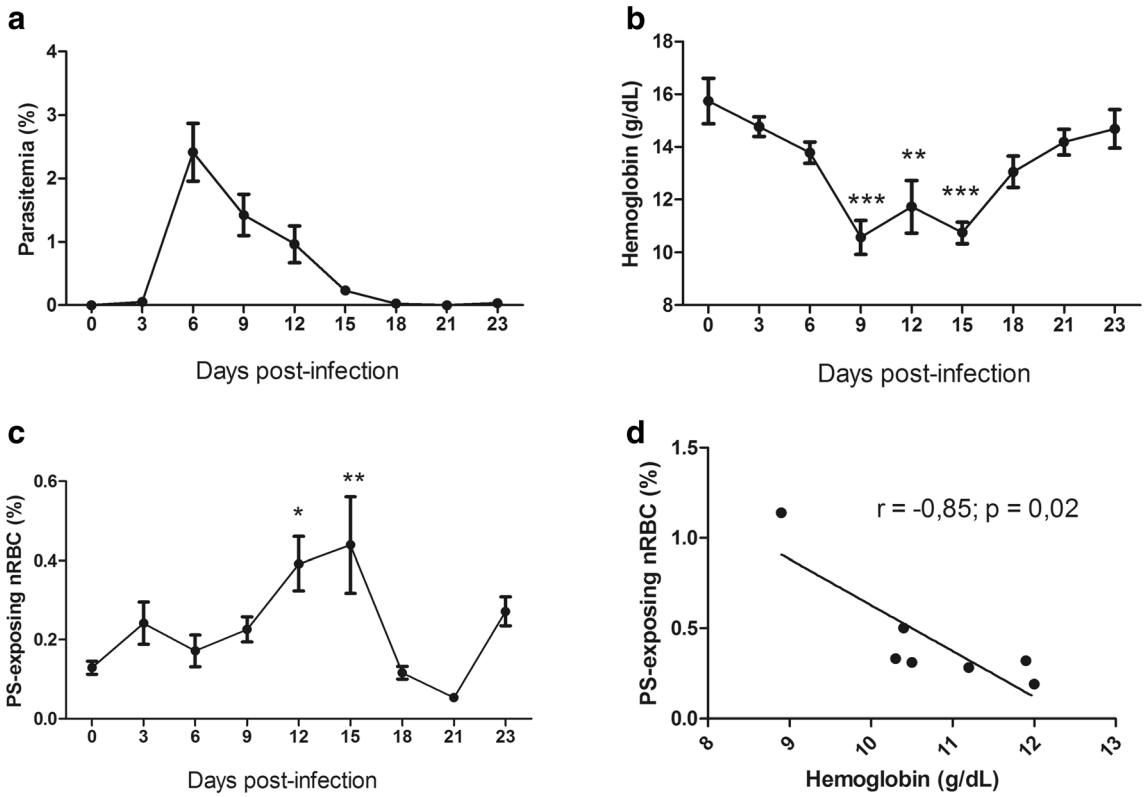

Fig. 2 Phosphatidylserine (PS) externalization in non-parasitized red blood cells (nRBC) and relation to anemia in *P. berghei*-infected Wistar rats. Animals were infected with 1×10^6 red blood cells parasitized by GFP-expressing parasites and, then, parasitemia **(A)**, blood hemoglobin (Hb) concentration **(B)** and frequency of PS-exposing nRBC **(C)** were evaluated over 23 days post-infection. Parasitemia (GFP-positive RBCs) as well as PS externalization (annexin V-positive nRBCs) were determined by flow cytometry analysis and anemia was evaluated using a hemoglobinometer. Data in **(A-C)** are shown as mean ± standard error (SEM); statistical difference of Hb concentrations **(B)** and PS-exposing nRBC levels **(C)** was tested by one-way ANOVA and Tukey posttests comparing with pre-infection values (day 0). As shown in **(D)**, a negative correlation between individual frequency of PS-exposing nRBC and Hb concentration was observed in day 15 post-infection, as tested by Spearman correlation coefficient. Data represent one of two independent experiments performed with 7 to 10 animals. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

caspases are not key components of eryptosis pathways induced by *P. berghei* infection in Wistar rats.

Increased intracellular influx of Ca^{2+} promoted by activation of plasma membrane Ca^{2+} permeable channels has been

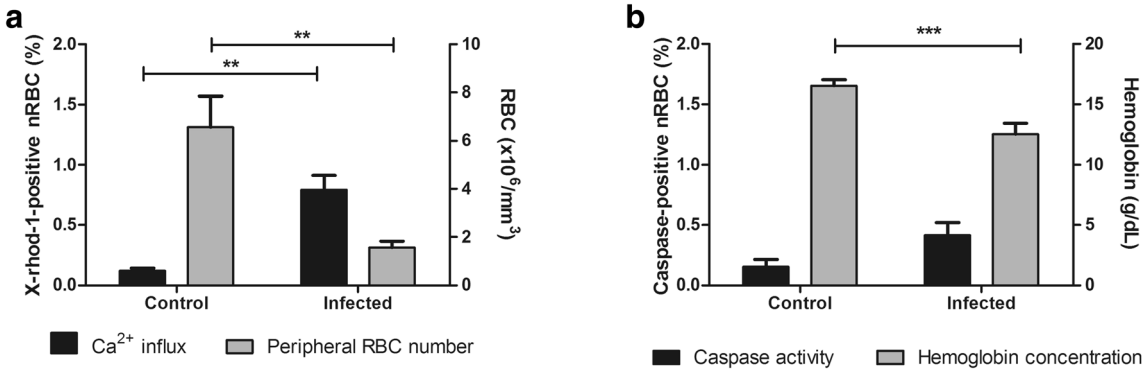

Fig. 3 Calcium influx and caspase activity in non-parasitized red blood cells (nRBC) at anemic stage of *P. berghei* ANKA infection. Wistar rats were infected with 1×10^6 red blood cells parasitized by GFP-expressing parasites and, then, Ca^{2+} influx **(A)** and activity caspase **(B)** in nRBCs were evaluated at day 12 post-infection by flow cytometry, using X-rhod-1 and Red-VAD-FMK staining, respectively. Anemia was evaluated by quantification of peripheral RBC **(A)** or hemoglobin (Hb) concentration **(B)**. In parallel, non-infected animals were used as control. Data are shown as mean ± standard error (SEM) and represent one of two independent experiments performed with 5 to 10 animals in each group (control or infected). Statistical difference of hematological parameters (Hb or RBC number), caspase activity and Ca^{2+} influx were tested comparing control and infected animals by Mann Whitney test. **: $p < 0.01$; ***: $p < 0.001$

identified as a common trigger of eryptosis (Lang and Qadri 2012), but the involvement of caspases as executers of eryptosis is not consensually demonstrated. It is well-known that caspases are central proteases in apoptotic pathways of nucleated cells (Chang and Yang 2000) and their activity has also been detected during in vitro induction of eryptosis by *Schistosoma mansoni* antigens (Kasinathan and Greenberg 2010), staurosporine (Qian et al. 2012), phytoestrogen ferutinin (Gao et al. 2013) and tyrosine kinase inhibitor dasatinib (Chan et al. 2018). However, treatment with caspase inhibitors has not been able to prevent eryptosis triggered by different types of inducers and, therefore, it is believed that eryptosis can be carried out through caspase-independent pathways (Jilani et al. 2011; Lupescu et al. 2012; Al Mamun Bhuyan et al. 2017). In agreement with this view, earliest studies on RBC susceptibility to programmed cell death demonstrated that, despite functional caspases are present in RBC cytoplasm, they failed to become activated under eryptotic stimuli, while activation of calpains—the major Ca^{2+}-activated cysteine proteases of RBC—was implicated in induction of eryptotic process (Berg et al. 2001; Bratosin et al. 2001). Thus, it is possible that calpains rather than caspases are required to trigger calcium-mediated eryptosis in nRBC during malaria infections, in which the mechanisms of eryptosis induction has not yet been elucidated, but potentially involve adsorption of parasite antigens on the cell surface, autoantibodies, and complement sensitization as well as oxidative stress generated by both parasite itself and host immune responses (Totino et al. 2016).

In conclusion, we showed for the first time that increase of eryptotic nRBC rates stimulated by plasmodial infection is related to anemia degree in a model of low parasite burden, supporting our initial hypothesis of eryptosis participation in the pathogenesis of malarial anemia. Moreover, we observed that *P. berghei*-induced eryptosis occurred independently of caspase activation, pointing to well-known involvement of calpain-dependent pathways in eryptotic processes. Further investigations evaluating calpain activity are required to improve our understanding of eryptotic pathways triggered during malaria and, thus, help to delineate eryptotic inhibition studies to better address the clinical significance of suicidal erythrocyte death in malaria pathogenesis.

Funding information This work received financial support from the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, Brazil) and Instituto Oswaldo Cruz (Fiocruz, Brazil). CTDR is recipient of a Research Productivity Fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and CTDR and MFFC received grants from FAPERJ as "Cientistas do Nosso Estado".

Compliance with ethical standards All animal experiments were approved by the Ethical Committee of Animal Experiments of Instituto Oswaldo Cruz-Fiocruz (protocol: L-040/2015).

Conflict of interest The authors declare that they have no conflicts of interest.

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