Apoptosis of non-parasitised red blood cells in *Plasmodium yoelii* malaria

Paulo Renato Rivas Totino[1,2]/+, Raquel Alves Pinna[3], Ana Cecilia Amado Xavier De-Oliveira[4], Dalma Maria Banic[3], Cláudio Tadeu Daniel-Ribeiro[1,2], Maria de Fátima Ferreira-da-Cruz[1,2]

[1]Laboratório de Pesquisas em Malária [2]Centro de Pesquisa, Diagnóstico e Treinamento em Malária
[3]Laboratório de Simulídeos e Oncocercose, Instituto Oswaldo Cruz [4]Laboratório de Toxicologia Ambiental,
Escola Nacional de Saúde Pública-Fiocruz, Rio de Janeiro, RJ, Brasil

Recently, while studying erythrocytic apoptosis during Plasmodium yoelii *infection, we observed an increase in the levels of non-parasitised red blood cell (nRBC) apoptosis, which could be related to malarial anaemia. Therefore, in the present study, we attempted to investigate whether nRBC apoptosis is associated with the peripheral RBC count, parasite load or immune response. To this end, BALB/c mice were infected with* P. yoelii *17XL and nRBC apoptosis, number of peripheral RBCs, parasitaemia and plasmatic levels of cytokines, nitric oxide and anti-RBC antibodies were evaluated at the early and late stages of anaemia. The apoptosis of nRBCs increased at the late stage and was associated with parasitaemia, but not with the intensity of the immune response. The increased percentage of nRBC apoptosis that was observed when anaemia was accentuated was not related to a reduction in peripheral RBCs. We conclude that nRBC apoptosis in* P. yoelii *malaria appears to be induced in response to a high parasite load. Further studies on malaria models in which acute anaemia develops during low parasitaemia are needed to identify the potential pathogenic role of nRBC apoptosis.*

Key words: malaria - anaemia - red blood cells - apoptosis

Severe anaemia is a common complication of *Plasmodium falciparum* infection and is strongly related to malarial mortality, particularly in children and pregnant woman living in malaria-hyperendemic regions of sub-Saharan Africa (Schantz-Dunn & Nour 2009, Muoneke et al. 2012). The pathophysiology of severe anaemia in malaria is not fully understood. However, the premature elimination of non-parasitised red blood cells (nRBCs) may play an important role in the genesis of this malarial complication, together with a decreased production of RBCs by the bone marrow and mechanical destruction of parasitised RBCs (pRBCs) (Casals-Pascual & Roberts 2006, Lamikanra et al. 2007).

Recently, in studying the susceptibility of RBCs to apoptosis during *Plasmodium yoelii* 17XL infection, we reported increased levels of nRBC apoptosis and we hypothesised that this event could contribute to acute anaemia (Totino et al. 2010) because cells undergoing apoptosis are cleared by phagocytosis (Fadok & Henson 2003). Indeed, RBC apoptosis can occur in anaemia-associated conditions, such as sepsis and visceral leishmaniasis, in which microbial factors and the host immune response appear to act together to cause pathology (Kempe et al. 2007, Chowdhury et al. 2010).

A variety of inducers and inhibitors of erythrocytic apoptosis have been identified in vitro (Lang & Qadri 2012). These factors include endogenous stimuli present in parasitic infections, such as anti-RBC antibodies, oxidative stress, nitric oxide (NO) and microbial antigens (Mandal et al. 2005, Attanasio et al. 2007, Nicolay et al. 2008, Kasinathan & Greenberg 2010). Thus, in the present study, we attempted to investigate the association of nRBC apoptosis with total RBC counts, parasite load, cytokines, NO and anti-RBC antibodies during the early and late stages of anaemia in experimentally infected *P. yoelii* 17XL BALB/c mice.

MATERIALS AND METHODS

Experimental infection - Female BALB/c mice aged six-eight weeks, provided by the Centre for Laboratory Animal Breeding of the Oswaldo Cruz Foundation (Fiocruz) (Rio de Janeiro, RJ, Brazil), were intraperitoneally inoculated with 1×10^6 *P. yoelii* 17XL-pRBC in 0.2 mL of phosphate-buffered saline (PBS). At the earlier (day 4) and later (day 7) stages of anaemia, blood from each animal was collected in heparinised tubes and RBCs and plasma were separated by centrifugation (350 *g*, 10 min) for the evaluation of apoptosis and the plasmatic levels of cytokines, NO and anti-RBC antibodies. Parasitaemia and anaemia were determined using non-heparinised whole-blood samples. Blood samples from non-infected age-matched mice were also obtained and used as controls.

Ethics - All animal experimentation protocols were approved by the Fiocruz Animal Ethical Committee.

Determination of parasitaemia - Parasitaemia was determined by counting the number of pRBCs among

1,000 RBCs in thin blood smears stained using Romanowski's method (Panótico Rápido, Laborclin®, Pinhais, PR, Brazil).

Determination of anaemia - Anaemia was evaluated by counting the number of RBCs per mm^3 of blood. A 2-μL aliquot of whole blood was suspended in 0.5 mL of heparinised PBS and diluted 1:10 in the same buffer. Subsequently, the number of RBCs was estimated using a haemocytometer.

Apoptosis assay - Apoptotic nRBCs were identified ex vivo through the detection of phosphatidylserine exposure at the cell surface using Syto 16 and annexin V-PE double staining, as previously described (Totino et al. 2010). Briefly, RBCs isolated from heparinised blood were washed twice with PBS (350 *g*, 10 min) and subsequently incubated at 37ºC for 40 min at a density of 1×10^5 cells/400 μL in PBS containing 100 nM Syto 16 (Invitrogen, Carlsbad, CA, USA). After incubation, the staining buffer was discarded and the RBCs were stained with 5 μL of annexin V (BD Pharmingen, San Diego, CA, USA) for 15 min at room temperature (RT) in 100 μL of annexin-binding buffer (BD Pharmingen, San Diego, CA, USA) containing 100 nM Syto 16. Finally, the cells were diluted five times in annexin-binding buffer containing Syto 16 and analysed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Measurement of NO - NO production was estimated by measuring total nitrite in the plasma using the Griess method (Schmidt et al. 1989). Briefly, 40 μL of each plasma sample was incubated overnight at 37ºC in a 96-well plate with equal volumes of a cocktail containing 500 μL of nicotinamide adenine dinucleotide phosphate NADPH (5 mg/mL) (Sigma, St. Louis, MO, USA), 1,000 μL of potassium phosphate buffer (0.5 M KH_2PO_4, pH 7.5), 50 μL of nitrate reductase (Sigma, St. Louis, MO, USA) (20 U/mL in potassium phosphate buffer) and 950 μL of deionised Milli-Q water. After incubation, the samples were centrifuged at 400 *g* for 5 min and transferred to a new plate. Subsequently, 80 μL of Griess reagent [1:1 mixture of 0.1% N-(1-naphthyl)ethylenediamine in deionised water and 1% sulphanilamide in 5% phosphoric acid] was added. The absorbance was measured using a spectrophotometer (Spectra Max, Molecular Devices, Sunnyvale, CA, USA) at 540 nm and the results were expressed as the concentration (μM) of nitrite.

Measurement of cytokines - The plasmatic levels of the cytokines tumour necrosis factor (TNF), interferon (IFN)-γ, interleukin (IL)-5, IL-4 and IL-2 were determined using the BD Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Briefly, a 25-μL plasma sample was incubated for 2 h at RT with 25 μL of cytokine capture beads and 25 μL of PE detection reagent. After incubation, the samples were washed once with wash buffer by centrifugation (200 *g*, 5 min). The supernatants were discarded and the pelleted beads were resuspended in 300 μL of wash buffer for analysis on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The plasmatic concentration of each cytokine in pg/mL was determined based on standard curves for the recombinant cytokines provided in the kit.

Detection of anti-RBC antibodies - Anti-RBC antibodies in the plasma were detected by flow cytometry using normal RBCs obtained from a non-infected control mouse. Briefly, plasma samples were diluted five times in PBS containing 1% bovine serum albumin (BSA) and 0.125% RBCs and incubated for 1 h at RT. After washing three times with PBS-1% BSA, the RBCs were incubated for 1 h at RT with anti-mouse polyvalent immunoglobulins (G, A and M) conjugated to fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO, USA) diluted 1:100 in PBS-1% BSA. Finally, the RBCs were washed three times with PBS and resuspended in the same buffer. Bound antibodies were detected using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The results were expressed as the mean fluorescence intensity of the RBCs.

Statistical analysis - Statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA, USA). Differences between groups were determined by the nonparametric Mann-Whitney *U* test or the Kruskal-Wallis test with Dunn's post-test. To determine correlations between apoptosis and other variables, the nonparametric Spearman's rank correlation test was applied. Statistical tests were considered significant at p values less than 0.05.

RESULTS

To study the relationship between nRBC apoptosis and anaemia, we infected BALB/c mice with a lethal strain of rodent malaria parasite (*P. yoelii* 17XL). We then evaluated the levels of apoptotic nRBCs and the number of peripheral RBCs during the early (day 4) and late (day 7) stages of anaemia, using non-infected mice as a control. As previously reported by our group, *P. yoelii* 17XL infection led to a significant increase in the levels of nRBC apoptosis at the late stage (Fig. 1A) and this increase was followed by a significant decline in the number of RBCs (Fig. 1B). However, a significant correlation between apoptosis and RBC count was not observed (Fig. 2).

To gain insight into the involvement of parasites and the immune response in nRBC apoptosis, we also evaluated parasitaemia and the plasmatic levels of cytokines (TNF, IFN-γ, IL-5, IL-4 and IL-2), NO and anti-RBC antibodies during the early and late stages of anaemia. As expected, parasitaemia significantly increased at the late stage compared with the early stage (Fig. 1C), confirming the typical progressive replication of *P. yoelii* 17XL parasites. Levels of the proinflammatory cytokines IFN-γ and TNF also increased during infection (Fig. 1D, E), whereas IL-5, IL-4 and IL-2 were not significantly detected in the plasma of either control or infected mice (data not shown). IFN-γ levels increased at both the early and the late stages, with no significant difference between these two phases of infection (Fig. 1D). Inversely, the TNF concentration and the levels of anti-RBC antibodies only significantly increased during the late stage (Fig. 1E, F). In contrast to what was observed for parasitaemia,
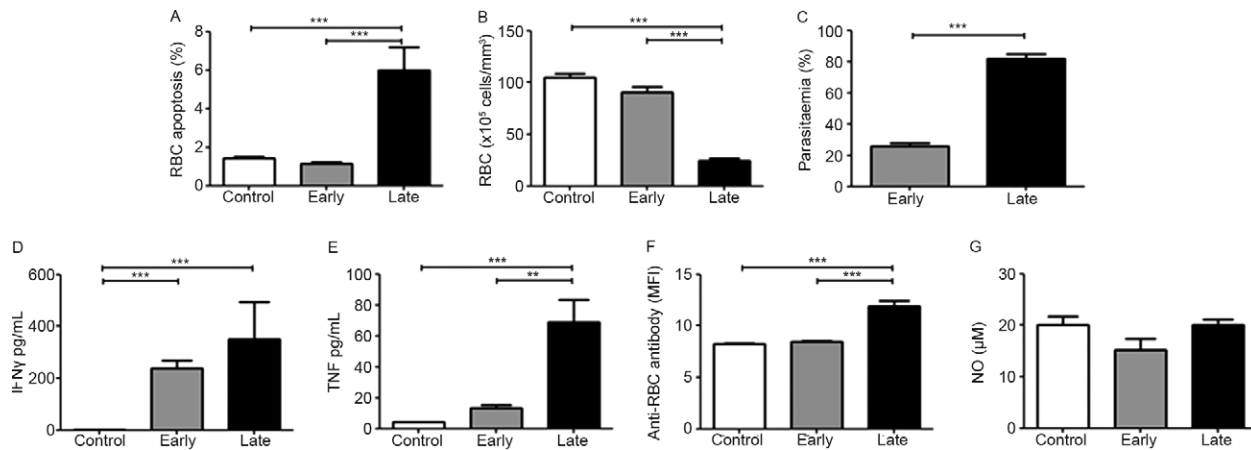
Fig. 1: profile of non-parasitised red blood cell (RBC) apoptosis (A), anaemia (B), parasitaemia (C), interferon (IFN)-γ (D), tumour necrosis factor (TNF) (E), anti-RBC antibodies (F) and nitric oxide (NO) (G) in *Plasmodium yoelii* 17XL infection. BALB/c mice (n = 7-12) were infected with *P. yoelii* 17XL and, then, at days 4 (early) and 7 (late) post-infection evaluations were paralely performed. Data are results from three separate experiments and are presented as mean ± standard error of the means. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

proinflammatory cytokines and anti-RBC antibodies, no significant change in NO concentration was observed on day 4 or day 7 ($p > 0.5$) (Fig. 1G).

Considering that parasitaemia, TNF levels and anti-RBC antibodies were increased during the late stage of infection, we attempted to correlate these factors with nRBC apoptosis (Fig. 3). Whereas the antibodies and TNF were not correlated with apoptosis (Fig. 3A, C), parasitaemia was positively associated with this process (Fig. 3B).

DISCUSSION

Augmented levels of erythrocytic apoptosis have been reported in different anaemia-associated clinical disorders, suggesting that apoptosis is involved in this haematological complication (Lang & Qadri 2012). In malaria, the relationship between erythrocytic apoptosis and anaemia has not yet been explored. We therefore investigated whether nRBC apoptosis detected in experimental malaria could be associated with the number of peripheral RBCs, parasitaemia or plasmatic levels of NO, cytokines and anti-RBC antibodies. Our present data confirm our previous results concerning increased levels of apoptotic nRBCs during the late stage of *P. yoelii* 17XL infection in BALB/c mice and also suggest that the degree of anaemia is not related to the extent of apoptosis. We also observed that nRBC apoptosis was associated with parasite load, but not with the components of the immune response that were assessed herein.

It is known that parasite-derived factors can participate in the physiopathology of many parasitic infections, including the induction of host cell apoptosis (Bienvenu et al. 2010). In malaria, several studies have demonstrated that parasitic antigens can stimulate the apoptosis of brain endothelial and neuroglia cells, cardiomyocytes and erythroblasts in vitro (Wennicke et al. 2008, Wilson et al. 2008, Lamikanra et al. 2009). In our study, a high parasite load correlated with increased levels of nRBC

apoptosis. This finding, together with the previous identification of nRBCs as target cells for the incorporation of plasmodial molecules (Layez et al. 2005, Omodeo-Sale et al. 2005, Bratting et al. 2008), suggested that malarial antigens could also have apoptogenic effects on nRBCs. In fact, it has previously been shown that nRBCs undergo erythrocytic apoptosis in *P. falciparum* culture in vitro (Koka et al. 2007, Pattanapanyasat et al. 2010), similar to RBCs following treatment with bacterial toxins and *Schistosoma mansoni* antigens in vitro (Lang et al. 2004, Föller et al. 2007, Kasinathan & Greenberg 2010). Thus, in addition to the pathogenic effects of hyperparasitaemia mediated through the lysis and cytoadherence of pRBCs (Lamikanra et al. 2007), parasite load could induce nRBC apoptosis through a massive adsorption of parasitic antigens on nRBC membranes.

In addition to parasitaemia, the basal levels of NO during *P. yoelii* 17XL infection could also contribute to parasite-induced apoptosis. NO is a biological mediator with several roles and this mediator's reduced bioavailability in malaria has been associated with disease severity (Sobolewski et al. 2005, Zanini et al. 2011). In visceral leishmaniasis, the depletion of NO promotes RBC apoptosis and the administration of NO to infected animals can prevent this apoptosis (Chowdhury et al. 2010). Therefore, it is possible that the limited availability of NO described herein could potentiate the induction of nRBC apoptosis mediated by parasites because NO is a potent regulator of RBC survival and an inhibitor of erythrocytic apoptosis (Nicolay et al. 2008, Chowdhury et al. 2010).

Parasites and NO can influence RBC apoptotic processes, but the same does not appear to be true for cytokines. Although proinflammatory cytokines have been implicated in apoptosis of nucleated cells (Roeske-Nielsen et al. 2010), their pro-apoptotic effects were not observed when fresh or pRBCs were maintained in the presence of TNF and/or IL-1 (Pattanapanyasat et al.
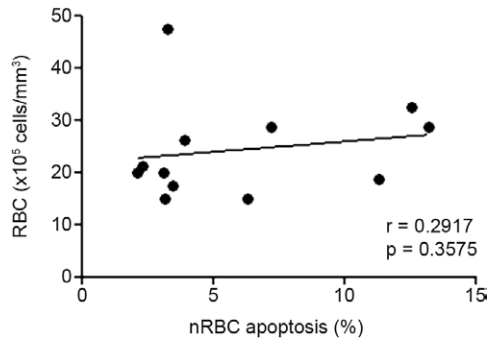
Fig. 2: correlation of non-parasitised red blood cell (nRBC) apoptosis and anaemia in *Plasmodium yoelii* 17XL infection. BALB/c mice (n = 12) were infected with *P. yoelii* 17XL and, then, both the levels of apoptotic nRBC and the number of RBC/mm³ of blood were measured during the late stage of infection (day 7). Data represent the results of two separate experiments.
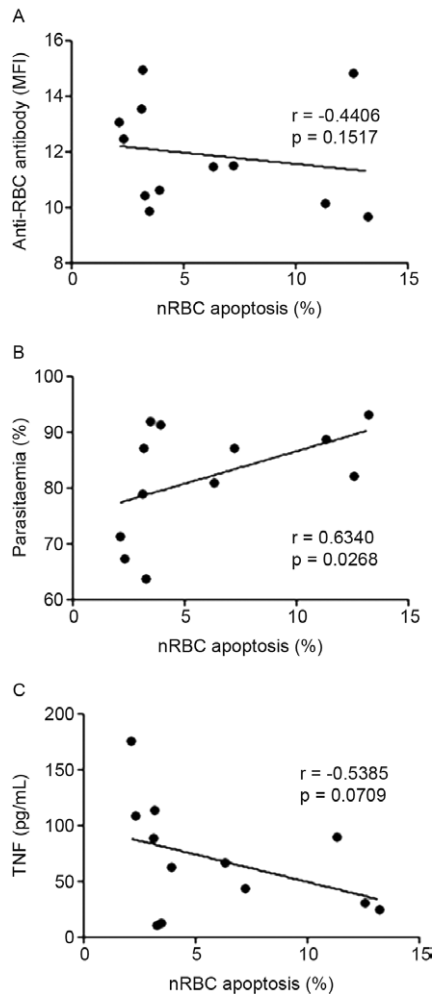

Fig. 3: correlation of non-parasitised red blood cell (nRBC) apoptosis with anti-RBC antibodies (A), parasitaemia (B) and tumour necrosis factor (TNF) (C) in *Plasmodium yoelii* 17XL infection. BALB/c mice (n = 12) were infected with *P. yoelii* 17XL and analysed at late stage of infection (day 7). Data represent the results of two separate experiments. MFI: mean fluorescence intensity.

2010). Our results are also in accordance with the possible refractoriness of RBCs to cytokine-induced apoptosis (Neote et al. 1994, Daniels 2007) because IFN-γ levels were elevated at stages of infection that were related (late stage) and not related (early stage) to nRBC apoptosis. Moreover, the augmented levels of TNF at the late stage tended to be negatively correlated with the percentage of apoptotic RBCs.

Our data also suggest that anti-RBC antibodies, which levels are increased at the late stage of infection, do not participate in nRBC apoptosis induced by *P. yoelii* 17XL parasites. The ability of antibodies to stimulate erythrocytic apoptosis has been demonstrated using antibodies targeted against certain antigens expressed on the RBC surface; this antibody-antigen interaction likely mimics the receptor-ligand interaction that occurs during apoptotic induction (Head et al. 2005a, b, Lang et al. 2005, Attanasio et al. 2007). Thus, it is possible that the lack of an association between anti-RBC antibodies and apoptosis is due to the ability of the antibodies to recognise RBC surface antigens that are unrelated to apoptosis. The purification of anti-RBC antibodies coupled to apoptotic nRBCs could help to clarify this possibility.

Regarding the putative role of erythrocytic apoptosis in anaemia associated with different clinical disorders, we hypothesised that nRBC apoptosis could be involved in the anaemia observed during the late stage of *P. yoelii* 17XL infection (Totino et al. 2010). Although the percentage of nRBC apoptosis increased at the later stage of infection, this increase was not associated with a reduced number of peripheral RBCs in the present study. However, we cannot rule out the pathogenic effect of nRBC apoptosis in human malaria, in which, in contrast to *P. yoelii* malaria, anaemia can occur in the presence of low parasitaemia levels (Casals-Pascual & Roberts 2006, Bouyou-Akotet et al. 2009).

We conclude that further studies on malaria models in which acute anaemia develops during a low parasite burden, such as semi-immune mice (Evans et al. 2006) and New World monkeys (Carvalho et al. 2003), could improve our understanding of the role of nRBC apoptosis in malarial anaemia.

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