Critical contribution of endothelial protein C receptor in experimental malaria-associated acute respiratory distress syndrome

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Abstract

*Plasmodium falciparum* malaria severity is associated with parasite cytoadherence, but limited knowledge about its effect in malaria-associated acute respiratory distress syndrome (ARDS) is notorious. Our objective was to evaluate cytoadherence of infected red blood cells (iRBC) in a murine model of ARDS and appraise the role of endothelial protein C receptor (EPCR) in ARDS pathogenesis. Lungs from ARDS-developing mice showed evidences of iRBC accumulation in lungs besides increase of EPCR and TNF concentrations. Furthermore, TNF increased iRBC adherence *in vitro*. Dexamethasone-treated infected mice showed lower levels of TNF and EPCR mRNA expression and, finally, decreased the vascular permeability, protecting mice from ARDS. In addition, EPCR knockdown decreased the capacity of iRBC adherence *in vitro*. In conclusion, we identified that increased iRBC cytoadherence in lungs underlies malaria-associated ARDS in DBA/2 infected mice and that inflammation increased cytoadherence capacity through EPCR expression suggesting a potential target for drug development.

Author summary

It’s important to understand the development of malaria-associated acute respiratory distress syndrome (ARDS) to help in better prognosis and therapy. Carrying out a model using rodents suffering from ARDS, which is similar to that in humans, we were able to report, *in vivo* and *in vitro* approaches, that *P. berghei* ANKA, a parasite from mice, adhere in lungs endothelial cells of infected mice. We show that a receptor
named EPCR is important in this adhesion. This receptor is modulated by TNF, an pro-inflammatory factor, being decreased by dexamethasone, an anti-inflammatory drug. This treatment, which improves the infected mice respiratory parameters and lung lesions, due to less adherence, inflammation and pulmonary vascular permeability, could be considered as a co-treatment with antimalarial drugs.

Introduction

Malaria infections by \textit{Plasmodium falciparum} are responsible for the largest number of severe and fatal cases in the tropics [1,2]. The main complications of \textit{P. falciparum} include cerebral malaria, pulmonary complications, acute renal failure, severe anemia, bleeding and placental malaria [3]. An important aspect of the pathogenesis of severe malaria results from the ability of infected red blood cells (iRBC) to adhere in the microvasculature. This interaction between the iRBC and the endothelium can cause blocking blood flow and/or local inflammatory response [3–5]. Furthermore, these adhesions promote the disappearance of asexual forms of the parasite in the peripheral circulation, thus, preventing them from being destroyed in the spleen [3,5,6]. Pulmonary complications caused by severe malaria include Acute Respiratory Distress Syndrome (ARDS), which has been associated with different diseases and not only being a result of severe malaria [7–9]. Even though malaria-associated ARDS often causes high mortality rate, slight research has been done. Murine models have been used to study malaria-associated ARDS [10,11] and DBA/2 mice infected with \textit{P. berghei ANKA} (PbA) develop ARDS and die between the 7th to 12th days post infection (dpi) without signals of cerebral malaria. In addition, we established a predictive criteria to distinguish which mice would die from ARDS, on the 7th dpi, using respiratory and parasitemia date [12]. Using this model, we found that
recruitment of neutrophils and VEGF are essential to the pathogenesis of malaria-associated ARDS [13,14] and the induction of heme oxygenase-1 (HO-1) has a protective effect in the development of ARDS in mice [15]. Additionally, it has already been demonstrated that PbA-iRBC adhere to MVECS (microvascular lung endothelial cells from CBA/Ca mice) and that TNF-stimulated cells express more ICAM-1 and VCAM [16]. In P. chabaudi model, the absence of ICAM-1 in infected mice, showed less anemia and weight loss, reduced parasite accumulation in both spleen and liver and higher peripheral blood parasitemia during acute stage malaria, which presented the slight importance of ICAM-1 in adhesion and pathogenesis of P. chabaudi [17]. Endothelial receptors have been studied to understand the iRBC adherence in the microvasculature of different organs in severe malaria and some of these receptors are well established such as ICAM-1 in cerebral malaria and CSA in placental malaria [18]. However, both the adhesion and consequences of PbA-iRBC into DBA/2 lung endothelial cells remains unknown. In 2013, Endothelial protein C receptor (EPCR) was shown to be a new receptor for Plasmodium falciparum erythrocyte protein 1 (PfEMP1) in P. falciparum severe malaria [19] which may result in a new branch of research in severe malaria. EPCR has, as its primary function, to bind with activated protein C (APC) and to cleave protease activated receptor 1 (PAR-1), in a specifically RAC1 pathway, which inhibits the activation of the nuclear factor-κB and provides barrier protection [20]. EPCR facilitates the activation of protein C (PC) by the thrombin-thrombomodulin complex; the cytoprotective effects of EPCR results in vascular and tissue protection in brain, lungs, kidneys and liver [21]. Also, APC cytoprotective activities provide neuroprotective effects as anti-inflammatory and anti-apoptotic, protecting the blood-brain barrier, kidneys and lungs, and thus, may be
directly relevant to the complications associated with severe malaria [22]. Indeed, in vitro studies with purified CIDRα1 domains and iRBCs with selected *P. falciparum* laboratory strains confirm the loss of EPCR functionality upon CIDRα1 or iRBC binding that includes loss of PC and APC binding to EPCR, inhibition of EPCR-mediated PC activation and obstruction of APC-mediated endothelial barrier protective effects. Consequently, the binding of PfEMP1 to EPCR results in an acquired functional PC system deficiency which supports new evidences that EPCR plays a central role in the pathogenesis of severe malaria [22]. EPCR binding is mediated through the amino-terminal cysteine-rich interdomain region (CIDRa1) of DC8 and group PfEMP1 subfamilies, and that CIDRa1 interferes with protein C binding to EPCR [23]. However, it has unexpectedly been shown that parasitized erythrocytes expressing the DC13 HB3var03 or IT4var07 variants of PfEMP1 do not bind to the EPCR of brain endothelial cells in vitro. On the other hand, it has been evidenced that the DC8 variant IT4var19 may bind to the EPCR, but this interaction was inhibited when standard human serum or plasma was added to the assays. Therefore, the discrepancy in the binding activity of the EPCR and the recombinant PfEMP1 proteins indicates the need for further studies to understand the current physiological significance resulting from this PfEMP1-EPCR interaction [24]. Although there are some suggestions that PfEMP1 can bind to EPCR with important effects on the severe malaria, these data are controversial, and, in addition, there are any evidences of *P. berghei* ANKA binding on EPCR and the effects of EPCR on ARDS pathogenesis must be elucidated. Our study herein was developed in a murine model, which mimics various human ARDS, [12] and used primary culture of microvascular lung endothelial cells from DBA/2 mice (PMLEC), to clarify not only the adhesion of infected erythrocytes to murine lung microvascular endothelial cells, but also
understand the relevant aspects of EPCR modulation by the immune response which can bring important contribution to understand malaria-associated ARDS.

Results

ARDS-developing mice show higher load pulmonary parasite than HP-developing mice

In our previous study, it was shown that a proportion of DBA/2 mice infected with PbA-iRBC developed singular characteristics of ARDS and died between the 7th and 12th dpi [12–15]. To investigate if adherence is essential in the evolution of this phenotype, we observed that PbA-luciferase-infected erythrocytes were distributed in the peripheral blood and tissues of DBA/2 mice. However, when they were perfused with PBS 1x, the bioluminescence (luciferase/luciferin) signal remained concentrated in spleen and lungs, especially in ARDS-developing mice (Fig 1 A-C), corroborating with our published data [14]. We demonstrated that ARDS-developing mice showed higher levels of 18s subunit PbA rRNA expression (Fig 1D) and higher hemozoin concentration in the lungs compared to HP-developing mice, on the 7th dpi (Fig 1 E-G). In addition, we analyzed histological lung section of ARDS-developing mice, finding several iRBC in close contact with endothelial cells (Fig 1H). These results together indicated that ARDS-developing mice accumulated a considerable amount of infected red blood cells (iRBC) in the lungs, suggesting the essential participation on ARDS pathogenesis.

TNF increases Plasmodium berghei adherence in ARDS experimental model
ARDS-developing mice, on the 7th dpi, displayed higher TNF levels in serum compared to HP-developing mice (Fig 2A), suggesting that this inflammatory cytokine may be critical to ARDS development. For this, we further investigated if iRBC could contribute to TNF release by endothelial cells. First, we demonstrated that PMLEC stimulated with iRBC can directly contribute to TNF production (Fig 2B). Then, we checked the influence of TNF in adhesion of iRBC in PMLEC through static and flow conditions. It was observed that TNF-stimulated cells increased the capacity of iRBC adherence into PMLEC after 24 and 72 hours in static assay (Fig 2 C-E). In the flow adherence assay, which mimics the physiologic conditions, the iRBC had more cytoadherence with TNF than without TNF stimulation (Fig 2 F-H). In addition, PMLEC were seeded in transwell plates in indirect contact with peritoneal macrophages (Mφ), stimulated or not by red blood cells (RBC) or iRBC (Fig 2I). Mφ stimulated by iRBC produced more TNF than Mφ without previous contact with iRBC (Fig 2J). These Mφ also produced IFN-γ and IL-10 but with no difference between groups (data not shown). Additionally, PMLEC that was submitted to indirect contact with Mφ stimulated with iRBC demonstrating more adherence of iRBC than PMLEC with no previous contact with Mφ (Fig 2K). Finally, in order to evaluate the contribution of TNF in the adhesion of iRBC in PMLEC, cells were blocked with TNF antibody and, as a result, the blockage reduced the capacity of iRBC to adhere in PMLEC (Fig 2L). All these data show that ARDS-developing mice have more TNF, which contributes to iRBC adherence in endothelial cells than in HP-developing mice.

EPCR acts directly in *P. berghei* cytoadherence
Looking for adhesion molecules that could be involved in PbA-iRBC cytoadherence in ARDS pathogenesis, we observed that ARDS-developing mice showed higher levels of VCAM and ICAM-1 in lungs than HP-developing or non-infected mice (NI), analyzed by immunohistochemistry (Fig S2 A-C). Also, recombinant TNF upregulated the mRNA expression of ICAM-1 and VCAM in PMLEC (Fig S2 D-I).

However, the most interesting findings were the mRNA EPCR expression upregulated in the lungs of ARDS-developing mice compared to NI mice (28.48-fold increase) and HP-developing mice (13.16-fold increase) (Fig 3A). There was also an increase in soluble EPCR (sEPCR) (Fig 3B) and EPCR protein (Fig 3C) in ARDS-developing mice serum compared to HP-developing mice. We further investigated the influence of TNF in the regulation of EPCR expression in PMLEC. TNF-stimulated cells showed an upregulation in EPCR expression in 48 and 72 hours, but not with PbA-iRBC stimulus (Fig 3 D-F). Moreover, EPCR knockdown with siRNA transfection in PMLEC, reduced EPCR expression compared to non-stimulated PMLEC (Fig 3G) and reduced the adherence of iRBC (Fig 3H), suggesting an important contribution of TNF and EPCR to adherence of iRBC in PMLEC.

Dexamethasone reduces TNF and EPCR, protecting mice lungs and PMLEC from increased vascular permeability

Once TNF seems to be important to induce PbA-iRBC adherence, and consequently, ARDS development, DBA/2 mice were infected with PbA-iRBC and treated with dexamethasone (80 mg/kg) to investigate if it could protect them from lung injury.

Dexamethasone treatment decreased TNF concentrations (Fig 4A) apart from IL-6 and IL-33 concentrations in treated mice, compared to non-treated mice (Fig S3 A and B). Dexamethasone also downregulated EPCR expression in lungs of infected mice (Fig...
4B) and sEPCR concentration in serum and bronchoalveolar lavage (BAL) (Fig 4C and D). VEGF concentration, an essential factor to increase vascular permeability and ARDS development, also decreased after dexamethasone treatment in the BAL of infected mice, on the 7th dpi (Fig 4E).

Consequently, we analyzed the lung vascular permeability and dexamethasone-treated infected mice showed to be protected compared to non-treated mice (Fig 4F-H). To clarify the mechanism that reduced the lung permeability, the opening of interendothelial junctions in PMLEC through actin analyzes was evaluated. The PMLEC stimulated with iRBC increased the spaces between the interedothelial junctions and leads to the disruption and reorganization in the actin filaments which was protected by the treatment with dexamethasone (Fig 4I and J). iRBC-stimulated PMLEC also demonstrated more permeability of Evans Blue in a transwell assay, compared to dexamethasone-treated PMLEC (Fig 4K). Additionally, dexamethasone-treated PMLEC showed a reduction in VEGF concentration compared to cells not treated with dexamethasone (Fig 4L). Everything considered, these data suggest that dexamethasone is capable to reduce inflammation, decreasing EPCR and vascular permeability in PMLEC and lungs of DBA/2 mice.

**Anti-inflammatory drug protects mice from ARDS but not from malaria infection**

Dexamethasone-treated mice improved their respiratory parameters [Respiratory Frequency, Tidal Volume and Enhanced Pause (Penh)] compared to infected non-treated mice, on the 7th dpi (Fig 5 A-C). On the other hand, parasitemia levels increased in peripheral blood in dexamethasone-treated mice on the 7th dpi (Fig 5D) and these mice succumbed between 8 and 20 dpi with characteristics of HP, without edema and lower inflammatory infiltration according to the histopathological and count of iRBC.
Finally, we proposed the essential mechanisms of protection of dexamethasone in experimental malaria (Fig 6) suggesting that PbA-iRBC induce TNF release by endothelial cells and by Mφ, then the TNF up-regulates EPCR expression in PMLEC, increasing the iRBC adhesion through EPCR pathway. The adhesion of iRBC in PMLEC leads to gap formations increase in the interendothelial junction, raising vascular permeability and consequently, edema formation. Activated alveolar macrophages also produce TNF, which contributes to the activation of endothelial cells, possible recruitment of neutrophils [14] and alveolar damage. The drastic reduction in inflammation with dexamethasone may be responsible for the increase in parasitemia and mortality. All things considered, these data suggest that TNF is essential to increase expression of EPCR and consequently, increase adhesion of iRBC in malaria-associated ARDS.

Discussion

We describe, in the present study, the cytoadherence of PbA-iRBC in microvascular lungs endothelial cells in mice which develop ARDS as well as the importance of the EPCR in this mechanism and, consequently, in the ARDS pathogenesis. A relevant advantage of this study is that our animal model reproduces physiopathological aspects from those found in human ARDS [12,13,25]. Moreover, the pathogenesis of ARDS in this experimental model is only related to pulmonary manifestation development, because there are no inflammatory signs associated to cerebral malaria [13], differently from what occurs in some other animal models [26]. It is also important to note that most ARDS-associated malaria models have their syndrome aspects compared with those in non-infected mice. Here, we compared
aspects of ARDS mice with those also infected but had not developed the syndrome and
died from hyperparasitemia and anemia.

It is well known that mature forms of *P. falciparum*-iRBC can adhere to the
microvasculature of different organs which is an important feature in the pathogenesis
of severe malaria in humans [5,27]. Animal models have been used to study severe
malaria pathogenesis which is well established, especially for cerebral malaria [28].

However, as there are just few studies focusing ARDS murine models [11,26], the
pathogenesis is not completely enlightened. Recently, PbA-iRBC has been
demonstrated, *in vitro*, to adhere to MVECS (microvascular lung endothelial cells from
CBA/Ca mice) and that TNF-stimulated cells have expressed more ICAM-1 and VCAM
[16], and in our results. Nonetheless, there are not studies about the expression of EPCR
in acute respiratory distress syndrome in human beings or murine malaria. It is known
that iRBC adhere to endothelial cells, *in vivo* and *in vitro*. Nevertheless, the study herein
is the first showing PbA-iRBC adherence using DBA/2 mice that developed ARDS.

Additionally, our findings provide the first evidence that PbA-iRBC adhere more in
ARDS-developing mice than in those HP-developing. Moreover, we found that ARDS-
developing mice have about 28.5 times more EPCR expression than those not infected,
implying that EPCR can be essential in ARDS progress. Finally, we demonstrated that
TNF-stimulated cells induced EPCR expression while iRBC did not.

Previously, we had found that ARDS-developing mice showed more parasites load in
lungs, which can be important to induce the syndrome, than those HP-developing. On
the other hand, it is mostly unknown whether the parasite can directly induce ARDS
development or not.

Quite a few works have been presenting the adherence of PbA Luciferase (*PbAluc*) *in
vivo* [29,30] and our work reinforces it. The *PbAluc*-iRBC was accumulated in the
lungs of DBA/2 mice, especially in those ARDS-developing, when the organs were
perfused we confirmed that the PbA-iRBC was adhered mainly in the lungs and spleen.
On the other hand, in our experimental model, there was no adhesion in the brain as in
other models of ARDS [11,26].
Also, ARDS-developing mice showed more hemozoin (Hz) area in lungs compared to
those HP-developing on the 7th dpi, the onset of ARDS development, and some previous
works suggest that Hz, which is released from the food vacuole into circulation during
erthrocyte lysis is rapidly taken up by circulating monocytes and tissue macrophages,
inducing the production of pro-inflammatory mediators [31,32]. In addition Hz linked
by Plasmodium DNA is a ligand for TLR 9, which activates innate immune responses in vivo and in vitro, resulting in the production of cytokines, chemokines, and up-
regulation of costimulatory molecules [33,34] and proinflammatory mediators like IL-1β, via NLP3 inflammasome complex activation [32].
Interestingly, due to the fact that some mice develop ARDS and others do not, and HP-
developing mice also had some Hz and parasite adhered in lungs, we further
investigated the adhesion of PbA-iRBC in endothelial cells with different stimuli. Other
studies show that endothelial cells stimulated with TNF had an increase in ICAM-1
expression [35,36], corroborating with our results in vivo and in vitro, as recently
demonstrated that TNF and ICAM-1 markers were increased in the lungs of the patients
who developed the syndrome [37]. It is interesting to highlight that the parasites by
themselves (PbA-iRBC or PbA-iRBC extract) cannot upregulate the ICAM-1, VCAM
or EPCR expression in DBA/2-PMLEC. Actually, PbA-iRBC adhere more in TNF-
stimulated DBA/2-PMLEC.
With the hypothesis that inflammation is essential to expose adherence molecules and
consequently ARDS pathogenesis, we treated DBA/2 mice with dexamethasone to
reduce inflammation, observing that these mice were protected from ARDS and had a
downregulation in EPCR expression, which indicates that EPCR may be important to
iRBC adherence and to the pathology progress of the disease. However, in a recent
study, the expression of EPCR decreased in the lungs of *P. falciparum*-infected patients
who developed ARDS when compared to those who did not develop, analyzed by
immunohistochemistry. Although, the authors suggest that the change in EPCR,
together with thrombomodulin in association with the deposition of hemozoin in the
lungs, play an important role in the pathogenesis of ARDS [37]. Besides that,
dexamethasone-treated mice are less susceptible to increase the vascular permeability.
Likewise, iRBC-stimulated PMLEC and treated with dexamethasone showed lesser
permeability compared to not treated cells. These results are similar to those found in an
experimental model of C57bl/6 mice infected with *P. berghei* NK65 and treated with
the same dose of dexamethasone. The authors showed an increase in the peripheral
parasitemia and ARDS protection [26], but not an effect in the pulmonary vascular
permeability. On the other hand, the increase in vascular permeability was previously
found in our ARDS model [12].

*P. falciparum*-infected patients present elevated levels of circulating nitrogen oxide
reactive intermediates and by cytokines, such as IFNα/γ, TNF, IL-1, IL-6, and the
chemokine IL-8. Furthermore, these cytokines and chemokines levels have been found
to correlate with different manifestations of severe malaria [32]. Additionally, although
ARDS of different etiologies is characterized by a local inflammatory response, the
inflammatory mediators can spread into the general circulation. The severity of ARDS
depends on of the magnitude of the resultant systemic inflammatory response [38].
Based on our results, the treatment with dexamethasone, a potent anti-inflammatory
drug, decreased the serum concentrations of IL-33, IL-6 and TNF in PbA-infected mice.
IL-33 is a tissue-derived nuclear cytokine from the IL-1 family and it’s highly expressed during homeostasis and inflammation in endothelial, epithelial and fibroblast-like cells, working as an alarm signal released upon cell injury or tissue damage to alert immune cells to express the ST2 receptor (IL-1RL1). The major targets of IL-33 in vivo are tissue-resident immune cells such as mast cells, group 2 innate lymphoid cells (ILC2s) and regulatory T cells (Tregs). IL-33 is emerging as a crucial immune modulator in regulatory immune responses and infectious inflammatory diseases [39]. Bronchial IL-33 expression is significantly increased in severe malaria patients with pulmonary edema [40]. Moreover, in PbA-induced experimental cerebral malaria (ECM), IL-33 expression is increased in brain, and ST2-deficient mice were resistant to PbA-induced neuropathology [41,42]. On the other hand, another publication demonstrated that PbA-infected C57BL/6 mice treated with recombinant IL-33 presented no signs of neurological pathology associated with CM and had reduced production of pro-inflammatory cytokines and chemokines[43].

The IL-6-type cytokines are released in response to tissue injury and/or an inflammatory stimulus. They act locally and systemically to generate a variety of physiologic responses, especially in the acute phase response [44]. Circulating IL-6 levels are elevated in nearly all infectious, traumatic, and inflammatory states including ARDS. Elevated levels of IL-6 are found in BAL and plasma of patients with ARDS and those at risk [45,46]. Finally, the TNF is an inflammatory mediator strongly implicated in the development of ARDS [47], signal through two receptors, p55 and p75, which play differential roles in pulmonary edema formation during ARDS. It was shown that p55 by a novel domain antibody (dAb™) attenuated ventilator-induced lung injury [48] and also lung injury and edema formation in models of ARDS induced by acid aspiration [47]. Recently, a selective blockage of TNFR1 (GSK1995057 antibody) inhibited
cytokine and neutrophil adhesion molecule expression in activated HMVEC-L monolayers in vitro and attenuated inflammation and signs of lung injury in primates. Moreover, treatment with this antibody attenuated pulmonary neutrophilia, inflammatory cytokine release and signs of endothelial injury in BAL and serum sample in healthy humans challenged with a low dose of inhaled endotoxin [49].

In conclusion, our data suggest that P. berghei infection induces TNF production by inflammatory and endothelial cells, leading to the expression of adhesion molecules, such as ICAM-1, VCAM and, especially EPCR. These results allow us to infer that those factors are essential for PbA-iRBC cytoadhesion, and, thus, strongly suggest the participation of these mechanisms in the pathogenesis of malaria-associated ARDS. Being known that inhibition of cytoadhesion mechanisms may improve the prognosis of infection [50], we emphasize that the processes described above may be important targets in the treatment of ARDS due to Plasmodium infection, acting as adjunctive therapy in association with the use of antimalarials, not only making the treatment more efficient, but also reducing the morbidity and mortality of these patients.

Methods

Experimental outline

Ten to twelve DBA/2 mice per group were infected with 10⁶ infected red blood cells (iRBC) of Plasmodium berghei ANKA (PbA).

To classify PbA-infected mice in ARDS-developing or HP-developing before the death, on the 7th dpi, we used parameters such as respiratory patterns (enhanced pause and
respiratory frequency) and parasitemia as predictive, according to previously described [12]. Lung tissue, blood and BAL samples were collected from mice on the 7th day post infection (dpi).

**Ethics statement**

All experiments were performed in accordance with the ethical guidelines for experiments with mice, and the protocols were approved by the Animal Health Committee of the Biomedical Sciences Institute of the University of São Paulo (CEUA nº 24, page 16, book 03). The guidelines for animal use and care were based on the standards established by National Council for Control of Animal Experimentation (CONCEA: Conselho Nacional de Controle de Experimentação Animal), The Brazilian College of Animal Experimentation (COBEA) and Brazilian Federal Law nº 11.794. Infected mice were monitored daily and euthanized with (150 mg/kg)/xylazine (15 mg/kg) when they presented any symptoms of suffering. Animals for the experimentation were euthanized with ketamine and xylazine on the 7th after infection according to the approved ethics committee.

**Mice, parasites and euthanasia**

Male DBA/2 mice between 6-10 weeks old (purchased from the Department of Parasitology, University of São Paulo, Brazil) were infected with $1 \times 10^6$ *P. berghei* ANKA (clone 1.49L) iRBCs, as previously defined [12]. Parasitemia and mortality were monitored daily. Parasitemia was determined by Giemsa staining and expressed as the percentage of infected red blood cells. The euthanasia of mice was made using ketamine (150 mg/kg)/xylazine (15 mg/kg).
**Determination of Respiratory Pattern**

Respiratory patterns (respiratory frequency [RF], tidal volume [TV] and enhanced pause [Penh]) were monitored on the 7th dpi by an unrestrained whole-body plethysmography chamber (WBP, Buxco Electronics, USA) for 10 minutes (basal level), according to formerly described [12].

**Histopathological analyses and hemozoin count**

Lung tissue fragments were fixed with 10% buffered formalin for 24 hours and kept in 70% ethanol until embedding in paraffin and 4–5 μm sections were stained with hematoxylin-eosin (HE). To perform the count of hemozoin (Hz) area, lungs of mice euthanized (ARDS-developing or HP-developing mice, on the 7th day after-infection according to the predictive model [12]) were stained with H&E, 10 images were captured from each tissue using polarized light (400x magnification) using a Zeiss color camera (cam Axio HRc), connected to a light microscope Zeiss (Axio Imager Program. M2). The corresponding percentage of Hz in each image area was identified using the program Image J in which the points of Hz were distinguished by brightness adjustment.

**Quantification of gene expression**

Quantitative RT-PCR was performed for relative quantification of gene expressions from lungs of non-infected and infected mice. RNA extraction was performed according to the "Animal Cell I" protocol from RNeasy Mini (Qiagen, USA). cDNA synthesis was performed with 1μg RNA sample, using First Strand cDNA Synthesis kit RT-PCR (Roche, USA) according to manufacturer's instructions. Finally, to gene expression, SYBR Green PCR Master Mix (Applied Biosystems, USA) and relative quantification
2 (-ΔΔCT) method were used as described before (51). The qRT-PCR reactions were performed in the 7500 Fast instrument (Applied Biosystems, USA) using oligonucleotides: PbA 18s (forward 5'-agcattaaataaagegaatacatcttc-3'; reverse 5'-ggagatgtgggtttagtgtg-3'); HPRT (forward 5'-aagcttgctggtgaaaagga-3'; reverse 5'-ttgcgctcatcttaggcttt-3'); ICAM-1 (forward 5'-cgaaggttcttcttcttctc-3'; reverse 5'-gttcgcccaatgtg-3'); VCAM (forward 5'-agtccgttctgacctcgag-3'; reverse 5'-tgtctggagccactcctg-3'); EPCR (forward 5'-gacgaagtttctgccgctac-3'; reverse 5'-ctggaggatgtgggtttcttt-3'); TNF (forward 5'-aatggcctcctcctcagtt-3'; reverse 5'-ccacttggtgtgtcagga-3').

Parasite localization by bioluminescence

DBA/2 mice were infected with *P. berghei* ANKA expressing luciferase. On the 7th day post-infection, luciferin (VivoGlo™ Luciferin, In Vivo Grid, Catalog #: P1041, Promega) was injected allowing the emission of parasite luminescence. Then the localization of the parasite was analyzed with IVIS Spectrum (PerkinElmer). For this purpose, mice were sedated with isoflurane for pictures (approximately 6 minutes after the injection of luciferin). Later, they were euthanized and perfused. After the perfusion, a new image of the mice was captured, the organs were collected and placed in sterile petri dishes for observation of bioluminescence of each tissue.

Cytokines quantification

The quantities of TNF, IL-6, IL-33 in serum and culture supernatants were determined by ELISA commercial kits eBioscience (San Diego, CA, USA) *mouse Elisa Ready-Set-Go*: TNF (ref 88-7324-88), IL-6 (ref 88-7064-88) and IL-33 (ref 88-7333-88), according to the manufacturer protocols.
Isolation of the primary microvascular lung endothelial cells

Primary microvascular lung endothelial cells (PMLEC) were obtained from DBA/2 mice, as described before (15, 52). Briefly, after euthanasia, the animal body was disinfected with iodine alcohol. Then, the mice had all its blood taken by cutting the carotid artery. In a laminar flow chamber, the lung tissue was cut into fragments of approximately 1mm² and distributed among 6-well polystyrene plates in 20% of fetal bovine serum (FBS) and antibiotics supplemented DMEM culture medium at 37°C and 5% CO₂. After, 72 h, the tissue fragments were removed and 50% of the medium was replaced. After 7 days of incubation, the cells were removed with trypsin 0.25% EDTA (Gibco) and replaced in a 75 cm² culture flask. The trypsinization procedure was repeated every 5 to 7 days. Finally, the cells were cultured for 15 to 20 days (3rd and 4th passage) until being used in the trials. The isolated DBA/2-PMLEC were characterized by immunofluorescence with Lectin from *Ulex europaeus* (Sigma-aldrich, USA–l9006) and the antibodies anti-VWF (Santa Cruz Biotechnology, USA – sc14014), anti-CD31 (Abcam, UK – ab28364), anti-ACE (Abcam, UK – ab85955), anti-CD62E (Abcam - ab18981), anti-eNOS (Abcam, UK – ab87750) and anti-VE-cadherin (Abcam, UK – ab205336) (Fig S1).

Plasmodium synchronization and enrichment of parasitized erythrocytes

To obtain mature forms of *P. berghei* ANKA, iRBCs were synchronized as described previously (53). Succinctly, iRBCs were collected from infected mice exhibiting 10 to 20% parasitemia through cardiac puncture and transferred to RPMI 1640 culture medium (Gibco-Thermo, USA) supplemented with 25% fetal bovine serum (FBS). The iRBCs were subsequently maintained in vitro at 37°C for 14 h in an atmosphere
containing 5% CO₂, 85% N₂, and 10% O₂. The parasitized erythrocytes were then
enriched using a magnetic separation column (Miltenyi Biotec, USA) to generate cell
populations consisting of approximately 95% iRBCs, as assessed by thick blood smears.
P. berghei extract was obtained from iRBC subjected to several freeze-thawing cycles.

Transwell assay with endothelial cells and peritoneal macrophages
Sterile 13 mm diameter cover slip-knittel glass plates were placed in 24-well culture
plates. The coverslips were treated with 0.2% gelatin (gelatin from bovine skin - Sigma-
Aldrich) diluted in 1x PBS. PMLECS were seeded in the culture plate (7 x 10⁴ cells /well) in 400 μl of complete DMEM medium and 1x10⁵ peritoneal macrophages (Mφ)
were seeded in the transwell membrane (6.5 mm membrane diameter, 0.4 μm
membrane pore, Corning, Costar 3470). After 24 hours, 2.5 x 10⁶ mature forms of
iRBC or RBC were added over the Mφ and incubated for 24 hours. After this time, the
supernatant between Mφ and endothelial cells was collected for the quantification of
cytokines by ELISA. Subsequently, endothelial cells were stimulated with 1.75 x 10⁶
iRBC for adhesion assay.

Plasmodium berghei ANKA adhesion assay under static conditions
DBA/2-PMLEC were plated in Lab-Tek slides "chamber slide" permanox of 8 wells
(3x10⁴ cells/well) (Thermo Fisher Scientific - 177 455). Afterwards, the stimuli with
TNF-α (50ng/ml) are added and kept for 24, 48 or 72 hours. After stimulation,
synchronized PbA-iRBC were added for 1 hour in culture at a ratio of 25 iRBC/
PMLEC at 37 and 5% CO₂. After the incubation period, the culture medium was
removed and detached from the chamber slides, washed, fixed with methanol, stained
with Giemsa and then observed under an optical microscope immersion (magnification
1000 times). The counting pattern used was the observation of the number of adhered parasites at 100 DBA/2-PMLEC and expressed as a percentage.

**Parasite adhesion assay in flow conditions**

PMLEC between 3rd and 5th passage was seeded (8x10^4/well) in 2-wells permanox chamber slides labteck (Thermo Scientific, Nunc). The cells were incubated at 37 °C and 5% CO² for 24 hours. After this time, TNF might have been added for 24 hours or not (control). In sequence, the stimulus was removed and mature forms of EP-PbA (25 per cell) in DMEM supplemented with 20% FBS were added and allowed to interact with the endothelial cells for 1 hour at 37°C in 5% CO₂.

Subsequently, the wells were detached from the polystyrene slides and the slides were added to the flow system composed of a chamber (Cell Adhesion Flow chamber, Immunetics) which kept the slide adhered through the formation of a vacuum (coupled by a vacuum pump), a syringe pump (insight Inc.), an inverted microscope (Zeiss Vert. A1) connected to a camera (Axio Cam E Rc 5s Zeiss) and to a computer with an image capture system (program Zen 2011, AxioVision Rel 4.8.2 SP2).

The chamber was initially filled with DMEM medium, then an initial photo with the parasites adhered to the CEPP-DBA/2 was taken, after, a medium continuous stream was run through the chamber and maintained at 2 ml/hour with a pump syringe (Insight Inc). Five images, every 3 minutes, of each well were captured, maintaining the same field as the initial image, to determine the erythrocyte binding efficiency.

Image processing and analysis were performed by ImageJ (Version 1.46r). The images were opened individually in ImageJ and analyzed through the "cell counter" plugin, where all EP-PbA images were selected on each image. After counting all the images, the total of EP-PbA that remained in the final image (15 minutes) was recorded.
experiment was repeated with two independent cultures, each with two technical replicates. Image processing and analysis was done in Image J.

Treatment with dexamethasone

80mg/kg of dexamethasone (Decadronal, ache) was inject intraperitoneally (ip) on days 5 and 6 after infection 80 mg/kg, according to previously described [26].

Lung permeability and edema

To investigate lung permeability, non-infected or PbA-infected mice (dexamethasone-treated or not), on the 7\textsuperscript{th} dpi, were injected intravenously with 0.2mL of 1\% Evans Blue (Sigma). The mice were euthanized 45 minutes later, and the lungs were weighed immediately and placed in 2mL of formamide (Merck) for 48 hours at 37\textdegree C [12]. The absorbance of the formamide was then measured at 620 nm and 740 nm. The amount of Evans Blue staining per gram of lung tissue was calculated from a standard curve.

Measure of primary microvascular lung endothelial cells permeability.

The increased lung vascular permeability was analysed in DBA/2-PMLEC plated on inserts of permeable membranes with 0.4\textmu M pores (Transwell Corning), pre-treated with gelatin and coupled in 24-well polystyrene plates at a concentration of $2.2 \times 10^4$ cells per insert and maintained in DMEM culture at 37\textdegree C, as previously described (15). After 96 hours, until the cells reach confluency, the extract was applied for 1h after incubation with dexamethasone (500 ng/ml during 24 h) or solely with 20\% FBS supplemented DMEM culture medium. Subsequently, the culture medium was replaced by Hank’s balanced salt solution and in the upper compartment of each insert, in contact with the cells; 200\textmu l of Evans Blue was incubated at 2mg/mL concentration at 37\textdegree C.
After 30 min, the liquid from the lower compartment was collected and analysed in a spectrophotometer at 650 nm wavelength (NanoDrop 2000, Thermo Scientific). Finally, the concentration of Evans Blue was determined from a standard curve (0.2mg/mL to 0.0031mg/mL) as previously known.

**Actin Microfilaments Identification by Immunofluorescent and Morphometric Analysis of the Opening of Interendothelial Junctions.**

In order to analyse the area of interendothelial junctions, PMLEC were plated in 24 well plates (7x10^4 cells/well), adhered to gelatin on glass coverslips, and maintained at 37°C and 5% CO2. The cells were stimulated with either iRBC or RBC for 1 h, after incubation with dexamethasone (24 hours), or solely with DMEM culture medium, supplemented with 20% FBS, in triplicate. Subsequently, the cells were fixed with 3.7% formaldehyde, permeabilized with acetone at −20°C, and blocked with bovine serum albumin solution (1% BSA). Actin was marked with Texas Red Phalloidin (T7471, Life Technologies) by 20 minutes. The cell nuclei were marked with Hoechst (H33342, Life Technologies). Each slide, with fully confluent cells, was chosen randomly and ten to twenty pictures were taken and scanned in a “zig-zag” way, from top to bottom. The images were acquired in the fluorescence Axio Imager M2 microscope (Zeiss) using the Axio Cam HRc (Zeiss) and the software Axio Vision, version 4.9.1.0. The total area of OIJ was measured in each picture using the software ImageJ.

**TNF-blockage**

TNF was blocked in DBA-PMLEC using Mouse TNF Neutralizing (D2H4) Rabbit mAb #11969 (Cell signaling®) in a concentration of 1 μg/ml for 24 hours in PMLEC.
Soluble EPCR quantification

Soluble endothelial protein C receptor (sEPCR) was measured with an ELISA kit (Elabscience®, E-EL-M1073), according to the manufacturer’s instructions.

EPCR Western Blot

Fresh frozen mice lung tissues collected on the 7th dpi were sonicated and homogenized at 4°C, using the Radio-Immunoprecipitation Assay (RIPA) buffer composed of 50 mM TrisHCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulphate (SDS), 1 mM sodium orthovanadate, 1 mM NaF, and a protease inhibitor tablet. The total protein concentration was determined using a SpectraMax® Plus 384 (Molecular Devices) spectrophotometer with a 562 nm filter using the Pierce™ BCA Protein Assay kit (Thermo Scientific) according to the manufacturer instructions. Then, 30 μg of protein from each sample was electrophoretically separated in 10% polyacrylamide mini-gel (Bio-Rad) with SDS-PAGE at 100V for 2 hours, in a Mini-PROTEAN® Tetra cell apparatus (Bio-Rad). The Kaleidoscope commercial standard Precision Plus Protein (Bio-Rad), standard molecular weight, was used. Subsequently, proteins separated on the gel were transferred to PVDF (Polyvinylidene Difluoride, 0.2 μm, Bio-Rad) membranes in transfer buffer (0.1 M glycine, 0.02 M Tris, 20% methanol and MilliQ water) overnight at 4°C in a wet transfer apparatus at 70mA constant voltage (Bio-Rad). Detection of chemiluminescence immunochemical reaction was performed on a ChemiDoc™ XRS + Molecular Imager® (Bio-Rad) transilluminator with ImageLab™ program (Bio-Rad). Blocking of non-specific sites was carried out after the transfer; (Tris-buffer-saline-Twin-20 buffer, 0.05 M TRIS, 0.1 M NaCl, pH 7.3 and 0.1% Twin-20, Synth), BSA 3% under a 2 h stirring at room temperature (RT). The
membrane was then incubated with the primary anti-EPCR antibody (# 151403, abcam) 1: 1,000 stirring overnight at 4°C. After washing in TBS-T, the membrane was incubated with the peroxidase-conjugated rabbit anti-rabbit IgG (HRP) secondary antibody (# AP307P, Millipore) at 1: 6,000 concentration in TBS-T for 1 hour (RT). Bands were detected by the chemiluminescence method Clarity Western ECL (Bio-Rad) for EPCR and the housekeeping protein β-actin (mouse IgG # NB600-501, Novus, at the 1: 40,000) and finally, densitometrically measured by ImageJ 1.6.0 software.

**Bronchoalveolar lavage and VEGF quantification**

On the 7th dpi, infected mice (not-treated or treated with dexamethasone) were anesthetized, and for bronchoalveolar lavage (BAL) collection, the trachea was cannulated, and lungs were washed once with 1.0 ml of PBS 1X. An ELISA kit (R&DSystems, USA) was used to quantify VEGF levels in BAL and in culture supernatant according to the manufacturer instructions.

**Transfection of EPCR siRNA**

To transfect EPCR to interference RNA assay, Lipofectamine® RNAiMAX Reagent (Invitrogen, by life technologies) and a poll of 3 different oligonucleotides silencer select pre-designed and validated siRNA (Ambion, by life technologies) were used according to the manufacturing protocol. Also, a Silencer Select Negative Control (Ambion 4390843) and a silencer select GAPDH positive control (Ambion 4390849) were employed.

siRNA1: forward: 5’ CAACCGGACUCGGUAUGAATT 3;
reverse: 5’UUCAUACCAGUCCCGGUUGta3’
siRNA2: forward: 5’ACGCAAAACAUGAAAGGGATT 3’
reverse: 5’UCCCUUUAUGUUUUGCGUGG 3’
siRNA3: forward: 5’ CGCCCUUUGUAACUCCGAUTT 3’
reverse: 5’AUCGGAGUUACAAAGGGCGCA3’

Statistical Analysis

The statistical analyses were performed using the GraphPad Prism® 5.0 software for analysis and graphing. The data were analysed for normality by Kolmogorov-Smirnov test or Shapiro-Wilk normality test and the variance with Bartlett test. Non-parametric variables for two groups were compared by using Mann-Whitney test. For analysis of three groups, we used Kruskal-Wallis test followed Dunn post-test. In order to compare parametrical variables for two groups, T-test was employed and for three or more groups, One-way ANOVA followed by Bonferroni post-test were used. For the survival curves, Log-rank and Gehan-Breslow Wilcoxon tests were applied. The differences between the groups were considered significant when $p \leq 0.05$ (5%). To establish a cut-off from data, ROC curves were generated by using the results of the control group obtained in MedCalc version 8.2.1.0.

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Figure Captions

Fig 1. ARDS-developing mice showing higher load pulmonary parasite than HP-developing mice. (A) Infected mice had luminescence signal in the peripheral blood and tissues and (B and C) perfused infected mice showing luciferase/luciferin signal on the 7th day after infection. (B and C) The luminescence was more concentrated in lungs and spleen. Images taken in IVIS® Spectrum in vivo imaging system [Kruskal-Wallis test and post-test Dunn’s, n= 3. Bars represent mean ± SEM (*, p<0,05)]. (D) rRNA gene expression of P. berghei ANKA (subunit 18s) in lung tissue of euthanized mice on the 7th day after infection, using 2-ΔΔCT method. ARDS-developing mice (n= 15) have more P. berghei mRNA than HP mice (n= 12 HP); (E-G) Hemozoin quantification in polarized light [(magnification: 400x, scale bar: 20μm n=3 mice/group, (10 images for each tissue) bars represent mean ± SEM (*, p<0,05 Student’s T-test)]. (H) Lung histological analyses showing iRBC in close contact to endothelial cells in a mouse that died by ARDS (magnification: 630x, scale bar: 10 μm). HP: hyperparasitemia; ARDS: acute acute respiratory distress syndrome; iRBC: infected red blood cells. Infection of DBA/2 mice with P. berghei Luciferase (PbA-luciferase). Lu: lung; Sp: spleen; Ki: kidney; Li: liver; Br: brain; He: heart.
Fig 2. TNF increases Plasmodium berghei adherence in ARDS experimental model.

(A) ARDS-developing mice (n= 10) have more TNF in serum compared to HP (n= 8) and NI mice (n= 6 NI); (B) PMLEC stimulated with iRBC secrets more TNF compared to NS cells within 24 hours (ELISA, n= 4/group). PMLEC stimulated with TNF have more adhesion of iRBC compared to not stimulated cells (NS) in (C) 24 hours (n= 4 NS, 4iRBC), (D) 48 hours (n= 7 NS, 6 TNF) and (E) 72 hours in static conditions and (n= 7 NS, 6 TNF) (F-H) in flow conditions (n= 2 NS, 4 TNF). (I-K) A transwell assay with peritoneal macrophages (Mφ) in indirect contact with endothelial cells shows that Mφ stimulated with iRBC for 24 hours produce a large quantity of (J) TNF compared to non-stimulated cells. (K) Moreover, PMLEC, in indirect contact with Mφ with previously stimulus with iRBC, showed more capacity of iRBC adherence then cells without previously contact with Mφ (J-K: n= 3-4/group). (L) TNF neutralizing reduced iRBC adherence in PMLEC (n= 8/group). Bars represent mean ± SEM, Graphics A, J. K and L: Nonparametric ANOVA, Kruskal-Wallis test, Post Test Dunns. Graphics B, C, D, E and H: Mann-Whitney T test (*p<0.05; ** p<0.01; *** p<0.001). ARDS: acute respiratory distress syndrome; HP: hyperparasitemia; PMLEC: primary culture of microvascular lung endothelial cells from DBA/2 mice; Mφ: peritoneal macrophage; NS: non-stimulated cells; RBC: red blood cell; iRBC: infected red blood cells.

Fig 3. EPCR acts directly in P. berghei cytoadherence. (A) ARDS-developing mice have more EPCR mRNA expression in lungs (n= 17 ARDS, 11 HP), (B) more soluble EPCR (sEPCR) in sera (n= 4/group), and (C) more EPCR protein in lungs compared to HP mice (n= 6 ARDS, 5 HP). PMLEC stimulated with TNF show upregulation in EPCR expression compared to non-stimulated cells (NS) or cells stimulated with iRBC in (D) 24 hours (n= 4 NS, 6 iRBC, 6 TNF), (E) 48 hours (n= 5 NS, 6 iRBC, 6 TNF) and (F) 72 hours after treatment (n=4 NS, 5 iRBC, 6 TNF). (G) EPCR knockdown reduced
EPCR mRNA expression in PMLEC (n= 6/group) and (H) reduced iRBC adherence in PMLEC compared to TNF stimulated cells (n= 7/ group). Bars represent mean ± SEM. Graphics A and B: Mann-Whitney T test; Graphics D-H: Nonparametric ANOVA, Kruskal-Wallis test, Post Test Dunns (*p<0.05; **p<0.01; ***p<0.001). ARDS: acute respiratory distress syndrome; HP: hyperparasitemia; PMLEC: primary culture of microvascular lung endothelial cells from DBA/2 mice; NS: non-stimulated cells; RBC: red blood cell; iRBC: infected red blood cells. Red dashed lines: non-infected mice mean.

Fig 4. Dexamethasone reduces TNF and EPCR, protecting mice lungs and PMLEC from increased vascular permeability. (A) TNF mRNA expression is reduced in Dexamethasone-treated mice (n= 5/group). (B) EPCR mRNA expression in lungs (n= 9/group) and (C) sEPCR concentration in serum (n= 4 Infected, 8 Infected+Dexa) and in (D) BAL is down regulated in Infected+Dexa (n= 4) compared to infected-not-treated mice (n= 5). (E) VEGF concentration is also decreased in Infected+Dexa mice (n= 5/group). (F-G) Infected+Dexa mice have less Evans blue accumulated in lungs compared to NI or infected mice (after perfusion) (n=4/group) and (H) reduced lung weight (n= 4/group). (I-J) Actin microfilaments staining in PMLEC (grey) and nuclei stained with Hoechst (blue) showing that endothelial cells stimulated with dexamethasone have less opening in the interendothelial junctions (indicated by yellow arrows) compared to non-treated cells. (K) PMLEC stimulated with iRBC and treated with dexamethasone (n=12) have less Evans blue flow-through compared to non-treated cells (n= 11) in a transwell assay and (L) decreased VEGF concentration. iRBC: infected red blood cells; Infected+Dexa: mice infected with P. berghei ANKA and treated with dexamethasone (n= 4/group); BAL: Bronchoalveolar lavage; iRBC: infected red blood cells; Red dashed lines: non-infected mice mean. Bars represent
mean ± SEM (*, p<0.05; ***, p<0.001) (A-D; F-G; J-K Mann-Whitney test; I and L kruskal-Wallys test).

**Fig 5. Dexamethasone protects mice from ARDS but not from malaria infection.**

(A) Respiratory frequency, (B) tidal volume and (C) enhanced respiratory pause improved in Infected+Dexa mice compared to those infected-control. (D) Parasitemia of Infected+Dexa mice is increased but (E) they survive more compared to infected-not-treated mice (A-E: n= 9 infected, 8 Infected+Dexa). Representative image of histological analyze of (F) infected mice and (G) Infected+Dexa mice (Scale bar: 50 µm). Infected+Dexa: mice infected with *P. berghei* ANKA and treated with dexamethasone. Red dashed lines: non-infected mice mean. Bars represent mean ± SEM. Graphics A-D: Mann-Whitney T test. (*p<0.05; ** p<0.01).

**Fig 6. Schematic representation of ARDS development involving adhesion of iRBC to EPCR.** *Plasmodium berghei* ANKA infected red blood cells (iRBC) induce TNF release by endothelial cells. The stimulus with TNF up-regulate EPCR expression in PMLEC, which increases the adhesion of iRBC in PMLEC through EPCR pathway. The adhesion of iRBC in PMLEC lead to the increase of gap formations in the interendothelial junction, increase in vascular permeability with infiltration of inflammatory cells, red blood cells and edema. Activated alveolar macrophages also produce TNF, which contribute to the activation of endothelial cells, recruitment of neutrophils and alveolar damage. Mice treatment with dexamethasone decrease inflammatory cytokines release, including TNF and consequently downregulate EPCR expression in PMLEC; the dexamethasone also decreases VEGF released by endothelial cells, protecting mice from gap formations, vascular permeability, inflammatory cells infiltration and alveolar damage. PMLEC: primary culture of microvascular lung endothelial cells from DBA/2 mice.
Supporting information

S1 Fig. Primary microvascular lung endothelial cell characterization. PMLEC were stained by immunofluorescence with Lectin from *Ulex europaeus*, and anti- vWF, CD31, ACE, CD62E, eNOS and Ve-cadherin antibodies. Nuclei stained with Hoechst (blue). PMLEC: primary culture of microvascular lung endothelial cells from DBA/2 mice. (scale bar: 50 µm).

S2 Fig. ARDS-developing mice show more ICAM-1 and VCAM expression in lungs compared to HP-developing or non-infected mice. (A) Immunohistochemistry of ICAM-1 and VCAM in lungs of NI, ARDS-developing and HP-developing mice on the 7th day post infection (scale bar: 100 µm). There was a decrease in (B) ICAM-1 area in HP-developing mice compared to ARDS-developing and NI mice (n= 11 NI, 39 ARDS, 38 HP) and an increase in (C) VCAM area of ARDS-developing mice compared to NI mice (n= 12 NI, 53 ARDS, 33 HP). (D-I) There was an intensification of ICAM-1 and VCAM mRNA expressions in PMLEC stimulated with TNF for 24, 48 and 72 hours compared to NS or cells stimulated with iRBC, measured by qRT-PCR. (D-I: n= 9 NS, 6 iRBC, 6 TNF). Bars represent mean ± SEM; Kruskal-Wallis test, *p<0.05; ** p<0.01; *** p<0.001. ARDS: acute respiratory distress syndrome; HP: hyperparasitemia; NI: non-infected mice; NS: non-stimulated cells; iRBC: infected red blood cells; PMLEC: primary culture of microvascular lung endothelial cells from DBA/2 mice.

S3 Fig. Dexamethasone reduced inflammation, but not decreased ICAM-1 expression in *P. berghei*-infected mice. Infected+Dexa mice show a decrease in serum levels of (A) IL-6 and (B) IL-33 compared to infected and non-treated mice (A-B: n= 5
Infected, 5 Infected+Dexa). (C) There was no difference in ICAM-1 mRNA expression between Infected+Dexa and infected and non-treated mice lungs and there was a decrease in VCAM mRNA expression in lungs of Infected+Dexa compared to infected-non-treated mice (C-D: n= 9 Infected, 8 Infected+Dexa). Samples collected on the 7th day post infection. Infected+Dexa: mice infected with *P. berghei* ANKA and treated with dexamethasone. Red dashed lines: non-infected mice. Bars represent mean ± SEM. Mann-Whitney T test (** p<0.01).
The figure shows the effects of dexamethasone (Dexa) on respiratory frequency, tidal volume, enhanced pause, and parasitemia in infected animals. The survival rates are also depicted over days after infection.
