Disruption of parasite *hmgb2* gene attenuates *Plasmodium berghei* ANKA pathogenicity

Running title: effect of *hmgb2* gene in experimental cerebral malaria

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Abstract

Eukaryotes High Mobility Group Box (HMGB) proteins are nuclear factors involved in chromatin remodelling and transcription regulation. When released into the extracellular milieu, HMGB1 acts as a pro-inflammatory cytokine that plays a central role in the pathogenesis of several immune mediated inflammatory diseases. We found that the *Plasmodium* genome encodes two genuine HMGB factors, *Plasmodium* HMGB1 and HMGB2 that encompass, as their human counterparts, a pro-inflammatory domain. Given that these proteins are released from parasitized red blood cells, we then hypothesized that *Plasmodium* HMGB might contribute to the pathogenesis of experimental cerebral malaria, a lethal neuroinflammatory syndrome that develops in C57BL/6-susceptible-mice infected with *P. berghei* ANKA and that resembles in many aspects human cerebral malaria elicited by *P. falciparum* infection. The pathogenesis of experimental cerebral malaria was suppressed in C57BL/6 mice infected with PbANKA lacking the *hmgb2* gene (*Δhmgb2 PbANKA*), an effect associated with reduction of histological brain lesions and with lower expression of several pro-inflammatory genes. Incidence of ECM in *pbhmgb2* deficient mice was restored by the administration of recombinant *Pb*HMGB2. Protection from experimental cerebral malaria in *Δhmgb2 PbANKA*-infected mice was associated with reduced sequestration in the brain of CD4+ and CD8+ T-cells including CD8+ Granzyme B and CD8+ IFN-γ cells, and to some extent neutrophils. This was consistent with a reduced parasite sequestration in the brain, lungs and spleen even though to a lesser extent when compared to wild-type *PbANKA*-infected mice. In summary, *Plasmodium* HMGB2 acts as an alarmin that contributes to the pathogenesis of cerebral malaria.
Introduction

Malaria, the disease caused by Plasmodium infection, accounts for an estimated 660000 deaths per year mainly in sub-Saharan regions (WHO, last World Malaria Report). Among the five Plasmodium subspecies infecting humans, P. falciparum, is the most prevalent in these regions where it can cause the development of clinical symptoms ranging from asymptomatic or flu-like illness to the so called severe forms of malaria that include severe anaemia, respiratory distress, acidosis, multi-organ failure and eventually cerebral malaria (1, 2). Of these clinical outcomes, cerebral malaria (CM) is the most common and fatal, occurring mainly in children under the age of 5 years (see for review (2) (3). There is currently no cure for CM and therefore, identification and functional characterization of host and parasite molecules involved in the pathogenesis of this disease is of major importance as it could lead to the development of new therapeutic interventions.

The molecular mechanisms underlying the pathogenesis of CM remain poorly understood. Presumably, sequestration of parasitized red blood cells (pRBC) in the brain micro-vasculature, associated to a systemic inflammation with production of free-radicals and pro-inflammatory cytokines are critical to the onset of this disease. While several host genes encoding, e.g. cytokines, chemokines, adhesion molecules (4-7) and product of haemoglobin degradation have been implicated in the pathogenesis of CM (8-10) related to the pro-inflammatory nature of the immune host response, the individual contribution of genes encoded by Plasmodium onto the CM onset is less clear. Recently, some Plasmodium proteins were reported to play a role in severe malaria. First, in human malaria, the level of expression of several specific PfEMP1 promote cytoadherence to brain endothelial cells and correlate with the severity of cerebral malaria (11, 12), suggesting that they constitute a major virulence factor for cerebral malaria. Second, in rodents, the plasmepsin-4 (pm4) gene an aspartic protease involved in haemoglobin digestion was shown to be implicated in experimental cerebral malaria ECM (13, 14). Finally, several studies reported also a crucial role of ICAM-1 in malaria pathogenesis and, notably, ICAM-1-deficient mice are protected from ECM (15). The combined effect of these pathological events...
is associated with disruption of the physical integrity of the blood brain barrier (BBB) and the development of brain oedema, leading to coma and ultimately to death (1, 16-19). One of the characteristic features of the ECM is the increased sequestration of CD4+ and CD8+ T-cells which were reported to be associated with disease pathogenesis (20, 21). More recently, a subpopulation of CD8+ T-cells expressing Granzyme B was found to be critically involved in ECM (22) and was associated to the parasite burden within the brain of C57BL/6 infected with PbANKA parasite (23). This finding underlines a direct link occurring in the brain between parasite load and CD8+ T-cell recruitment and accumulation of products of haemoglobin oxidation in the pathogenesis of ECM. Pro-inflammatory cytokines such as IFNγ was shown to be implicated in the pathogenesis of ECM (24, 25). Production of IL-6 in response to TNFα by endothelial cells of brain capillaries was also reported in mice genetically sensitive to CM development (26). More recently various reports showed that IFN type 1 governed cerebral malaria (27). High mobility group box (HMGB) proteins are nuclear proteins originally shown to be loosely associated with DNA and to participate, to at least some extent, in the regulation of gene transcription [see for review (28, 29)]. Subsequent studies have shown that the human HMGB proteins composed of two HMGB domains, Box A and Box B in tandem and in particular the HMGB1 isoform are actively secreted from activated innate immune cells, namely macrophages (30) and can be released from injured cells (31) as well. In humans, HMGB1 has an important role as extracellular soluble protein that signals tissue injury and initiates inflammatory response. This “alarmin” protein is considered as a danger signal that alerts the innate immune system to trigger a defensive and an inflammatory response. Indeed, once released, extra-cellular HMGB1 becomes associated with a variety of endogenous ligands that are recognized by several pattern recognition receptors, including members of the toll like receptor (TLR) family as well as the receptor for advanced glycation end products (RAGE) (32). This explains the ability of HMGB1 to trigger inflammatory responses (33) contributing to the pathogenesis of inflammatory disorders (34). Plasma or serum HMGB1 levels were increased in patients with sepsis (35, 36), haemorrhagic shock (37), trauma (38) and high circulating levels of
HMGB1 is often associated with critical illness (39, 40). This pro-inflammatory effect has been mapped to a single 20 amino acid domain of Box B of the mammal protein that alone bears the pro-inflammatory activity of this protein (41). Moreover, huHMGB1 can promote vascular permeability leading to oedema (42). Presumably, the combined pro-inflammatory and vasoactive effects of HMGB1 participate subsequently to the establishment of an amplification loop (43) and explain the involvement of the protein in the pathogenesis of a variety of immune mediated inflammatory diseases. In malaria, the huHMGB1 serum level evaluated in *P. falciparum* infected children developing fatal cases of CM was significantly higher when compared to uncomplicated malaria cases (44, 45) suggesting that as for other immune mediated inflammatory diseases, HMGB1 might be also involved in the pathogenesis of CM.

Considering the established role of human HMGB proteins in the pathogenesis of several inflammatory diseases, we hypothesized that *Plasmodium* HMGB proteins might contribute to inflammation during severe malaria. Among these disorders, ECM is defined as a lethal outcome of *Plasmodium* infection frequently associated with multiple organ failure (46).

We have previously shown that several *Plasmodium* genes encoding transcription-associated proteins (TAP) implicated in chromatin remodelling (47) include the HMGB isoform orthologues *Pf*HMGB1 and *Pf*HMGB2 (48). As for mammalian HMGB, in *Plasmodium* there are two HMGB1 and HMGB2 which consist of only one HMGB domain also comprising a 20 amino acid peptide potentially bearing the pro-inflammatory activity. In keeping with this notion, *P. falciparum* HMGB proteins are released in culture medium and present in the plasma of infected C57BL/6 mice. The recombinant protein was showed to induce TNFα and IL-6 production (49). Since we consider that cerebral vascular obstruction and systemic inflammation are probably combined to trigger cerebral malaria, we studied the role of parasite HMGB proteins in the onset of cerebral malaria. On the basis of these observations we hypothesized that *Plasmodium* HMGB proteins might act in a pro-inflammatory and vasoactive manner being implicated in the pathogenesis of severe forms of malaria, including CM. We showed that the involvement of *hmgb2* gene in the pathogenicity of the parasite is governed by the parasite itself -
PbANKA or PbNK65 - in addition to the mouse strain - C57BL/6 or BALB/c - highlighting the importance of the host/parasite context. Most importantly, we provide herein evidence showing that this is indeed the case using an ECM model comprising the ECM sensitive (ECM-S) C57BL/6 mice infected either with the highly lethal Plasmodium berghei ANKA (PbANKA) or with its pbhmgb2 deficient cognate Δhmgb2 PbANKA. The implication of pbhmgb2 in ECM was also studied via supplementation experiments with the recombinant PbHMGB2 protein.

Materials and Methods

Ethics Statement. All animal care and experiments described in the present study involving mice were approved by the "Direction Départementale des Services Vétérinaires" de Paris, France (Permit Number N° A75-13-01) and performed in compliance with institutional guidelines and European regulations (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimize suffering.

Materials

Mice. C57BL/6 and BALB/c mice were purchased from Janvier and Charles River Laboratories, respectively. All animal care and procedures were performed in accordance with institutional guidelines and European regulations.

Parasites. Mice were inoculated with red bloods cells (RBCs) infected with either GFP-transgenic P. berghei ANKA (MRA-867) or Δhmgb2PbANKA parasites and occasionally with PbNK65 (MRA-268) and its hmgb2 deficient counterpart.

Methods

Murine model of ECM
In all experiments, RBCs infected with PbANKA or their knock-out counterparts were used to infect five to ten weeks old C57BL/6 or BALB/c mice. Parasites, before every experiment, were reactivated by previous passage in susceptible C57BL/6 mice to ECM (ECM-S). According to the experiment, C57BL/6 mice were then infected by intravenous (i.v.) or intraperitoneal (i.p.) inoculation of 10^5 pRBC. Parasitaemia was determined by flow cytometry and the results expressed in percentage of pRBCs. C57BL/6 mice infected with PbANKA or Δhmgb2 PbANKA supplemented with recombinant PbHMGB2 were monitored for clinical symptoms of ECM: hemi- or paraplegia, deviation of the head, tendency to roll over on stimulation, ataxia and convulsions.

**Preparation of the proteins**

*Escherichia coli* BL21 (DE3) p-LysS (Stratagene) strain was used to express PbHMGB2 protein from pEXP5-CT/TOPO (Invitrogen) construct. Pbhmgb2 ORF was amplified by PCR from *P. berghei* ANKA cDNA using the following oligonucleotides: pbhmgb2 forward 5’ATGGCAACTAAAAACACAAAA3’ and pbhmgb2 reverse 5’ttaTTCCTTTGTCTATATTTCT3’. The stop codon was removed from the reverse primer to allow cloning into the pEXP5-CT/TOPO vector in which the 6 His-tag is added in C-terminus of the protein. Overnight cultures were used to inoculate 2YT medium containing appropriate antibiotics. Cultures were shaken at 230 rpm until the A_600nm reached 0.6. The protein expression was then induced for 4 hr at 37 °C by the addition of 1 mM of IPTG (Isopropyl-β-D-thiogalactopyranoside). The bacteria were then harvested by centrifugation 20 min at 4000 rpm and stored frozen at -20 °C. PbHMGB2 was purified using a protocol adapted from (50). The bacteria were suspended in 1mL of lysis buffer (PBS 1X, β-mercaptoethanol 10 mM, Triton X-100 0.5%, imidazole 10 mM) for 0.5 g of bacteria pellet with a pinch of lysozyme. Bacteria were disrupted by sonication (Branson sonifier 450) at 10 W, 10 times 20 sec with 20 sec pauses and centrifuged 25 min, 10000 rpm at 4 °C. The supernatant was collected, loaded onto Ni-NTA Agarose beads (Qiagen) which were previously equilibrated in 5 mL of washing buffer 1 (PBS 1X, imidazole 20 mM, Triton X-114 0.1 %). Triton X-114 was added to this washing buffer to remove contaminating LPS. The mixture was rotated on a wheel for 2 hr at 4 °C. Wet
beads, 0.2 mL for 1 mL of lysate, were incubated for 2 hr. Beads were centrifuged thereafter at 4 °C, 3000 rpm for 3 min, and washed 3 times for 10 min with 25 vol of washing buffer 1 then 2 times for 10 min with 10 vol of washing buffer 2 (PBS 1X, imidazole 30 mM) to remove the residual Triton X-114. The protein elution was achieved by PBS 1X containing 150 mM to 200 mM of imidazole. The recombinant protein concentration and purity were assessed by spectrophotometric quantitation (BioRAD Protein Assay) and 12 % SDS-PAGE. The presence of residual endotoxin in the proteins was checked by the E-toxate kit (Sigma) following the manufacturer’s instructions.

Disruption of the pbhmg2 gene

We described here the strategy for hmbg2 gene disruption (PBANKA_071290) that was used for the two parasites PbANKA and PbNK65. Two PCR fragments flanking the HMGB2 open reading frame (ORF) were amplified from the genomic DNA using oligonucleotides listed in Supplementary Table S1. Amplification of the 5’ untranslated region (UTR) with the primer combination 5’-hmgb2-for and 5’-hmgb2-rev including Apal and Smal restriction sites, respectively resulted in a 526-bp fragment and was cloned upstream to the positive selection marker human dihydrofolate reductase (hudhfr) previously introduced into the pBC SK- vector (Stratagene) under the control of EF1α promoter and of dhfr/ts 3'UTR (dihydrofolate reductase/thymidylate synthase). Next, the 3'UTR region was amplified with 3'-hmgb2-for and 3'-hmgb2-rev primers including NotI and Ascl restriction sites. This fragment was inserted downstream to the hudhfr box resulting in the hmgb2 targeting vector pBC-5'B2hudhfr3'B2 allowing replacement of the endogenous hmgb2 locus in PbANKA upon a double cross-over homologous recombination and subsequent selection with the antifolate pyrimethamine (51). P. berghei parasite transfections were performed with 5 µg Apal/Ascl-digested pBC-5'B2hudhfr3'B2 and gradient-purified P. berghei ANKA schizonts as described in (52). Positive selection for successful integration of the targeting plasmid was carried out by providing 70 µg/mL of pyrimethamine in drinking water for a period of 8 days. Transfer of the emerging P. berghei population into naive animals confirmed pyrimethamine resistance. Genomic DNA for selected parasite populations were genotyped by an
integration-specific PCR using primers dhfr-for and hmgb2-anarev (see Supporting information Table 2). Clonal hmgb2 deficient parasite populations were obtained by limiting dilution into 20 naive Swiss mice and confirmed by diagnostic PCR and southern blotting. A probe spanning 5’-hmgb2 portion was amplified by PCR and digoxigenin labelled dUTP (DIG) using the DIG random prime labelling kit (Roche Applied Science). Southern blot was performed after running 4 µg of MstI digested genomic DNA on a 0.8 % agarose gel and passive transfer on a nitrocellulose membrane and revealed using an anti-DIG antibody coupled to peroxydase (Roche Applied Science).

**Supplementation with the recombinant proteins**

We assessed the best concentration of protein capable to restore ECM in these mice according to the data reported for recombinant HMGB1 (53). C57BL/6 mice infected with 10^6 Δhmgb2 PbANKA pRBCs were injected by i.p. inoculation, from day 4 to day 8 post-infection, with 25 mg/kg of PbHMGB2, twice a day (every 12 hr). According to the LPS contamination of the protein preparations (25 ng or 50 ng per protein injection), control infected C57BL/6 mice were inoculated following the same procedure, with the protein elution buffer containing same concentrations of LPS. Also, uninfected C57BL/6 mice were injected with recombinant PbHMGB2 alone to assess the lack of toxicity of the recombinant protein.

**Evaluation of the parasite burden by cytometry and Giemsa counting**

Growth of WT PbANKA and derived mutant parasites was determined by flow cytometry using an EPICS XL Beckman as described in (52). Experiments were repeated three times and statistical analysis was performed using Student t-test. Growth of WT PbNK65 and derived mutant parasites was determined by microscopic examination of Giemsa-stained thin blood smears. Parasitaemia was measured by counting 3000 red blood cells and expressed as percentage of total parasitized erythrocytes.

**Histological analysis**
Brains from PbANKA and Δhmgb2 PbANKA infected C57BL/6 were removed at specific time points of infection and fixed in 4 % neutral buffered formalin for four days, then dissected and embedded in paraffin. For each brain, five transversal large sections, each 2 mm thick, from brain stem to olfactory bulb, were removed before embedding in five paraffin blocks. For each paraffin block serial 5 micron sections stained with Hemalun eosin and Giemsa staining were studied and this protocol correlated with a total of 20 sections for each mouse brain (modified from the protocol described in the Mouse brain in stereotaxic coordinates (54). Elementary lesions were studied according to the following criteria: hemorrhages, malaric pigment deposit, attachment of red and white blood cells to endothelium.

Hemorrhagic focus was defined as a minimal surface of a square 10 x 10 microns, malaric pigment was only observed in infected red cells, adhesion is observed in murine animal models only for white blood cells compared to human pathology.

**BBB permeability**

C57BL/6 infected with PbANKA were injected, retro-orbitally with 0.1 mL of 2 % (w/v) Evans Blue (EB) in PBS, when clinical symptoms of ECM were observed (head deviation, convulsions, ataxia and paraplegia) usually at day 6-8 p.i. Δhmgb2 PbANKA infected C57BL/6 were injected, following the same protocol, at the same time and later on (day 12 p.i.) in case of survival. One hour later, mice were perfused with 20 mL PBS under anaesthesia (0.5 mL Rompun, 1 mL Imalgène® 1000, qsp 4 mL PBS 1X) and brains were harvested and photographed.

In the protein supplementation experiments, the brain of mice infected with either WT or KO parasites, supplemented or not with recombinant HMGB2, were harvested as depicted above, weighted and incubated in 2 ml formamide for 48 h, at 37°C and in the dark. The quantification of the BBB disruption was evaluated by absorbance measured at 620 nm with a correction at 740 nm. The amount of Evans Blue that infiltrates the brain was measured by comparison with a standard curve of Evans Blue in formamide.

**Preparation of total RNA and RTqPCR transcript analysis**
At different times post infection, total RNA was extracted from brains, spleens and lungs removed from C57BL/6 infected with either PbANKA or Δhmgb2 PbANKA parasites. RNA preparation was performed, between day 6 and 8 (coma stage) and at day 12 post infection the last day analysed (survival). Total RNA was extracted from the samples by mechanical grinding using Trizol (Invitrogen), following the manufacturer’s instructions. Contaminant DNA and proteins were removed following Qiagen’s protocol for RNA clean-up (Qiagen RNeasy Kit) and the integrity of the RNAs was controlled by the Bioanalyzer 2100 (Agilent Technologies).

The expression level of diverse transcripts was analysed by real-time RT-qPCR in an MX 3005P Cycler (Stratagene) using SYBR Green Jumpstart™ TaqReadyMix™ (Sigma) and various primer sets listed in Supporting information Table 2. Murine hmgb transcripts as well as transcripts of diverse pro and anti-inflammatory cytokines and cellular adhesion molecules (cams) were monitored as follows. Reverse transcription of 1 µg total RNA was performed using Superscript VILO™ cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. Five µl of a 1/10 dilution of each cDNA were amplified with 200 nM of every primer set and 10 µl of SYBR Green, in a final volume of 20 µl. Amplification conditions comprised initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation step 30 sec at 95 °C and annealing-elongation step 60 sec at 60 °C. The specificity of the reaction was controlled by the generation of a dissociation curve with 36 cycles of 30 sec, starting at 60 °C with an increment of 1 °C every cycle. The relative expression level of transcripts was evaluated against that of uninfected C57BL/6 and normalized for each RNA sample with hypoxanthyl-guanine phosphoribosyl tranferase (hprt) transcript.

Preparation of brain and spleen cell suspensions

The brains and spleens were obtained from wild-type PbANKA or Δhmgb2 PbANKA infected C57BL/6 mice at the coma stage of CM (d6). Briefly, mice anesthetized with ketamine (600 mg/kg) and xylazine (20 mg/kg) were perfused with 50 ml of PBS. Then, each brain was removed and homogenized in RPMI 1640 medium (BioWhittaker, Walkersville, Maryland) by passing through
sterile meshes to obtain a single-cell suspension. To harvest leukocytes from brain tissue, Percoll (Pharmacia Biotech, Uppsala, Sweden) was added at a final concentration of 35% to the cell pellet and centrifuged at 400 g for 20 min at 20°C. The cell pellet was washed twice and analysed via flow cytometry.

Flow cytometric analysis of brain and spleen leukocytes

Spleen and brain cells were stained for FACS analysis according to standard protocols in cold PBS containing 2% FCS and 0.01% sodium azide (FACS buffer) with the following Abs: APC-labeled CD4, FITC-labeled anti-CD8α, FITC-labelled anti-F4/80 antibody, and PE-labeled anti-Ly6G antibody, PE-labeled anti-granzyme B antibody, and PE-labeled anti-IFNγ antibody. All antibodies were purchased from eBioscience, San Diego, CA. RBC were eliminated using cell lysis buffer, and cells were washed in FACS buffer. A total of 4 x 10^4 and 10^5 living cells for brain and spleen, respectively, were analysed using a four-color FACSCalibur flow cytometer with ProCellQuest software (BD Biosciences, Mountain View, California).

ELISA determination of PbHMGB1 and PbHMGB2 proteins in the sera of mice

Serum samples were obtained from naive or infected C57BL/6 mice inoculated with WT or Δhmgb2 PbANKA strain. Titration of PbHMGB1 and PbHMGB2 was performed using the protocol described by Barnay-Verdier et al. (55) with slight modifications. The majority of serum proteins other than PbHMGB1 and PbHMGB2 may be removed by precipitation in the presence of 3% perchloric acid (PCA) as follows. A stock solution of 13.7% PCA was prepared by mixing 1.26 vol. PCA of 70% (the concentration of PCA as sold commercially) with 8.69 vol. H2O. The stock solution was stored in a PCA tinted bottle sealed at room temperature. To 1 vol. serum (or other test sample) with ice, add ¼ volume PCA 13.7% and mix well. Solution of 3% PCA led to the immediate formation of an abundant precipitate. The sample was immediately centrifuged for 5 min. at 13000 x g at 4°C. A known volume of the supernatant, which contains the PbHMGB proteins, was collected and neutralized by addition of 1/5 of its volume of 1.5 M
NaOH. By experience, 200 μl of supernatant were neutralized with 17 μl of 10 M NaOH. Since the
process of precipitation/neutralization led to the increase in volume of 50% due to the addition of the
reagents, 1.5 μl of the supernatant neutralized is tested to measure the concentration of PbHMGB in 1
μl of serum. A volume (50 μl) diluted ¼ was added to a 96-well plate pre-coated with rabbit IgG anti-
PbHMGB1 or anti-PbHMGB2 antibody and incubated for 2 h at 37°C. After saturation with 100 μl of 1%
BSA, wells were washed and Biotin-labelled rabbit IgG anti HMGB1 or anti HMGB2 was added at a pre-
defined dilution of 1/250 and incubated for 2h at 37°C. After washing, 50 μl were added to the wells
followed by Streptavidin-peroxydase. The reaction was revealed by adding ortho-phenylene-diamine
plus H2O2.

Statistical analysis

Differences in mouse survival were evaluated by the generation of Kaplan-Meier survival plots and Log-
rank analysis. Differences in growth rates (peripheral parasitaemia) and in parasite load were analysed
by Student t-test. Kruskal-Wallis analysis was performed to compare means between the 3 or 4 groups
of data for haemorrhagic foci numbering, survival curves, BBB disruption evaluation, brain or spleen-
infiltrating T cells numbering and transcript expression level. Post-test of Dunn was applied to analyse
the effect of pbhmgb2 deletion. P-value<0.05 was considered statistically significant in each test.

Gene identifications

PlasmoDB, [http://plasmodb.org/plasmod/](http://plasmodb.org/plasmod/): *P. berghei* high mobility group protein (*pbhmgb2*)
PANKA_071290; *P. berghei* ANKA rRNA encoding gene berg06_18s.

NCBI Genbank: *M. musculus* hypoxanthine guanine phosphoribosyltransferase (*Hprt*) NM_013556.2;
*M. musculus* high mobility group protein 1 (*muhmgb1*) NM_010439.3; *M. musculus* high mobility group
protein 2 (*muhmgb2*) NM_008252.3; *M. musculus* tumor necrosis factor alpha (*tnfα*) NM_013693.2; *M.
musculus* interleukin 6 (*il-6*) NM_031168.1; *M. musculus* interferon gamma (*ifnγ*) NM_008337.3; *M.
musculus* interleukin 10 (*il-10*) NM_010548.2; *M. musculus* intercellular adhesion molecule 1 (*icam1*)
Results

The Plasmodium HMGB proteins are released in the extracellular milieu.

To explore the systemic biological effect of HMGB2 on the Plasmodium pathogenicity our working hypothesis was based on the secretion of the “alarm in” protein from the parasite. We determined the occurrence of the *P. berghei* rodent parasite protein (*Pb*HMGB2) in the sera of C57BL/6 infected with wild type *Pb*ANKA (WT *Pb*ANKA) via ELISA titration (Fig. 1). As expected the protein was not found in the sera of naive non-infected mice but present in the mice infected by the WT *Pb*ANKA parasite, the rodent model of ECM (as *Pb*HMGB1, data not shown).

Parasite multiplication and ECM occurrence are differently generated according to host/parasite strain context.

We studied implication of the HMGB2 protein in several parasite and murine strains. We used two highly pathogenic *P. berghei* parasites, *Pb*ANKA and *Pb*NK65 parasites leading to the death of mice within 7 days +/- 1 per ECM or 20-25 days per hyperparasitaemia, respectively. Actually, two strains of mice known to be susceptible to ECM (ECM-S) C57BL/6 or resistant (ECM-R) BALB/c were infected by the two WT parasites and the addition of the recombinant HMGB was investigated (Fig. 2A-D). The occurrence of ECM is dependent on the host/parasite combination (summarized in Table 1).

In the ECM-R BALB/c mice, *Pb*ANKA parasite developed with complete absence of neurological symptoms associated to ECM leading thereafter to the death via hyper-parasitaemia (Fig. 2B).
However, injection of recombinant PbHMGB2 (see details in materials and methods and indicated by a dashed line) in these ECM-R mice induced the death of mice (60%) within 7-9 days p.i. with neurological symptoms (Fig. 2A). We verified that the protein alone was not pathogenic therefore ECM did not rely on the toxicity of the proteins.

In the ECM-S C57BL/6 mice, the PbNK65 parasite developed in absence of neurological symptoms (Fig. 2C, vehicle control). Supplementation with the PbHMGB2 protein did not induce ECM (Fig. 2C) and PbNK65 multiplication was not affected (Fig. 2D).

We generated hmgb2 deficient PbANKA and PbNK65 parasites (Δhmgb2 PbANKA and Δhmgb2 PbNK65) as described in Fig. S1. The asexual development of the two PbANKA and PbNK65 as well as their hmgb2 deficient parasites was studied in the two mouse strains. In ECM-R BALB/c mice the multiplication of the WT was similar to that of the KO PbANKA parasite (Fig. 2E). In ECM-S C57BL/6 the multiplication of the PbNK65 and Δhmgb2 PbNK65 appeared different, whereas the WT parasitaemia increased up to 15 days and more, the multiplication of Δhmgb2 PbNK65 parasite increased and decreased thereafter up to 15 days where it is no longer detected in the peripheral blood (Fig. 2F). Indeed, the hmgb2 gene disruption modified the fitness of the parasite since from similar multiplication early p.i. the hmgb2 deficient PbNK65 parasites were ultimately cleared in the peripheral blood.

Finally, in the ECM-S C57BL/6 mice PbANKA and Δhmgb2 PbANKA parasites developed differently (see next paragraph). Only PbANKA infection triggered ECM in C57BL/6 mice in contrast to all combinations just mentioned (see Table 1). We therefore decided to focus our study on the implication of HMGB2 in ECM occurrence by the use of C57BL/6 mice infected by PbANKA WT and hmgb2 deficient parasites.

**Deletion of Plasmodium hmgb2 protects C57BL/6 mice from ECM outcome.**
As just mentioned the rodent ECM model used in the following experiments showed the death of C57BL/6 mice within day 6-7 post-infection (p.i.) when i.p. injected with $10^5$ red blood cells infected with PbANKA (Fig. 3A). We investigated the role of HMGB2 protein in experimental cerebral malaria via deletion of the gene in the parasite. A targeting construct was generated, i.e. pBC-5′B2hudhfr3′B2 that, after double crossover homologous recombination, inserts the huDHFR selectable cassette in place of the *Plasmodium berghei* ANKA *hmgb2* locus. After transfection of the linearized plasmid pBC-5′B2hudhfr3′B2 into PbANKA merozoites, recombinant parasites were selected and cloned (Fig. S1).

Pbhmgb2 gene disruption in PbANKA parasites was verified in three independent clones. When the ECM-S C57BL/6 mice were infected with these three clones, we observed that they differ from the wild type parasites for their lethality and ECM occurrence (Fig. S2). Indeed, a marked survival from ECM was observed up to 100% at d20 p.i. according to the clone tested. We then selected clone F, named thereafter Δhmgb2 PbANKA, to perform all subsequent analyses. Actually, PbHMGB2 was not detected in the sera of mice infected with Δhmgb2 PbANKA in contrast to the sera of mice infected with WT PbANKA (Fig. 1) neither the *hmgb2* transcript (Fig. S1). Using the same procedure no Δhmgb1 parasites were obtained after three independent transfections, suggesting that the gene is essential for parasite development.

The survival and peripheral parasitaemia of mice infected by either WT PbANKA or Δhmgb2 PbANKA were monitored in three independent experiments. As expected, all mice infected (p.i.) with WT PbANKA died within 6-7 days (d6-7). In contrast, deletion of *hmgb2* gene was associated with a reduction of ECM incidence with 65% ± 10.67 SEM (p<0.0001) not developing ECM and succumbing 20 days after infection from hyper-parasitaemia a lethal outcome of PbANKA unrelated to ECM (Fig. 3A). Both strains developed similar peripheral parasitaemia up to d6, evaluated by the percentage of parasitized red blood cells (Fig. 3B). The Δhmgb2 PbANKA parasite maintained its development up to d20 leading to the death of C57BL/6 mice later by severe anaemia and hyper-parasitaemia. To control that the lack of ECM onset in Δhmgb2 PbANKA infected mice was not due to an alteration of the
parasite growth we compared the asexual multiplication rate of both parasites. The cloning procedure described by Janse and colleagues (52) was achieved at d6 by counting Giemsa stained blood smears. The mean of asexual multiplication rate of 24 hr was calculated assuming a total of 1.2 x 10^{10} erythrocytes/mouse and resulted in 11.94 ± 0.7 SEM and 11.66 ± 0.2 SEM for WT PbANKA or Δhmgb2 PbANKA, respectively. The asexual multiplication of the KO parasite was not reduced when compared to the WT parasite underlining identical growth rate of the knockout parasites.

Deletion of Plasmodium hmgb2 protects C57BL/6 mice from blood brain barrier damages.

Suppression of ECM in C57BL/6 mice infected with Δhmgb2 PbANKA was associated with reduced brain damages as assessed by the extent of microvascular haemorrhages and with reduced parasite sequestration. As early as d5 p.i., prior to ECM manifestation, no apparent brain damage was observed in Δhmgb2 PbANKA infected C57BL/6 mice whereas minimal haemorrhages were already noticeable in 20% of mice infected with the WT parasite (data not shown). PbANKA infected C57BL/6 exhibited extended and severe haemorrhagic foci at d6-d8 after infection (Fig. 3C, top line) while mice infected with the mutant parasite displayed moderate haemorrhagic foci (Fig. 3C, middle line). In an additional experiment, there was a marked decrease in the number of haemorrhagic foci per histological brain section at d6 and 7 p.i. (p<0.05) for each time point between PbANKA and Δhmgb2 PbANKA (Fig. 3D). Finally, at d12 p.i., mice surviving Δhmgb2 PbANKA infection did not exhibit either haemorrhages or cerebral lesions (Fig. 3C, bottom line). These observations were confirmed by BBB disruption, illustrated by Evans blue leakage in the brain of WT PbANKA infected mice, whereas this was not apparent in brains of Δhmgb2 PbANKA infected mice (Fig. 3C, right column).

Exogenous PbHMGB2 protein restores Δhmgb2 PbANKA virulence.
To ascertain that the increased resistance to ECM in C57BL/6 mice infected with $\Delta hmgb2$ PbANKA versus WT PbANKA was due at least in part to the lack of extracellular PbHMGB2, we assessed whether recombinant PbHMGB2 was sufficient *per se* to re-establish ECM susceptibility in $\Delta hmgb2$ PbANKA infected mice.

Recombinant PbHMGB2 restored ECM incidence in $\Delta hmgb2$ PbANKA infected mice, i.e. 60% of mice died of ECM at d10 with cerebral symptoms (Fig. 4A). This experiment analysed by Kruskal-Wallis (p=0.0025) was repeated 3 times with 10 mice per group. A statistical difference was observed at d10 up to d20 post-infection between mice infected with the $\Delta hmgb2$ PbANKA parasite in absence or presence of PbHMGB2 protein when analysed by Student's t-test (p=0.0012). The pathologic effect of PbHMGB2 was not associated with modulation of peripheral parasitaemia (Fig. 4B) since the parasite fitness was not affected. Moreover, a low ECM incidence was observed in vehicle treated C57BL/6 mice infected with $\Delta hmgb2$ PbANKA ($\leq 30\%$ death) like in control suggesting that the low LPS contamination did not account for the ECM increase observed when PbHMGB2 was injected. These observations were confirmed by BBB permeability. Figure 4C shows the histogram of Evans blue evaluation in brains of mice infected with $\Delta hmgb2$ PbANKA complemented with the recombinant protein as regard to the brain of control mice infected with hmgb2 deficient parasite alone or in presence of vehicle. The recombinant PbHMGB2 preparations produced in *E. coli* contained less than 100 ng LPS per mg of recombinant protein. This residual LPS contamination was used as vehicle control in these experiments. A statistical difference of BBB disruption was observed between the brains of $\Delta hmgb2$ PbANKA and complemented $\Delta hmgb2$ PbANKA infected mice (p<0.01).

The resistance of mice to the development of ECM following infection with $\Delta hmgb2$ PbANKA correlates with a reduction of brain-infiltrating T-cells.

Development of CM is strongly associated with the activation and recruitment of T-cells in the brain (20, 18).
Accordingly, we examined whether such T-cell recruitment occurred when mice were infected with \( \Delta hmgb2 \) PbANKA. Brain- and spleen-infiltrating leukocytes were isolated from naive C57BL/6 or mice infected with WT or \( \Delta hmgb2 \) PbANKA 7 days after infection (Fig. 5). Compared to uninfected control, C57BL/6 mice infected with WT PbANKA displayed in brain (Fig. 5A and Fig. 5B), as expected a marked increase in the number of CD4\(^+\) T-cells (7 fold) and of CD8\(^+\) (64 fold). In addition, CD8\(^+\) GzmB\(^+\) and IFN\(\gamma\) T cell sequestration (22, 23) was also increased (18 and 40 fold, respectively) as regards to the uninfected mice. Interestingly, the recruitment of CD4\(^+\) was lower (2 fold) and CD8\(^+\) T cells (4 fold) in brains of mice infected with the \( \Delta hmgb2 \) PbANKA when compared to those infected with the WT PbANKA parasite as well as the recruitment of the CD8\(^+\) T cells expressing both GzmB or IFN\(\gamma\) (3.5 fold), respectively, (Fig. 5C and 5D). To verify whether the lack of \( pbhmgb2 \) gene alters the CD4\(^+\) and CD8\(^+\) GzmB\(^+\) and IFN\(\gamma\) T cell sequestration not only in the brain, similar analysis was performed in spleens of naive and infected mice. All data in the spleen were consistent with those observed in the brain (Fig. 5E, F, H) except for CD8\(^+\) GzmB\(^+\) T cells which numbers did not differ significantly in the two groups (Fig. 5G).

In search of other leukocytes that exert their effector functions during ECM, we surmised that neutrophils might represent inflammatory cells which under particular circumstances cause the disease. These cells were indeed shown to be associated with the disease pathogenesis (56). Accordingly, although the difference was not statistically significant, the percentage of Ly6G\(^+\) neutrophils in the brain was lower in \( \Delta hmgb2 \) PbANKA-infected mice than in WT infected mice (Fig. S3A). Analysis of sequestered F4/80\(^+\) macrophages in the brain did not show any significant difference (Fig. S3B). Similarly, no difference could be detected in Ly6G\(^+\) neutrophils (Fig. S3C) and in F4/80\(^+\) macrophages (Fig. S3D) within the spleen of the two groups.

Are these cellular alterations, part of the modulation of the host immune response by HMGB2 protein, correlated to parasite density (22) (23)? Parasite burden was measured in the periphery (parasitaemia)
and in different tissues including the brain, spleen and lung (Fig. 6). We evaluated the parasite load by RT-qPCR measuring relative levels of parasite 18S RNA in different tissues of C57BL/6 infected by both parasites in organs taken 6-7d p.i. Even though the peripheral parasitaemia of \( \Delta \text{hmgb2} \ P_b \text{ANKA} \) was comparable to that of WT \( P_b \text{ANKA} \)-infected mice (Fig. 6D), lesser parasite sequestration was noticed in mouse organs at d6 p.i. In the brain (Fig. 6A) and lung (Fig. 6B), the parasite level of \( \Delta \text{hmgb2} \ P_b \text{ANKA} \) was lower than that observed for WT parasites, approximately 5 and 3.5 fold respectively. In spleen (Fig. 6C), the parasite load was also lessened but to a lower extent 2.5 fold as regard to the amounts in the brain and lung.

**Deletion of Plasmodium hmgb2 decreases cytokine and adhesion protein messenger expression in brains of infected C57BL/6 mice**

The brains of infected C57BL/6 harvested at d6 and d12 (see figure 3C) were analysed to evaluate the contribution of a variety of gene transcripts to the survival of \( \Delta \text{hmgb2} \ P_b \text{ANKA} \) infected C57BL/6. In addition, we performed an additional transcript analysis (corresponding to Fig. 3D) focussing our examination on d6 or d7 at the onset of ECM and these data were included in our statistical analyses. The transcripts of the murine host: \( \text{hmgb1} \) and \( \text{hmgb2} \), pro-inflammatory cytokines (\( \text{tnf} \alpha \), \( \text{ifn} \gamma \), \( \text{il-6} \)), anti-inflammatory (\( \text{il-10} \), \( \text{hmox-1} \)) and adhesion proteins (\( \text{icam-1} \), \( \text{vcam-1} \)) were analysed by RT-qPCR with the primer sets listed in Table S2. The level of all transcripts (except for those of \( \text{muhmg1} \) and \( \text{hmox-1} \)) was increased at d6 in the brain of WT \( P_b \text{ANKA} \) infected mice when compared to uninfected mice with the highest magnitude for \( \text{ifn} \gamma \) (Fig. 7). The level of these transcripts was also enhanced in the brain of mice infected by \( \Delta \text{hmgb2} \ P_b \text{ANKA} \), however to a lesser extent. It is of note that the decreased expression of the aforementioned genes was statistically significant in these mice when compared to the WT parasite infected mice. In addition, this decrease was maintained at d12 post-infection. As already mentioned two transcripts did not follow this trend: the \( \text{hmox-1} \) transcript encoding an heme degrading...
enzyme eliciting a marked increase in $\Delta$hmgb2 PbANKA at d6 in contrast to WT PbANKA and in turn decreased at d12. Also, the muhmgb1 transcript which level was similar in non-infected and infected mice in contrast to the expression of muhmgb2 transcript that follows the common features of all genes (markedly increased and markedly decreased in mice infected with the WT PbANKA and $\Delta$hmgb2 PbANKA infected mice, respectively).

**Discussion**

The molecular mechanisms underlying the pathogenesis of cerebral malaria remain poorly understood. In humans, presumably, sequestration of parasitized red blood cells in the brain micro-vasculature, associated to the production of inflammatory cytokines and haemoglobin oxidation are critical for the onset of this disease. However, the resources and opportunities to explore the physiopathology in humans are limited and based mainly on post-mortem investigations. Although the murine model of cerebral malaria does not perfectly match the human disease, there is however a substantial number of evidence that both share common features [for review see (26) (57)]. Studies on the mechanisms underlying the pathophysiology of ECM revealed a number of pro-inflammatory proteins implicated in parasitized red blood cell sequestration and in brain pathology. Being aware that both host and parasite biological products might combine to trigger ECM we decided to focus our study on parasite proteins. Only one pathogen-associated molecular pattern (PAMP) the glycosylphosphatidylinositol (GPI) has been reported in several protozoan parasites including *Plasmodium* to elicit a deleterious host innate immune response. The *P. falciparum* GPI, via TLR2 and 4, signals the immune cells to produce pro-inflammatory cytokines and induce severe malaria.

We were interested in *Plasmodium* HMGB proteins to assess if they might be involved in the ECM development since their human counterparts are involved in a number of inflammatory disorders. In *P.*
*falciparum* genome, two orthologues of human HMGB are present and annotated as *Pf*HMGB1 and *Pf*HMGB2. As for the mammalian HMGB isoforms these two proteins encompass a TNF-α-activating domain, suggesting that in addition to their implication in chromatin remodelling (48) when released from the parasite in the extracellular milieu, they might as their mammalian counterparts, act as genuine inflammatory agonists. In Fig. S4A the multiple alignments of different eukaryotic HMGB protein domains underline their conserved trait and the strong conservation between the *Plasmodium* proteins with a higher conservation observed for the Box B of the mammal proteins. In addition, the alignment of the 20 aa long sequences of the TNF-α-activating domains (Fig. S4B) emphasizes the greater homology encountered between the *Plasmodium* sequences and that of Box B of the mammalian proteins (identity 45%, homology 80%) reported to bear the pro-inflammatory activity. In keeping with this notion, *P. falciparum* HMGB proteins are released in culture medium (data not shown) and the *P. berghei* proteins are detected in the sera of C57BL/6 mice infected with *PbANKA* (Fig. 1). In addition, the recombinant protein induced TNF-α and IL-6 production (49). This led to the hypothesis that *Plasmodium* HMGB might be implicated in the pathogenesis of severe forms of malaria, including cerebral malaria.

In order to study the role of HMGB in cerebral malaria we constructed *hmgb* deficient parasites (*PbANKA* and *PbNK65* in Fig. S1) and investigate their growth and their ability to drive ECM in C57BL/6 and BALB/c mice. Few studies report gene disruption at the level of erythrocytes in vivo (58-60). In mice, absence of the *muhmgb1* is lethal since *hmgb1-/-* mice are not viable and die a few hours after birth (61). The disruption of *hmgb2* is not lethal and *hmgb2-/-* mice displayed a reduced fertility and spermatogenesis defect (33, 62). On the same line, the two *P. falciparum* HMGB proteins displayed different cellular localization within the parasite. Whereas *PfHMGB1* is essentially nuclear all throughout the asexual erythrocytic cycle, *PfHMGB2* is expressed predominantly in gametocytes and present in the nucleus as well as in the cytoplasm. The two proteins also differ by their nuclear activities, *PfHMGB1* being more efficient and specific than *PfHMGB2* for architectural properties (DNA binding and DNA bending) suggesting that, of the two proteins, *PfHMGB1* has a major role in transcription regulation and
parasite development (48). This is consistent with the inability to generate Δhmgb1 P. berghei parasites whereas several clones of Δhmgb2 parasites were obtained.

The pbhmgb2 deletion in PbANKA parasite strain induced reduction of ECM incidence in infected ECM-S C57BL/6 mice. The pathogenic attenuation was not due to a decreased multiplication of the asexual stage since the rate was similar to that of the WT PbANKA as evaluated in the peripheral blood (Fig. 3B). Histological analysis of brain sections and EB dye leakage showed decreased brain damages already at d6 and d7 post-infection when compared to WT infected mice (Fig. 3C and Fig. 3D). A complete absence of haemorrhages was observed in the brains of surviving mice at d12 p.i. The injection of recombinant PbHMGB2 to Δhmgb2 PbANKA-infected mice increased the incidence of ECM (up to 60%), thus re-establishing the high mortality rate observed in susceptible mice (Fig. 4). Moreover, the protein restored ECM in the PbANKA-infected ECM-R BALB/c mice (Fig. 2A). In contrast, recombinant HMGB2 was not able to trigger ECM even in ECM-S mice when infected with the PbNK65 parasite (Fig. 2C), another lethal parasite that does not induce ECM. The multiplication of PbNK65 parasite increased up to the death of mice per hyperparasitaemia in contrast to the Δhmgb2 PbNK65 which is no more detected in the peripheral blood after d15 p.i. (Fig. 2F). In summary, the involvement of HMGB2 protein in the pathogenicity of the parasite is governed by the parasite as well as the mouse strain highlighting the importance of the host/parasite context.

In this ECM model a number of reports proposed that sequestration of CD8⁺ and CD4⁺ T-cells in the brain capillaries is a characteristic feature of the brain pathology (20, 21). Recent studies provided clear evidence that antigen-specific CD8⁺ T-cell-derived granzyme B (22) and CD8⁺ IFNγ⁺ T-cells (25) were critical in driving cerebral pathology. In addition, these authors provided a direct causal link between parasite load and CD8⁺ T-cell-mediated ECM pathology (23). Interestingly, the relationship between the absence of HMGB2 and the lack of ECM expression was associated with a significantly reduced recruitment of CD8⁺ GzmB⁺ and CD8⁺ IFNγ⁺ T-cells in Δhmgb2 mice. In addition, the recruitment of CD4⁺ T-cells was also decreased even though to a lower extent (Fig. 5). This suggests that the lower
level of CD4+ and CD8+ GzmB+, IFNγ+ T-cells sequestered within the brain capillaries of mice infected with Δhmgb2 PbANKA parasites could be the basis of the failure of these mice to develop ECM. It can also be inferred that HMGB2 exerts systemic effects, since it appears that sequestration of CD4+ and CD8+ T-cells rather than neutrophils and macrophages also occurred in inflamed spleens.

Very recently, Renia and colleagues (25) observed that CD8+ T cells and IFN-γ drive the rapid increase in total parasite biomass and accumulation of infected RBC in the brain and in different organs at the time when mice developed CM (23). Our data underline that in absence of HMGB2 the decrease in the recruitment of CD8+ GzmB+ and CD8+ IFNγ+ T-cells (Fig. 5B-D) was associated with a reduced recruitment of the Δhmgb2 PbANKA parasites in the brain (Fig. 6A) in contrast to the similar parasitaemia observed in the peripheral blood, reflecting their identical multiplication rates (Fig. 6D).

However, even if the brain is the organ where the CD8+ T-cells and KO parasite sequestration was the most markedly decreased, a decrease was also observed in the lung and spleen (Fig. 5B and C) indicating that HMGB2 exerts its pro-inflammatory effects at a systemic level.

When compared to the brain of WT PbANKA infected mice, the transcript level of most pro-inflammatory cytokines including Infεx and Infγ were decreased in Δhmgb2 PbANKA infected mice (Fig. 7). Also, the cell adhesion icam-1 transcript was decreased in contrast to vcam-1. In one study, vcam-1 was identified by microarray analysis as a gene candidate that discriminates between ECM-R and ECM-S mice in a P. berghei model (63). Our data do not support the assertion that elevated vcam-1 corresponds to ECM, as we observed slightly higher vcam-1 mRNA levels in Δhmgb2 PbANKA-infected mice at d6 post-infection as compared to WT PbANKA-infected C57BL/6 mice, suggesting an uncertain or, perhaps, protective role for this adhesion molecule in our model. In concordance with our observation of a predominant role for ICAM-1 as compared to VCAM-1 in malaria pathogenesis, infusion of anti-ICAM-1 but not anti-VCAM-1 mAb prevents cytoadherence of infected erythrocytes in a P. yoelii model of ECM (64) as well as in vivo evidence for the role of ICAM-1 in the sequestration of infected red blood cells in a mouse model of lethal malaria (65). In contrast, the transcript level of the cytoprotective
heme oxygenase (hmox-1) was markedly increased at d6 in Δhmgb2 PbANKA infected C57BL/6 and thus consistent with the literature (8-10). Our results showing a lower expression of il-10 transcript in brains of Δhmgb2 PbANKA infected C57BL/6 mice diverge from other reports using murine CM that suggest a protective effect for IL-10 in CM (66) but are in accordance with observations in humans that showed an increase in IL-10 in CM patients when compared to mild malaria patients (67). Finally, the level of muhmgb1 transcript did not vary in hmgb2 deficient and in PbANKA infected mice in contrast to the muhmgb2 transcript which highly increased in PbANKA infected mice and remained unchanged Δhmgb2 PbANKA infected mice.

Taken together, these data represent a breakthrough in that the Plasmodium parasite HMGB2 is ascribed a major pro-inflammatory cytokine-like function that is reminiscent of its mammalian HMGB counterparts shown to play a key role in several human diseases including sepsis, lupus, rheumatoid arthritis, and cancer (68).

Our data demonstrate that deletion of a single Plasmodium gene i.e. hmgb2 implicated in chromatin remodelling (48) and in inflammation (49) contributes to reduced host lethality, an effect due to suppression of the neuro-pathology. An infected host has two evolutionary conserved defence strategies that can limit its disease severity. One relies on the capacity of its immune system to reduce pathogen load, a defence strategy referred as resistance to infection. The other defence strategy acts irrespectively on pathogen load and relies instead on limiting the extent of tissue damage caused by the pathogen and/or by the immune response elicited by that pathogen. This defence strategy is referred as disease tolerance [reviewed in (69, 70)]. The lower virulence of hmgb2 deficient parasites vs. wild type controls is not associated with modulation of peripheral pathogen load suggesting that PbHMGB2 can compromise disease tolerance to the blood stage Plasmodium infection. The observation that protection from ECM in mice infected with hmgb2 deficient parasites is reversed by recombinant HMGB2 complementation, suggests that it is the soluble PbHMGB2 that compromises the disease tolerance.

This finding adds significantly to the previous observation that free heme generated as an end product...
of host haemoglobin oxidation also impairs disease tolerance to *Plasmodium* infection (71). Whether
free heme and *PbHMGB2* interact functionally to trigger the development of severe forms of malaria,
such as ECM remains to be established.

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References


Table 1. ECM occurrence according to host/parasite strain context.

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<th>BALB/c</th>
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<td>-</td>
<td>† 100% ECM</td>
<td>No ECM, † 100% per hyperparasitaemia</td>
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<tr>
<td></td>
<td>+</td>
<td>ND</td>
<td>† 65% ECM</td>
<td></td>
</tr>
<tr>
<td>KO PbANKA</td>
<td>-</td>
<td>65% ECM survival</td>
<td>No ECM, † 100% per hyperparasitaemia</td>
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<tr>
<td></td>
<td>+</td>
<td>† 65% ECM</td>
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<td>-</td>
<td>No ECM, † 100% per hyperparasitaemia</td>
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*† stands for death of mice

+ ND ND
Legend of figures

Figure 1. Release of HMGB2 protein in the extracellular milieu
Release of PbANKA HMGB2 in the sera of mice analysed by ELISA. C57BL/6 mice were inoculated with 10^5 erythrocytes infected with either Δhmgb2 or WT PbANKA parasites. Sera of four mice were taken at d7 post-infection and tested for their content in HMGB2 by ELISA. Sera from naive mice were taken as negative controls. Results shown in the figure are expressed as ng/ml as determined by a titration curve obtained using a range of concentrations of recombinant HMGB2. See insert for the code of mice.

Figure 2. Multiplication of diverse WT and hmgb2 deficient parasites according to the host-mouse strains.
A. Survival and B. parasitaemia of BALB/c mice infected with WT PbANKA and supplemented or not from day 4 to 8 i.p. (see dashed line) with either recombinant PbHMGB2 (25 mg/kg) or vehicle (protein buffer + 50 ng LPS that takes into account the residual LPS contamination of the recombinant protein), twice a day (every 12 hr). All mice were i.v. injected with 10^6 red blood cells infected with WT PbANKA. Differences in mortality/survival between WT supplemented or not with PbHMGB2 were analysed by Log-rank test. C. Survival and D. parasitaemia of C57BL/6 mice infected with WT PbNK65 and supplemented with recombinant PbHMGB2 as above mentioned. All mice were i.v. injected with 10^5 red blood cells infected with WT PbNK65. In Fig 2A-D, all sets of mice were n=5. E. Red blood cell development of PbANKA and F. PbNK65 as well as their Δhmgb2 counterparts in infected BALB/c and C57BL/6 mice, respectively. Parasitaemia was measured by flow cytometry (E) and Giemsa counting (F) in sets of five mice. Values represent mean ± s.d of one representative experiment from three. Statistical analyses of parasitaemia were analysed by Student t-test. The code of mice and parasites are indicated above or right to the plots.
Figure 3. Comparative analyses of C57BL/6 mice infected with the WT PbANKA and the Δhmgb2 PbANKA parasites

A. Kaplan-Meier survival plots C57BL/6 mice infected with either 10^5 WT PbANKA or Δhmgb2 PbANKA. Infected mice were monitored every day, starting on d5 p.i., for ECM symptoms. Difference in survival between WT and Δhmgb2 PbANKA infected C57BL/6 was analysed by Log-rank test. This experiment was performed 3 times (n=4, n=7, n=9). See insert parasite code.

B. Parasitaemia of both parasites measured by flow cytometry, values represent mean ± s.d.

C. Histological analyses of brains from WT and Δhmgb2 PbANKA infected C57BL/6 mice. The representative histological analysis of HE stained sagittal sections of brains harvested at d6 and d12 p.i. from WT (d6: 1-3) and Δhmgb2 PbANKA (d6: 5-7 and d12: 9-11) infected C57BL/6. N=5 in each experimental group. The black arrows indicate the hemorrhagic foci. For each line, two incremental magnifications (50 or 100 µm) of the same microscopic field are presented. Evans blue dye leakage in brains of WT d6: 4) and Δhmgb2 PbANKA (d6: 8 and d12: 12) infected C57BL/6 is presented in the far right column.

D. Microscopy counting of haemorrhagic foci per histological brain section at d6 and d7 p.i. (n=5 in each experimental group). The multiple comparisons of foci numbers were analysed by Kruskal-Wallis (Dunn’s multiple comparison test; P value =0.0008).

Figure 4. ECM occurrence in Δhmgb2 PbANKA-infected C57BL/6 mice supplemented with recombinant PbHMGB2 protein.

A. Survival analysis of C57BL/6 mice infected i.p. with 10^5 pRBCs Δhmgb2 PbANKA and thereafter injected from day 4 to 8 i.p. (see dashed line) with either PbHMGB2 (25 mg/kg) or vehicle (protein buffer + 50 ng LPS that takes into account the residual LPS contamination of the recombinant protein), twice a day (every 12 hr). N=10 in each experimental group and experiment was done 3 times. C57BL/6 mice were infected i.p. as well with 10^6 WT PbANKA pRBCs and stand for ECM positive control. Mice were monitored for ECM symptoms from d5 p.i. B. Parasitaemia was measured by flow cytometry, values...
represent mean ± s.d of one representative experiment from three. C. The amount of Evans Blue that
infiltrates the brain was measured as described in the methods from the three sets of mice (n=5 in each
experimental group). Multiple comparisons were analysed by Kruskall-Wallis followed by Dunn’s post-
test for the survival (p=0.0025) and BBB permeability (p=0.0008) between all sets of mice. The code of
mice is indicated above for figure A and B, and right for C. The code of mice is indicated above for figure
A and B, and right for C.

Figure 5. Reduced infiltration and reduced activation of CD4+ and CD8+ T cells in the brains of
\( \Delta hmgb2 \) PbANKA-infected mice.

At the coma stage (day 6 post-infection), brains from WT or \( \Delta hmgb2 \) PbANKA-infected C57BL/6 mice
with \( 10^5 \) infected erythrocytes per mouse were taken and leukocytes associated with cerebral tissue
were analysed by FACS for the presence of indicated leukocytes and expressed as absolute numbers
per brain. Six mice per group were used. Values represent the means ± s.d. of one experiment from
three. *p<0.05, ** p<0.01, *** p<0.001. A-D stand for CD4+, CD8+, CD8+ GzmB+ and CD8+ IFN\( \gamma \) in
brains and E-H in spleens as just mentioned. Multiple comparisons of brain-infiltrating T cells were
analysed by Kruskal-Wallis (Dunn’s post-test, *p<0.05, ** p<0.01, *** p<0.001).

Figure 6. Reduced parasite biomass in the organs of \( \Delta hmgb2 \) PbANKA-infected mice.

At d6 post-infection, RNA was extracted from brain, A; lung, B; and spleen, C of WT or \( \Delta hmgb2 
 PbANKA-infected C57BL/6 mice (10^5 infected erythrocytes per mouse). Bars represent the means ±
s.d. of parasite load evaluated by relative quantification of expression level of 18S mRNA normalized via
hypoxanthyl-guanine phosphorybosyl transerase hprt transcript. Gray stands for WT PbANKA infected
mice and black for \( \Delta hmgb2 \) PbANKA infected mice. D. Parasitaemia was measured by counting after
Giemsa staining, values represent mean ± s.d. Comparison of parasite biomass and parasitaemia was
analysed by Student t-test, *p<0.05, ** p<0.01, *** p<0.001.
Figure 7. Analysis of the expression level of cytokines, adhesion molecules and hmgb transcripts in the brain of WT and Δhmgb2 PbANKA infected C57BL/6. Brains were removed from uninfected (n=10), WT and Δhmgb2 PbANKA infected C57BL/6 at d6 (n=14 in each group) and d12 (n=3) p.i. and total RNA extracted. Bars represent the means ± s.d. of relative expression level of the transcripts analysed (GOI, gene of interest) normalized in each RNA sample via hprt transcript. The expression levels of cytokine, cam and hmgb transcripts were evaluated against those of naive C57BL/6. White stands for non-infected mice, light grey for WT PbANKA infected mice at d6, dark grey and black for Δhmgb2 PbANKA infected mice at d6 and at d12. Multiple comparisons of expression levels were analysed by Kruskal-Wallis (Dunn’s post-test, *p<0.05, ** p<0.01, *** p<0.001).
The diagram shows a bar graph comparing the concentration of PbHMGB2 (ng/ml) among different groups:

- **WT PbANKA**
- **Δhmgb2 PbANKA**
- **Non infected**

The y-axis represents the concentration levels, ranging from 0 to 200 ng/ml, and the x-axis denotes the groups. The graph indicates a significant difference in PbHMGB2 levels among these groups.