This is a postprint of an article published in
Bucher, K., Dietz, K., Lackner, P., Pasche, B., Fendel,
R., Mordmüller, B., Ben-Smith, A., Hoffmann, W.H.
Schistosoma co-infection protects against brain pathology
but does not prevent severe disease and death in a murine
model of cerebral malaria
(2011) International Journal for Parasitology, 41 (1),
pp. 21-31.

Accepted Manuscript

Schistosoma co-infection protects against brain pathology but does not prevent severe disease and death in a murine model of cerebral malaria

Kirsten Bucher, Klaus Dietz, Peter Lackner, Bastian Pasche, Rolf Fendel, Benjamin Mordmüller, Anne Ben-Smith, Wolfgang H. Hoffmann

PII: S0020-7519(10)00260-2
DOI: [10.1016/j.ijpara.2010.06.008](https://doi.org/10.1016/j.ijpara.2010.06.008)
Reference: PARA 3175

To appear in: *International Journal for Parasitology*

Received Date: 16 March 2010
Revised Date: 10 June 2010
Accepted Date: 12 June 2010

Please cite this article as: Bucher, K., Dietz, K., Lackner, P., Pasche, B., Fendel, R., Mordmüller, B., Ben-Smith, A., Hoffmann, W.H., *Schistosoma* co-infection protects against brain pathology but does not prevent severe disease and death in a murine model of cerebral malaria, *International Journal for Parasitology* (2010), doi: [10.1016/j.ijpara.2010.06.008](https://doi.org/10.1016/j.ijpara.2010.06.008)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

***Schistosoma* co-infection protects against brain pathology but does not prevent severe disease and death in a murine model of cerebral malaria**

Kirsten Bucher^a, Klaus Dietz^b, Peter Lackner^c, Bastian Pasche^d, Rolf Fendel^a, Benjamin Mordmüller^a, Anne Ben-Smith^e, Wolfgang H. Hoffmann^{a,*}

^a*Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany*

^b*Department of Medical Biometry, University of Tübingen, Tübingen, Germany*

^c*Department of Neurology, Innsbruck Medical University, Innsbruck, Austria*

^d*Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany*

^e*Maame Akua, P.O. Box 31563, Lilongwe, Malawi*

*Corresponding Author.

Institute of Tropical Medicine, University of Tübingen, Wilhelmstr. 27; 72074 Tübingen; Germany

Tel.: +49 7071 2982927; fax: +49 7071 295996.

E-mail address: w.hoffmann@uni-tuebingen.de

Abstract

Co-infections of helminths and malaria parasites are common in human populations in most endemic areas. It has been suggested that concomitant helminth infections inhibit the control of malaria parasitemia but down-modulate severe

malarial disease. We tested this hypothesis using a murine co-infection model of schistosomiasis and cerebral malaria. C57BL/6 mice were infected with *Schistosoma mansoni* and 8 to 9 weeks later, when *Schistosoma* infection was patent, mice were co-infected with *Plasmodium berghei* ANKA strain. We found that a concomitant *Schistosoma* infection increased parasitemia at the beginning of the *P. berghei* infection. It did not protect against *P. berghei*-induced weight loss and hypothermia, and *P. berghei*-mono-infected as well as *S. mansoni*-*P. berghei*-co-infected animals showed a high case fatality between days 6 and 8 of malarial infection. However, co-infection significantly reduced *P. berghei*-induced brain pathology. Over 40% of the *S. mansoni*-*P. berghei*-co-infected animals that died during this period were completely protected against haemorrhaging, plugging of blood vessels and infiltration, indicating that mortality in these animals was not related to cerebral disease. *Schistosoma mansoni*-*P. berghei*-co-infected mice had elevated plasma concentrations of IL-5 and IL-13 and on day 6 lower levels of IFN- γ , IL-10, monocyte chemoattractant protein-1 (MCP-1) and monokine induced by IFN- γ (MIG) than *P. berghei*-mono-infected mice. We conclude that in *P. berghei* infections, disease and early death are caused by distinct pathogenic mechanisms, which develop in parallel and are differentially influenced by the immune response to *S. mansoni*. This might explain why, in co-infected mice, death could be induced in the absence of brain pathology.

Keywords: Schistosomiasis; Cerebral malaria; Brain pathology; Hypothermia; Weight loss; Survival; IFN- γ ; Pro-inflammatory chemokines

1. Introduction

Polyparasitic infections in humans are common in many tropical regions of the world (Raso et al., 2004; Hamm et al., 2009). As helminths and malaria parasites are often co-endemic, helminth-malaria co-infections are frequently observed. Several studies have demonstrated that helminth infections can modulate the immune response to malaria parasites. Nevertheless, the outcomes of these studies have been contradictory, with some suggesting that co-infections can ameliorate (Briand et al., 2005; Lyke et al., 2005) and others showing that co-infections can exacerbate (Nacher et al., 2002; Le Hesran et al., 2004) disease severity in the host. Co-infection studies using well-defined animal models indicate that these contradictory results might be explained by variables such as the species and developmental stage of the parasites as well as the genetic background of the host (Lwin et al., 1982; Graham et al., 2005; Fernandez et al., 2009).

In malaria, a balance between pro- and anti-inflammatory cytokines plays a central role in the regulation of the immune response and pathogenesis. During early stage malaria, a strong Th1-like immune response to this intracellular parasite can prevent its uncontrolled multiplication, but an over-production of pro-inflammatory cytokines can lead to severe immunopathology, such as cerebral malaria (CM) (Hartgers et al., 2008). In humans, CM is a form of life-threatening malaria with CNS involvement. It is a major cause of death in *Plasmodium falciparum* infections with a high case fatality rate (Snow et al., 2001). Prominent histopathological features of CM are microhaemorrhages and oedema in the brain, and the cytoadhesion of host cells, such as parasitized red blood cells (pRBCs), leukocytes and platelets, to the brain microvasculature (Hunt and Grau, 2003).

The most important helminths in humans are trematodes, cestodes and nematodes. Although they live in extracellular body compartments and are exposed

to the immune system, they are able to avoid immune destruction and can persist in their host for decades (Chabasse et al., 1985). Their long-term survival is facilitated by immuno-regulatory mechanisms specific for the different parasitic life cycle stages. For example, schistosomal parasites in the egg-laying phase (patent infection) modulate dendritic cells and macrophages and induce regulatory T cells (reviewed in Dunne and Cooke (2005)). This generates Th2 and anti-inflammatory cytokine responses. Thus, a regulatory network evolves which protects the parasites from elimination, but also prevents severe inflammation and resulting immunopathology.

Immune responses in chronic helminth infections can dampen the immune response to third party antigens (Maizels and Yazdanbakhsh, 2003). It has been proposed that anti-inflammatory and Th2 responses to patent schistosomiasis might inhibit parasite control of early stage malaria, but down-modulate the malaria-induced pro-inflammatory response and limit severe pathology at later stages of malaria (Hartgers and Yazdanbakhsh, 2006). The present study investigates this hypothesis using a co-infection model of schistosomiasis (*Schistosoma mansoni*) and CM (*Plasmodium berghei* ANKA strain) in C57BL/6 mice. These mice were selected as they develop cerebral malaria at a high rate, probably due to their pro-inflammatory / Th1 predisposition (Lou et al., 2001). To investigate the impact of patent schistosomiasis on *P. berghei*-induced malaria, the course co-infection was followed longitudinally and quantified in terms of parasitemia, changes in body mass, body temperature, plasma lactate levels, brain histopathology and survival. To analyse the influence of co-infection on the systemic cytokine response, plasma concentrations of IFN- γ , TNF- α , IL-5, IL-10, IL-13, IFN-inducible protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), monokine induced by IFN- γ (MIG) and

macrophage inflammatory protein-1 (MIP-1 α) were determined at different time points before and after infection with *P. berghei*.

2. Materials and methods

2.1. Mice, parasites and infections

Female 3-week old C57BL/6 mice were obtained from Harlan-Winkelmann (Borchen, Germany). They were kept in the animal facility of Institute of Tropical Medicine, University of Tübingen, Germany. Mice were maintained in individually ventilated cages and supplied with heat-sterilized food and autoclaved water ad libitum. Mice were 4 weeks of age at the time of infection with *S. mansoni*.

The Puerto Rico PR-1 strain of *S. mansoni* is routinely bred at the Institute of Tropical Medicine, University of Tübingen. It is maintained by cyclical passage between pulmonate fresh water snails (*S. mansoni*-susceptible M-line of *Biomphalaria glabrata*) and BALB/c mice. Experimental mice were infected with *S. mansoni* by exposure to 50 cercariae percutaneously (Löhning et al., 1999).

The rodent malaria parasite, *P. berghei* ANKA strain, was originally obtained from D. Walliker, University of Edinburgh, United Kingdom. Parasites were stored as frozen stabulates at -80 °C. To obtain experimental inocula of *P. berghei* ANKA, pRBCs were sequentially passaged through three homologous donor mice. Experimental mice were inoculated i.p. with 10⁶ pRBCs. Control mice were injected with blood from an uninfected mouse. All animal experiments were conducted in accordance with German laws after approval by the local authorities.

2.2. Recovery of adult worms and verification of liver granulomas

Establishment of helminth infection was confirmed by the presence of worms and liver granulomas. Each mouse that had been exposed to cercariae underwent necropsy. Whenever possible, worms were obtained via portal perfusion (Smithers and Terry, 1965), and livers were examined for the presence of granulomas under a stereo microscope.

2.3. Determination of reticulocyte counts and erythrocyte counts

Reticulocytes were counted microscopically using brilliant cresyl blue-stained (Merck, Darmstadt, Germany) thin-blood smears of tail blood. To determine erythrocyte numbers, tail blood diluted was 1:200 in Hayem's solution (Merck, Darmstadt, Germany) and erythrocytes counted in a Neubauer chamber.

2.4. Malaria parasitemia and disease severity

Parasitemia was quantified as the percentage of pRBCs by microscopic examination of Giemsa-stained (Merck, Darmstadt, Germany) thin-blood smears of tail blood. Slides were coded and pRBCs were counted microscopically in at least five microscopic fields, each approximately 300 cells.

Disease severity after infection with *P. berghei* ANKA was measured in terms of change in body mass, body temperature and in terms of behavioural and functional alterations in the animals (Lackner et al., 2006). Body mass was measured using a top-pan electronic balance. Rectal body temperature was measured with a digital thermometer (GTH 1170, Greisinger electronic GmbH, Regenstauf, Germany). Additionally, the colour of ears and feet of the animals were examined daily. White ears and pale colour of plantar surface and digits of forelimbs served as signs for severe anaemia and were recorded accordingly. To evaluate clinical experimental CM and terminal illness, mice were monitored for the following signs: hypothermia

below 31 °C, abnormal respiration, abnormal body position (hunching, laying prone, laying on side), impaired motility (limited movement, incapacity of movement), paralysis, convulsions and coma. Alternative behaviour listed after body position and motility indicates increasing disease severity (Lackner et al., 2006) and was assessed with increasing score values ranging from 1 to 3 and from 1 to 2, respectively. Animals with cumulative scores of ≥ 4 were sacrificed. For survival analysis, the day of death was recorded as the day when the animal was killed.

2.5. Histology

Immediately after death, the brains of the mice were removed and fixed in 4% buffered formalin for at least 48 h. After that, brain tissue was dehydrated by a series of graded alcohols and xylene, and embedded in paraffin. Then, 7- μ m-thick coronal sections from two different brain regions with the stereotaxic co-ordinates bregma 0 and bregma -6 were cut on a microtome (Jung RM 2045, Leica, Bensheim, Germany). The serial sections were placed onto SuperFrost/Plus slides (Microm International, Walldorf, Germany), deparaffinized and hydrated, and routinely stained with haemalaun and eosin (both products Merck, Darmstadt, Germany). Slides were blinded and eight randomly selected sections per brain region were subjected to histological examination. Analysis was done on a Nikon Eclipse E800 research microscope (Nikon, Melville, NY, USA). At least 100 visual fields per section were analysed at 400-fold magnification. They were examined for haemorrhage, sequestration of blood cells to brain microvasculature and infiltration of immune cells into the perivascular space in a semi-quantitative manner. Cerebral score values (CSV) ranging from 0 to 2 indicate the degree of histopathology (0 = none; 1 = moderate; 2 = severe).

2.6. Multiplex analysis of plasma cytokine concentration

Mice with an 8-to-9-weeks-old *S. mansoni* infection were co-infected with *P. berghei* and were analysed together with mono-infected and uninfected controls. Plasma from four to seven mice per group and time point was obtained 3 days before and 1, 4 and 6 days after infection with *P. berghei*. Blood was collected from the orbital sinus of the mouse and plasma was prepared in plasma tubes (BD Microtainer, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Concentrations of IFN- γ , TNF- α , IL-5, IL-10, IL-13, IP-10, MCP-1, MIG and MIP-1 α were determined using a mouse cytokine Twenty-Plex kit (BioSource International Inc., Camarillo, CA, USA) according to the manufacturer's instructions. In brief, assays were performed in 96-well filter plates at room temperature. A mix of capture-molecule-coated microsphere beads was incubated with a standard or with samples, or left blank, in a final volume of 100 μ L for 2 h with continuous shaking. Plates were washed twice and incubated with a cocktail of biotinylated antibodies (100 μ L/well) for 1 h. After removal of excess biotinylated antibodies by two washes, streptavidin-R-Phycoerythrin was added and was incubated for 30 min. After three further washing steps the plates were analysed. Measurement was performed using a LiquiChip workstation (Qiagen, Hilden, Germany).

Prior to the generation of the standard curves and calculation of cytokine concentrations, fluorescence intensities (FIs) of blanks were subtracted from standard and sample values. FIs were transformed into cytokine concentrations (Conc) using the following formula:

$$\text{Conc} = \frac{K^{50}}{\left(\frac{A}{A} - 1\right)^{1/c}},$$

201

202 if $FI \neq 0$ and $Conc = 0$, if $FI = 0$,

203

204 where

205 A = maximum fluorescence intensity206 $K50$ = concentration at which the fluorescence intensity equals $A/2$ 207 c = a parameter, which determines the slope at $K50$.

208 The inverse of this function was fitted by least square means to the measured

209 standard values.

210

211 *2.7. Lactate measurement and determination of RBC parameters*

212 For lactate determination, plasma samples of seven *S. mansoni*-*P. berghei*-co-
 213 infected, seven *P. berghei*-mono-infected, four *S. mansoni*-mono-infected and seven
 214 uninfected control mice were obtained between days 6 and 7 after infection with
 215 *P. berghei* and were analysed by enzymatic methods using an ESAT 6661
 216 (Eppendorf, Hamburg, Germany).

217 To measure RBC parameters on day 4 after infection with *P. berghei*, the
 218 blood of six *S. mansoni*-*P. berghei*-co-infected, seven *P. berghei*-mono-infected, six
 219 *S. mansoni*-mono-infected and four uninfected control mice was analysed using a
 220 Vet ABC haematology analyser (Scil Animal Care Company, Veterinary Diagnostics,
 221 Viernheim, Germany).

222

223 *2.8. Statistics*

224 Statistical differences in reticulocyte counts and erythrocyte counts between *S.*
 225 *mansoni*-mono-infected and uninfected groups were determined by a Mann-Whitney
 226 (U) test. The Spearman rank correlation coefficient served to identify associations

between erythrocyte counts and reticulocyte counts and between body temperature and parasitemia. To compare concentrations in plasma lactate and RBC parameters between the four experimental groups, differences were tested for significance using a Kruskal-Wallis test, followed by a Bonferroni test for two by two comparisons. Survival data are presented as Kaplan-Meier graphs and were analysed with a Wilcoxon test.

For the analysis of brain histopathology, the cerebral score with the ordinal outcomes 0, 1 and 2 was described by an ordinal logistic regression model taking into account the presence or absence of an infection by *P. berghei* ANKA and *S. mansoni*. The interaction of the two factors was not significant, therefore the model included only the two factors in an additive way. The observed outcomes are shown in a mosaic plot (Fig. 6). The order of the four groups takes into account the estimated parameters of the model.

To analyse body temperatures on day 5 after *Plasmodium* infection, data were Box-Cox-transformed to stabilize variances and were compared using bi-factorial ANOVA followed by Tukey's Honestly Significant Difference (HSD) test for comparisons between the groups.

In order to compare parasitemia and body weight between the experimental groups at different days after the infection, ANOVA was performed. For both analyses, the model included the fixed factors "group" and "day" together with the interaction of "group" and "day" and the random factor "animal" nested under the factor "group". As parasitemias were originally expressed as percentages, they were transformed via the logit transformation in order to achieve normally distributed data. The estimation method for the model parameters was Restricted Maximum Likelihood (REML). To quantify the effects, the means for the individual groups and days were described together with their 95% confidence intervals. The logit

transformed parasitemias were back transformed in order to present the results on a logarithmic scale as percentages.

Differences in concentrations of cytokines over time and between the groups were determined by ANOVA. To stabilize variance of data, concentrations of cytokines were logarithmically transformed. As zero values occurred, a constant was added which was the minimum value out of those values, which were larger than zero.

ANOVAs of parasitemia and body weight were followed by post hoc *t*-tests for comparisons between the groups on different days after infection. ANOVAs of cytokine concentrations were followed by post hoc *t*-tests for comparisons between *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice, and for comparisons between different days after infection within the *S. mansoni*-mono-infected group and the control group. Due to the high number of comparisons, tests were performed using Bonferroni-Holm's *P* value adjustment, to reduce the number of false positive tests.

3. Results

3.1. *Schistosoma mansoni*-*P. berghei*-co-infection increases parasitemia in early stages of malaria

Mice with or without an 8-to-9-weeks-old *S. mansoni* infection were i.p. infected with 10^6 *P. berghei* ANKA-pRBC. *Plasmodium berghei* parasitemia was monitored continuously and compared between *S. mansoni*-*P. berghei*-co-infected and *P. berghei*-mono-infected mice. Reticulocytes and erythrocytes were counted immediately before *S. mansoni*-mono-infected and uninfected mice were inoculated

with the malaria parasite (day 0). On days 2 and 3 after infection with *P. berghei*, *S. mansoni*-*P. berghei*-co-infected mice had significantly higher parasitemia than *P. berghei*-mono-infected mice (day 2: 0.29%, 95% confidence interval [CI], 0.25% – 0.35% versus 0.08%, 95%CI, 0.03% - 0.26%; day 3: 2.06%, 95%CI, 1.61% - 2.63% versus 0.78%, 95%CI, 0.38% - 1.59%) (Fig. 1). Mean parasitemia in *S. mansoni*-*P. berghei*-co-infected mice was still higher on days 5 and 6, but differences were no longer significant. In those animals surviving the period of susceptibility to CM, a decline in parasitemia could be observed around day 7, which was followed by a rapid increase from day 8 onwards.

On day 0, percentages of reticulocytes were significantly increased approximately two-fold in *S. mansoni*-mono-infected mice compared with uninfected controls (Fig. 2A) and numbers of RBCs were significantly reduced ($P \leq 0.002$) (Fig. 2B). Reticulocyte percentages in *S. mansoni*-mono-infected mice were inversely correlated with erythrocyte counts (Fig. 2C).

3.2. *Schistosoma mansoni*-*P. berghei*-co-infected mice exhibit the most severe weight loss

To investigate whether a pre-existing patent *Schistosoma*-infection influences disease severity of *P. berghei* malaria, body mass and rectal temperatures were determined. Body mass was measured continuously following injection of malaria parasites. Data were analysed up to day 6, when the majority of the *P. berghei*-infected animals died with symptoms of severe disease. For comparisons between the four experimental groups, significance levels after Bonferroni-Holm's P value adjustment were $P < 0.0033$. On days 5 and 6, *S. mansoni*-*P. berghei*-co-infected and *P. berghei*-mono-infected animals were significantly lighter than *S. mansoni*-mono-infected and uninfected control mice ($P < 0.001$) (Fig. 3A). This weight loss

was more profound in the *S. mansoni*-*P. berghei*-co-infected group ($P \leq 0.003$). Interestingly, on day 2, *S. mansoni*-mono-infected mice tended to be lighter than *P. berghei*-mono-infected mice ($P = 0.026$), and on day 5 they tended to be lighter than uninfected controls ($P = 0.039$), indicating that the most severe weight loss in *S. mansoni*-*P. berghei*-co-infected mice probably resulted from a combined effect of the two parasites. While most of the uninfected controls had gained weight by day 6 (five out of seven mice), most of the *S. mansoni*-mono-infected mice had lost weight (four out of five mice). *Plasmodium berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected animals surviving day 8 also lost weight during the period of susceptibility to cerebral malaria, but temporarily regained some weight around days 8 and 9 (Fig. 3B).

Rectal temperatures were compared on day 5 after inoculation with *P. berghei*, when all *P. berghei*-mono-infected and all *S. mansoni*-*P. berghei*-co-infected mice were still alive. *Plasmodium berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice had significantly lower body temperatures than *S. mansoni*-mono-infected and uninfected controls ($P < 0.05$) (Fig. 4). There was no statistical difference either between *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice or between *S. mansoni*-mono-infected and uninfected controls.

3.3. Pre-existing schistosomiasis does not influence survival after co-infection with *P. berghei* ANKA

To establish whether a pre-existing patent *S. mansoni*-infection influences survival during the period of susceptibility to CM, mice were monitored after day 0 in relation to infection with *P. berghei*. Between days 6 and 8, the majority of the *P. berghei*-infected animals either died overnight or developed symptoms of terminal disease (clinical score value of ≥ 4) and were sacrificed. Concurrent patent

schistosomiasis did not alter the low survival rate, as only 11% of the *S. mansoni*-*P. berghei*-co-infected animals (two out of 18) and 25% of the *P. berghei*-mono-infected mice (four out of 16) survived the critical period (Fig. 5). This difference in survival was not significant ($P = 0.28$). In the *S. mansoni*-mono-infected group, one animal died 67 days after exposure to cercariae. Necropsy revealed severe intestinal inflammation and extensive bleeding, especially in the region of the caecum. In contrast, the caecum and colon of the other *S. mansoni*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice either seemed intact or displayed diffuse macroscopic haemorrhages.

3.4. Brain histopathology is decreased in *S. mansoni*-*P. berghei*-co-infected mice

To assess the effect of patent schistosomiasis on *P. berghei*-induced brain pathology, we analysed brain sections of *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice that had been sacrificed with symptoms of fatal disease during the period of susceptibility to CM. Results are presented in form of a mosaic plot (Fig. 6). Strikingly, in the *S. mansoni*-*P. berghei*-co-infected group no signs of brain pathology (CSV 0) could be detected in 42% of the mice that had been killed with symptoms of severe disease (Fig. 7A). In 50% of the animals pathology was moderate (CSV 1) and in 8% ($n = 1$) severe (CSV 2). In contrast, all *P. berghei*-mono-infected animals showed sequestration (Fig. 7B) and haemorrhage (Fig. 7C), and in all but one animal infiltration of immune cells could be observed. Brain pathology was moderate in 56% and severe in 44% of the *P. berghei* mono-infections. No histopathology was detected in *S. mansoni*-mono-infected mice and uninfected controls. Ordinal logistic regression analysis of the final score values resulted in three findings: (i) Infection, with both *P. berghei* ANKA as well as *S. mansoni*, had significant effects on brain pathology ($P < 0.0001$ and $P = 0.0063$,

respectively). (ii) The effects were additive but opposite: while *P. berghei* increased the severity of brain pathology (harmful effect), *S. mansoni* reduced it (protective effect). (iii) The harmful effect of *P. berghei* was larger than the protective effect of *S. mansoni*. In all mice that were killed with symptoms of severe disease, a mono-infection with *P. berghei* ANKA led to brain histopathology but the severity varied amongst the animals.

Interestingly, none of the *S. mansoni*-*P. berghei*-co-infected mice with a CSV of 0 showed any signs of neurological involvement such as paralysis or convulsions. As well as hypothermia, which was observed in all terminally-ill mice, they exhibited signs of general prostration (hunching, inability to walk) and an abnormal breathing pattern. In contrast, neurological signs were present in 56% of the *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice with a CSV of 1 and in 80% of the *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice with a CSV of 2. None of the animals that died between days 6 and 8 suffered from severe anaemia, as judged by skin colour (pink ears and pink plantar surface and digits of forelimbs). Moreover, on day 4 there were no significant differences in RBC counts, haemoglobin concentrations (Hb), and haematocrit levels (HCT) between the four experimental groups (median and interquartile range; *S. mansoni*-*P. berghei*-co-infected: RBC ($\times 10^6/\mu\text{L}$) 7.2 [6.7 - 7.9]; Hb (g/dL) 11.7 [10.9 - 12.6]; HCT (%) 36.2 [35 - 40.7]; *S. mansoni*-mono-infected: RBC ($\times 10^6/\mu\text{L}$) 7.8 [6.9 - 8.6]; Hb (g/dL) 13 [11.4 - 14.7]; HCT (%) 39.8 [36.1 - 44.7]; *P. berghei*-mono-infected: RBC ($\times 10^6/\mu\text{L}$) 9.5 [9.2 - 10.2]; Hb (g/dL) 15.3 [14.7 - 16.1]; HCT (%) 48.2 [45.1 - 50.9]; uninfected control: RBC ($\times 10^6/\mu\text{L}$) 9.2 [8.2 - 9.9]; Hb (g/dL) 14.9 [13.3 - 16.1]; HCT (%) 46.7 [41.6 - 50.8]).

382 3.5. *Parasitemia influences survival in the period of susceptibility to cerebral malaria*
 383 *and negatively correlates with body temperature*

384 Over 40% of *S. mansoni*-*P. berghei*-co-infected mice developed weight loss,
 385 hypothermia and terminal disease in the absence of brain histopathology and
 386 symptoms of neurological involvement. This indicates that the fatal outcome in these
 387 animals was not related to cerebral disease. To analyse whether other factors were
 388 involved in fatal outcomes during the period of susceptibility to CM, we investigated
 389 whether *P. berghei*-infected mice developed lactic acidosis, one of the key features
 390 of circulatory shock. We also compared parasitemias in mice that survived ('surviving
 391 mice') and died ('dying mice') during the CM vulnerable period.

392 On days 6 to 8 after infection with *P. berghei* we observed no differences in
 393 lactate concentrations between *S. mansoni*-*P. berghei*-co-infected, *P. berghei*-mono-
 394 infected, *S. mansoni*-mono-infected and uninfected control mice (Kruskal-Wallis test,
 395 $P = 0.17$) (data not shown). To investigate whether fatal outcomes in the period of
 396 susceptibility to CM were related to parasitemia, we compared the individual time
 397 courses of parasitemia between dying and surviving mice. As there was no statistical
 398 difference in survival between *S. mansoni*-*P. berghei*-co-infected and *P. berghei*-
 399 mono-infected mice, for these comparisons all *P. berghei*-infected mice were
 400 grouped together. As can be seen in Fig. 8A, the majority of dying mice had
 401 parasitemias above 6% on days 6 and 7 (27 out of 28 animals), whereas the majority
 402 of surviving mice had parasitemias below 6% during this period (five out of six mice).
 403 Body temperature was negatively correlated with parasitemia ($P < 0.001$) (Fig. 8B).

405 3.6. *Schistosoma mansoni*-*P. berghei*-co-infected mice have higher plasma
 406 concentrations of IL-5 and IL-13, and on day 6, lower plasma concentrations of IFN-
 407 γ , MCP-1 α , MIG and IL-10 than *P. berghei*-mono-infected mice

To assess the systemic immune response to co-infection, concentrations of nine different cytokines were analysed in *S. mansoni*-*P. berghei*-co-infected, *P. berghei*-mono-infected, *S. mansoni*-mono-infected and uninfected control mice. To evaluate potential effects of handling of mice due to pathogen injection and consecutive blood-taking, and to detect *S. mansoni*-induced alterations to cytokine levels, sham-treated uninfected control mice and *S. mansoni*-mono-infected mice were examined with respect to changes in cytokine concentrations. As in both groups no significant changes in cytokine concentrations over time were found (i. e. between 3 days before and 6 days after infection with *P. berghei*), data of these mice were not included in the following diagrams (Figs. 9 and 10). Cytokine concentrations in *S. mansoni*-*P. berghei*-co-infected and *P. berghei*-mono-infected mice were compared on different days after infection with *P. berghei*. Due to the large number of comparisons the standard *P*-value of 0.05 had to be adjusted according to Bonferroni-Holm and only *P*-values < 0.002 were considered significant. Plasma concentrations of IL-5 and IL-13 were significantly higher in *S. mansoni*-*P. berghei*-co-infected mice compared with *P. berghei*-mono-infected mice (Fig. 9). Mean concentrations of IFN- γ , TNF- α , IL-10, IP-10, MCP-1 and MIG increased during the course of the malaria infection and peaked on day 4, but mean concentrations of IFN- γ and TNF- α were lower in *S. mansoni*-*P. berghei*-co-infected mice (Figs. 9 and 10). *Schistosoma mansoni*-*P. berghei*-co-infected mice had significantly lower concentrations of IFN- γ , IL-10, MCP-1 and MIG on day 6 than *P. berghei*-mono-infected mice. No statistically significant differences in plasma levels of TNF- α , IP-10 and MIP-1 α were seen when comparing the two groups (Figs. 9 and 10).

4. Discussion

Studies investigating the impact of helminths on the pathogenesis of CM are limited and conflicting. In humans, co-infection with *Ascaris* was associated with protection against CM (Nacher et al., 2000). In mouse models, protection against experimental CM has been demonstrated in concomitant infections with irradiated larvae of *Brugia pahangi* (Yan et al., 1997), but a co-infection with *Heligmosomoides polygyrus* had no effect (de Souza and Helmbly, 2008). We analysed the impact of patent *S. mansoni* co-infection on severe malaria, using the well established C57BL/6 mouse model of CM. Our data demonstrate that *S. mansoni* diminishes the Th1 / pro-inflammatory response to *P. berghei* and protects C57BL/6 mice against CM-associated brain pathology but does not prevent malaria-induced severe disease and death.

Christensen et al. (1988) showed that the outcomes of experimental co-infections with protozoans and trematodes were dependent on the parasite species as well as the relative timing of infection. In co-infections with *S. mansoni* and malaria parasites, patent *S. mansoni* infection increased the susceptibility of resistant BL/6 mice to *P. chabaudi* (Helmbly et al., 1998), but abolished the susceptibility of A/J mice to the same *Plasmodium* sp. and protected against death from malaria (Yoshida et al., 2000). Moreover, in BALB/c mice, *S. mansoni* increased parasitemia and induced death from otherwise non-lethal *P. yoelii* infections (Sangweme et al., 2009). Thus, immunomodulation by *S. mansoni* might depend, at least partially, on interactions between host and concomitant *Plasmodium* parasites.

It has been hypothesized that regulatory responses associated with patent schistosomiasis might inhibit effective parasite control during early stage malaria (Hartgers and Yazdanbakhsh, 2006). In agreement with this, we observed that percentages of pRBCs were significantly increased in *S. mansoni*-*P. berghei*-co-infected mice early during malaria infection. In mice infected with *P. chabaudi*, a

concurrent infection with *H. polygyrus* led to an increased parasitemia and mortality, and this was associated with reduced Th1 cytokine responses (Su et al., 2005). Th1 cytokines might be necessary for the effective control of early phase parasitemia in murine malaria (Van Der Heyde et al., 1997; Yoneto et al., 1999). We observed that mean concentrations of the Th1 cytokine IFN- γ were generally lower in *S. mansoni*-*P. berghei*-co-infected mice, but differences in *P. berghei*-mono-infected mice failed to reach statistical significance at these early time points. We also observed that reticulocyte percentages were significantly elevated in *S. mansoni*-mono-infected mice before they were inoculated with *P. berghei* ANKA. These cells are preferentially invaded by *P. berghei* ANKA when parasitemia is low (Otten-Kuipers et al., 1997). Thus, by providing malaria parasites with more target cells, the *S. mansoni* infection might have initially facilitated malaria parasite growth.

We found that, in *S. mansoni*-mono-infected mice, erythrocyte numbers were significantly decreased on day 0 (ie the day of infection with *P. berghei*) and erythrocyte numbers were inversely correlated with reticulocyte counts. Therefore, elevated reticulocyte levels in *S. mansoni*-mono-infected mice could have been due to *Schistosoma*-induced mild anaemia; small amounts of blood in the faeces of these mice suggest that it was probably caused by intestinal bleeding induced by schistosomal eggs. Nevertheless, the healthy appearance of most of the animals and the pink colour of their extremities confirmed that this blood loss usually did not severely affect the mice.

Inoculation of C57BL/6 mice with *P. berghei* ANKA results in a lethal syndrome, commonly named CM, in which the majority of mice die 6 to 7 days after infection (Chang et al., 2001). As it has been suggested that the immune response to patent schistosomiasis might down-modulate the malaria-induced pro-inflammatory response (Hartgers and Yazdanbakhsh, 2006), we expected that a concurrent

infection with schistosomal parasites might delay or attenuate the development of severe *P. berghei* malaria and prolong the life-span of the animals. However, *S. mansoni*-*P. berghei*-co-infected mice were not protected against malaria-induced weight loss and hypothermia, and developed symptoms of terminal disease at the same time point and died at a similar rate to *P. berghei*-mono-infected animals. Moreover, while the only *S. mansoni*-mono-infected mouse that succumbed during the experimental period exhibited severe intestinal bleeding, dying co-infected mice showed no signs of such *Schistosoma*-related severe disease. Taken together, this suggests a similar malaria-related cause of death in *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice.

As early death of *P. berghei* ANKA-infected mice is commonly associated with severe brain histopathology (Lou et al., 2001), we analysed brains of *S. mansoni*-*P. berghei*-co-infected and *P. berghei*-mono-infected animals that developed symptoms of terminal disease in the acute phase of the infection. Strikingly, co-infection with *Schistosoma* had a significant protective effect on brain pathology. However, the protective effect of patent schistosomiasis was smaller than the harmful effect of *P. berghei*-induced malaria. Moreover, cerebral pathology varied among *P. berghei*-mono-infected mice, suggesting that the down-modulatory effect of schistosomiasis abrogated brain pathology in those malaria-infected mice that would otherwise have developed 'mild' pathology, and diminished pathology in animals that were prone to severe disease.

While CM is associated with a predominant Th1 / inflammatory immune response (Hartgers et al., 2008), helminths commonly generate Th2 and anti-inflammatory responses (Maizels et al., 2004). As plasma concentrations of the Th2 cytokines IL-5 and IL-13 were elevated in *S. mansoni*-*P. berghei*-co-infected mice at all time points, the *S. mansoni*-induced Th2 response appeared to prevail during the

concurrent acute malaria infection. It has been shown that in mice infected with *S. mansoni*, the anti-inflammatory cytokine IL-10 down-regulates IFN- γ and reduces mortality (Hoffmann et al., 2000). We found that, in *S. mansoni*-infected mice, systemic IL-10 was neither elevated before nor after inoculation with the malaria parasites, and on day 6, IL-10 was even lower in *S. mansoni*-*P. berghei*-co-infected mice. Similar results were found in *S. mansoni*-*P. berghei*-co-infected ICR HSD mice on day 7 (Waknine-Grinberg et al., 2010). Thus, it is unlikely that IL-10 contributed to the down-modulation of malaria-induced systemic inflammation in this co-infection model.

During CM, IFN- γ and TNF- α play an important role in up-regulation of cellular adhesion molecules (CAM) on brain endothelial cells (Weiser et al., 2007). This process leads to the sequestration of leukocytes in microvessels and is required for the development of murine CM (Favre et al., 1999). In *P. berghei* infections, leukocyte recruitment to the brain is dependent on pro-inflammatory chemokines such as IP-10, MCP-1, MIG and MIP-1 α (Belnoue et al., 2008; Campanella et al., 2008). In mice, MIG and IP-10 are essential for the development of CM (Campanella et al., 2008), and in humans the chemokine IP-10 was found to be the only biomarker in serum that predicted CM mortality in Ghanaian children (Armah et al., 2007). Significantly lower plasma concentrations of IFN- γ , MCP-1 and MIG in our *S. mansoni*-*P. berghei*-co-infected mice suggest that *S. mansoni* infection down-regulated the Th1 / inflammatory response induced by *P. berghei*. Thus, gradually lower levels of IFN- γ and inflammatory chemokines may have reduced the up-regulation of CAM and the recruitment of leukocytes, and this may have contributed to the observed protection against brain histopathology in *S. mansoni*-*P. berghei*-co-infected mice.

A comparable protective effect of patent schistosomiasis on inflammation of the CNS was also demonstrated in a murine model of multiple sclerosis (MS). Here, the number of inflammatory leukocytes in the brain and spinal cord was clearly reduced in *S. mansoni*-infected mice. This was accompanied by a down-regulation of pro-inflammatory cytokines (IFN- γ and TNF- α) and reduced nitric oxide (NO) production in response to MS-inducing antigens (La Flamme et al., 2003).

We found that over 40% of *S. mansoni*-*P. berghei*-co-infected mice developed weight loss, hypothermia and symptoms of terminal disease but showed no signs of cerebral pathology. Moreover, none of these animals showed any symptoms of neurological involvement such as paralysis or convulsions. Fatal outcome in the absence of brain pathology indicates that in the *P. berghei* ANKA model in C57BL/6 mice other causes of death must exist in parallel to CM. Our finding is consistent with other studies showing that reduction of *P. berghei*-induced brain pathology is not automatically associated with protection against coma and death (Engwerda et al., 2002; Piguet et al., 2002). The fatal outcome of infection in these experiments was instead attributed to immune-mediated metabolic disturbances (Piguet et al., 2002).

It has also been proposed that early death during *P. berghei* malaria might result from circulatory shock, a key feature of which is lactic acidosis (Chang et al., 2001). As no increase in lactate concentrations could be detected in any of our *P. berghei*-infected mice that succumbed around day 6, it seems unlikely that lactic acidosis played a role in the early death of *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice in the present experiments. As on day 4 there were no significant differences in RBC parameters between the four experimental groups, this indicates that *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice did not die due to severe anaemia. However, our results suggest a causal link between *P. berghei*-induced mortality and increased malaria parasite levels during

the CM vulnerable period. Moreover, inverse correlation of parasitemia with body temperature provides additional evidence for a direct influence of parasitemia on the pathogenesis of malarial disease. Thus, mice with low parasitemia were probably protected due to insufficient antigenic stimulation by *P. berghei* parasites.

In contrast to our experiments, Waknine-Grinberg et al. (2010) reported that a concomitant patent *S. mansoni* infection in ICR HSD mice led to a reduction of *P. berghei*-induced CM and increased survival. Protection against CM correlated with higher body temperatures, body weights and increased survival in co-infected mice. However, in these experiments CM was defined by death of mice at low parasitemia, accompanied by neurological symptoms, weight loss and hypothermia. Brain pathology in co-infected mice was not analysed. ICR mice were inoculated with low numbers of *P. berghei*-infected RBCs (5×10^4) and the majority of *P. berghei*-mono-infected animals developed CM around day 10. At this time point, systemic IL-10 concentrations in *S. mansoni*-*P. berghei*-co-infected ICR mice were higher than in *P. berghei*-mono-infected mice. In contrast, our C57BL/6 mice were infected with higher numbers of *P. berghei*-infected RBCs (10^6) and the majority of *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected animals developed fatal disease on day 6, a time point at which *S. mansoni*-*P. berghei*-co-infected C57BL/6 mice had significantly lower plasma concentrations of IFN- γ and IL-10. Thus, it is possible that the differences in the outcome of the two *S. mansoni*-*P. berghei* co-infection studies are related to the differences in the *P. berghei* infection dose and/or the genetic background of the host.

The disease outcome of *P. berghei* malaria could also vary with the stage of the concomitant schistosome infection. In the present experiments, we applied 8-to-9-week-old *S. mansoni* infections, which are marked by strong Th2 responses and are considered as acute stage infections. In contrast, schistosome infections at later

time points are characterized by an expansion of regulatory T cells and increased activation of immunosuppressive networks (chronic infections) (Singh et al., 2005). Therefore, it might be interesting to study whether different *P. berghei*-related disease patterns are found during chronic *S. mansoni* infections.

In conclusion, we have demonstrated that a concomitant 8-to-9-week-old *S. mansoni* infection conferred protection against brain pathology but not against severe disease and mortality in the C57BL/6 mouse model of CM. Fatal outcome was instead associated with parasitemia. Taken together, this indicates that in *P. berghei*-induced malaria severe disease and death can be caused by distinct pathogenic mechanisms, which develop in parallel and are differentially influenced by the immune response to *S. mansoni*. This might explain why in *S. mansoni*-*P. berghei*-co-infected mice death can be induced in a fashion independent of the presence of brain pathology. Further experimental studies using other species of helminths and/or other animal models of CM are needed to provide more information on the complex effects of helminth co-infections on the disease syndrome of CM.

Acknowledgements

This work was supported by the National Genome Research Network (NGFN) program of the Bundesministerium für Bildung und Forschung (BMBF), Germany (grant no. 01GSO403. NIE-S37T32), and by the European Union (project no. INCO-CT 2006-032321-SCOOTT). Kirsten Bucher was supported by the Deutsche Gesellschaft für Pneumologie. We thank Eric Kendjo (Institute of Tropical Medicine, University of Tübingen, Germany) for help with statistical analysis of RBC data.

References

- Armah, H. B., Wilson, N. O., Sarfo, B. Y., Powell, M. D., Bond, V. C., Anderson, W., Adjei, A. A., Gyasi, R. K., Tettey, Y., Wiredu, E. K., Tongren, J. E., Udhayakumar, V., Stiles, J. K. (2007). Cerebrospinal fluid and serum biomarkers of cerebral malaria mortality in Ghanaian children. *Malar. J.* 6, 147-164.
- Belnoue, E., Potter, S. M., Rosa, D. S., Mauduit, M., Gruner, A. C., Kayibanda, M., Mitchell, A. J., Hunt, N. H., Renia, L. (2008). Control of pathogenic CD8+ T cell migration to the brain by IFN-gamma during experimental cerebral malaria. *Parasite Immunol.* 30, 544-553.
- Briand, V., Watier, L., Le Hesran, J. Y., Garcia, A., Cot, M. (2005). Coinfection with *Plasmodium falciparum* and *Schistosoma haematobium*: protective effect of schistosomiasis on malaria in senegalese children? *Am. J. Trop. Med. Hyg.* 72, 702-707.
- Campanella, G. S., Tager, A. M., El Khoury, J. K., Thomas, S. Y., Abraszinski, T. A., Manice, L. A., Colvin, R. A., Luster, A. D. (2008). Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4814-4819.
- Chabasse, D., Bertrand, G., Leroux, J. P., Gauthey, N., Hocquet, P. (1985). [Developmental bilharziasis caused by *Schistosoma mansoni* discovered 37 years after infestation]. *Bull. Soc. Pathol. Exot. Filiales.* 78, 643-647.

- 635 Chang, W. L., Jones, S. P., Lefer, D. J., Welbourne, T., Sun, G., Yin, L., Suzuki, H.,
636 Huang, J., Granger, D. N., Van Der Heyde, H. C. (2001). CD8(+)-T-cell
637 depletion ameliorates circulatory shock in *Plasmodium berghei*-infected mice.
638 Infect. Immun. 69, 7341-7348.
- 639 Christensen, N. O., Furu, P., Kurtzhals, J., Odaibo, A. (1988). Heterologous
640 synergistic interactions in concurrent experimental infection in the mouse with
641 *Schistosoma mansoni*, *Echinostoma revolutum*, *Plasmodium yoelii*, *Babesia*
642 *microti*, and *Trypanosoma brucei*. Parasitol. Res. 74, 544-551.
- 643 de Souza, B., Helmby, H. (2008). Concurrent gastro-intestinal nematode infection
644 does not alter the development of experimental cerebral malaria. Microbes.
645 Infect. 10, 916-921.
- 646 Dunne, D. W., Cooke, A. (2005). A worm's eye view of the immune system:
647 consequences for evolution of human autoimmune disease. Nat. Rev.
648 Immunol. 5, 420-426.
- 649 Engwerda, C. R., Mynott, T. L., Sawhney, S., de Souza, J. B., Bickle, Q. D., Kaye, P.
650 M. (2002). Locally up-regulated lymphotoxin alpha, not systemic tumor
651 necrosis factor alpha, is the principle mediator of murine cerebral malaria. J.
652 Exp. Med. 195, 1371-1377.
- 653 Favre, N., Da Laperousaz, C., Ryffel, B., Weiss, N. A., Imhof, B. A., Rudin, W.,
654 Lucas, R., Piguet, P. F. (1999). Role of ICAM-1 (CD54) in the development of
655 murine cerebral malaria. Microbes. Infect. 1, 961-968.

- 656 Fernandez, R. D., Dubben, B., Saeftel, M., Endl, E., Deininger, S., Hoerauf, A.,
657 Specht, S. (2009). Filarial infection induces protection against *P. berghei* liver
658 stages in mice. *Microbes. Infect.* 11, 172-180.
- 659 Graham, A. L., Lamb, T. J., Read, A. F., Allen, J. E. (2005). Malaria-filaria coinfection
660 in mice makes malarial disease more severe unless filarial infection achieves
661 patency. *J. Infect. Dis.* 191, 410-421.
- 662 Hamm, D. M., Agossou, A., Gantin, R. G., Kocherscheidt, L., Banla, M., Dietz, K.,
663 Soboslay, P. T. (2009). Coinfections with *Schistosoma haematobium*, *Necator*
664 *americanus*, and *Entamoeba histolytica/Entamoeba dispar* in children:
665 Chemokine and cytokine responses and changes after antiparasite treatment.
666 *J. Infect. Dis.* 199, 1583-1591.
- 667 Hartgers, F. C., Obeng, B. B., Boakye, D., Yazdanbakhsh, M. (2008). Immune
668 responses during helminth-malaria co-infection: a pilot study in Ghanaian
669 school children. *Parasitology* 135, 855-860.
- 670 Hartgers, F. C., Yazdanbakhsh, M. (2006). Co-infection of helminths and malaria:
671 modulation of the immune responses to malaria. *Parasite Immunol.* 28, 497-
672 506.
- 673 Helmby, H., Kullberg, M., Troye-Blomberg, M. (1998) Altered immune responses in
674 mice with concomitant *Schistosoma mansoni* and *Plasmodium chabaudi*
675 infections. *Infect. Immun.* 66, 5167-5174.

- 676 Hoffmann, K. F., Cheever, A. W., Wynn, T. A. (2000). IL-10 and the dangers of
677 immune polarization: excessive type 1 and type 2 cytokine responses induce
678 distinct forms of lethal immunopathology in murine schistosomiasis. J.
679 Immunol. 164, 6406-6416.
- 680 Hunt, N. H., Grau, G. E. (2003). Cytokines: accelerators and brakes in the
681 pathogenesis of cerebral malaria. Trends Immunol. 24, 491-499.
- 682 La Flamme, A. C., Ruddenklau, K., Backstrom, B. T. (2003). Schistosomiasis
683 decreases central nervous system inflammation and alters the progression of
684 experimental autoimmune encephalomyelitis. Infect. Immun. 71, 4996-5004.
- 685 Lackner, P., Beer, R., Heussler, V., Goebel, G., Rudzki, D., Helbok, R., Tannich, E.,
686 Schmutzhard, E. (2006). Behavioural and histopathological alterations in mice
687 with cerebral malaria. Neuropathol. Appl. Neurobiol. 32, 177-188.
- 688 Le Hesran, J. Y., Akiana, J., Ndiaye, e. H., Dia, M., Senghor, P., Konate, L. (2004).
689 Severe malaria attack is associated with high prevalence of *Ascaris*
690 *lumbricoides* infection among children in rural Senegal. Trans. R. Soc. Trop.
691 Med. Hyg. 98, 397-399.
- 692 Lohning, M., Grogan, J. L., Coyle, A. J., Yazdanbakhsh, M., Meisel, C., Gutierrez-
693 Ramos, J. C., Radbruch, A., Kamradt, T. (1999). T1/ST2 expression is
694 enhanced on CD4⁺ T cells from schistosome egg-induced granulomas:
695 analysis of Th cell cytokine coexpression ex vivo. J. Immunol. 162, 3882-3889.

- 696 Lou, J., Lucas, R., Grau, G. E. (2001). Pathogenesis of cerebral malaria: recent
697 experimental data and possible applications for humans. Clin. Microbiol. Rev.
698 14, 810-820.
- 699 Lwin, M., Last, C., Targett, G. A., Doenhoff, M. J. (1982). Infection of mice
700 concurrently with *Schistosoma mansoni* and rodent malarias: contrasting
701 effects of patent *S. mansoni* infections on *Plasmodium chabaudi*, *P. yoelii* and
702 *P. berghei*. Ann. Trop. Med. Parasitol. 76, 265-273.
- 703 Lyke, K. E., Dicko, A., Dabo, A., Sangare, L., Kone, A., Coulibaly, D., Guindo, A.,
704 Traore, K., Daou, M., Diarra, I., Sztein, M. B., Plowe, C. V., Doumbo, O. K.
705 (2005). Association of *Schistosoma haematobium* infection with protection
706 against acute *Plasmodium falciparum* malaria in Malian children. Am. J. Trop.
707 Med. Hyg. 73, 1124-1130.
- 708 Maizels, R. M., Yazdanbakhsh, M. (2003). Immune regulation by helminth parasites:
709 cellular and molecular mechanisms. Nat. Rev. Immunol. 3, 733-744.
- 710 Maizels, R. M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M. D., Allen, J. E.
711 (2004). Helminth parasites - masters of regulation. Immunol. Rev. 201, 89-
712 116.
- 713 Nacher, M., Gay, F., Singhasivanon, P., Krudsood, S., Treeprasertsuk, S., Mazier,
714 D., Vouldoukis, I., Looareesuwan, S. (2000). *Ascaris lumbricoides* infection is
715 associated with protection from cerebral malaria. Parasite Immunol. 22, 107-
716 113.

- 717 Nacher, M., Singhasivanon, P., Yimsamran, S., Manibunyong, W., Thanyavanich, N.,
718 Wuthisen, R., Looareesuwan, S. (2002). Intestinal helminth infections are
719 associated with increased incidence of *Plasmodium falciparum* malaria in
720 Thailand. J. Parasitol. 88, 55-58.
- 721 Otten-Kuipers, M. A., Franssen, F. F., Nieuwenhuijs, H., Overdulse, J. P., Roelofsen,
722 B., Op den Kamp, J. A. (1997). Effect of tryptophan-N-formylated gramicidin
723 on growth of *Plasmodium berghei* in mice. Antimicrob. Agents Chemother. 41,
724 1778-1782.
- 725 Piguet, P. F., Kan, C. D., Vesin, C. (2002). Role of the tumor necrosis factor receptor
726 2 (TNFR2) in cerebral malaria in mice. Lab. Invest. 82, 1155-1166.
- 727 Raso, G., Luginbuhl, A., Adjoua, C. A., Tian-Bi, N. T., Silue, K. D., Matthys, B.,
728 Vounatsou, P., Wang, Y., Dumas, M. E., Holmes, E., Singer, B. H., Tanner,
729 M., N'goran, E. K., Utzinger, J. (2004). Multiple parasite infections and their
730 relationship to self-reported morbidity in a community of rural Cote d'Ivoire. Int.
731 J. Epidemiol. 33, 1092-1102.
- 732 Sangweme, D., Shiff, C., Kumar, N. (2009). *Plasmodium yoelii*: Adverse outcome of
733 non-lethal *P. yoelii* malaria during co-infection with *Schistosoma mansoni* in
734 BALB/c mouse model. Exp. Parasitol. 122, 254-259.
- 735 Singh, K. P., Gerard, H. C., Hudson, A. P., Reddy, T. R., Boros, D. L. (2005)
736 Retroviral Foxp3 gene transfer ameliorates liver granuloma pathology in
737 *Schistosoma mansoni* infected mice. Immunology 114, 410-417.

- 738 Smithers, S. R., Terry, R. J. (1965). The infection of laboratory hosts with cercariae of
739 *Schistosoma mansoni* and the recovery of the adult worms. *Parasitology* 55,
740 695-700.
- 741 Snow, R. W., Trape, J. F., Marsh, K. (2001). The past, present and future of
742 childhood malaria mortality in Africa. *Trends Parasitol.* 17, 593-597.
- 743 Su, Z., Segura, M., Morgan, K., Loredó-Osti, J. C., Stevenson, M. M. (2005).
744 Impairment of protective immunity to blood-stage malaria by concurrent
745 nematode infection. *Infect. Immun.* 73, 3531-3539.
- 746 Van Der Heyde, H. C., Pepper, B., Batchelder, J., Cigel, F., Weidanz, W. P. (1997).
747 The time course of selected malarial infections in cytokine-deficient mice. *Exp.*
748 *Parasitol.* 85, 206-213.
- 749 Wakine-Grinberg, J., Gold, D., Ohayon A., Flescher, E., Heyfets, A., Doenhoff M., J.,
750 Schramm, G., Haas, H., Golenser, J. (2010). *Schistosoma mansoni* infection
751 reduces the incidence of murine cerebral malaria. *Malar. J.* 9, 5-16.
- 752 Weiser, S., Miu, J., Ball, H. J., Hunt, N. H. (2007). Interferon-gamma synergises with
753 tumour necrosis factor and lymphotoxin-alpha to enhance the mRNA and
754 protein expression of adhesion molecules in mouse brain endothelial cells.
755 *Cytokine* 37, 84-91.

- 756 Yan, Y., Inuo, G., Akao, N., Tsukidate, S., Fujita, K. (1997). Down-regulation of
757 murine susceptibility to cerebral malaria by inoculation with third-stage larvae
758 of the filarial nematode *Brugia pahangi*. *Parasitology* 114, 333-338.
- 759 Yoneto, T., Yoshimoto, T., Wang, C. R., Takahama, Y., Tsuji, M., Waki, S., Nariuchi,
760 H. (1999). Gamma interferon production is critical for protective immunity to
761 infection with blood-stage *Plasmodium berghei* XAT but neither NO production
762 nor NK cell activation is critical. *Infect. Immun.* 67, 2349-2356.
- 763 Yoshida, A., Maruyama, H., Kumagai T., Amano, T., Kobayashi, F., Zhang, M.,
764 Himeno, K., Ohta, N. (2000). *Schistosoma mansoni* infection cancels the
765 susceptibility to *Plasmodium chabaudi* through induction of type 1 immune
766 responses in A/J mice. *Int. Immunol.* 12, 1117-1125.

Legends to figures

Fig. 1. Parasitemia in mice co-infected with *Schistosoma mansoni* and *Plasmodium berghei* ANKA strain. C57BL/6 mice with an 8-to-9-week-old *S. mansoni* infection were injected with 10^6 *P. berghei*-parasitized red blood cells (pRBC) (Sm+PbA; $n = 18$), and were analysed together with *P. berghei*-mono-infected animals (PbA; $n = 16$). Parasitemias were logit transformed and subjected to analysis of variance. Post hoc *t*-tests for two by two comparisons between the groups were performed with Bonferroni-Holm's *P* value adjustment, with significance levels of $P < 0.006$. The data shown were back transformed. They represent mean parasitemias and 95% confidence intervals. ($*P \leq 0.003$). The grey area indicates the period when the majority of *Plasmodium*-infected animals died.

Fig. 2. Reticulocyte percentages and erythrocyte numbers in *Schistosoma mansoni*-mono-infected (Sm) and uninfected control mice. Reticulocyte percentages (A) and erythrocyte numbers (B) were counted after 8 to 9 weeks of schistosomal infection, immediately before mice were inoculated with *Plasmodium berghei* parasites. Horizontal lines indicate the median. Data were compared using a Mann-Whitney (U) test ($*P \leq 0.002$). (Reticulocytes: Sm: $n = 24$; control: $n = 23$; erythrocytes: Sm: $n = 13$; control: $n = 13$). (C) In *S. mansoni*-mono-infected mice reticulocyte percentages were inversely correlated with erythrocyte counts. Figure shows scatter plot matrix and 95% density ellipse. ($r^2 = -0.63$; $P = 0.02$; $n = 13$).

Fig 3. Change of body mass after co-infection with *Schistosoma mansoni* and *Plasmodium berghei*. (A) Data of *S. mansoni*-*P. berghei*-co-infected (Sm+PbA; $n = 18$), *P. berghei*-mono-infected (PbA; $n = 16$), *S. mansoni*-mono-infected (Sm; $n = 6$), and uninfected control mice ($n = 7$) were compared using ANOVA. Post hoc *t*-tests for comparisons between the groups were performed with Bonferroni-Holm's *P* value adjustment, with significance levels of $P < 0.0033$. The data represent means and 95% confidence intervals. On days 5 and 6, *P. berghei*-mono-infected and *S. mansoni*-*P. berghei*-co-infected mice were lighter than *S. mansoni*-mono-infected and uninfected controls ($P < 0.001$). This weight loss was significantly more pronounced in the *S. mansoni*-*P. berghei*-co-infected group ($P \leq 0.003$). (B) Change in body mass in individual *S. mansoni*-*P. berghei*-co-infected and *P. berghei*-mono-infected mice that survived the period of susceptibility to cerebral malaria. Most of the animals became lighter between days 5 and 8 after *Plasmodium* infection, but temporarily regained weight afterwards.

Fig. 4. Rectal body temperatures after co-infection with *Schistosoma mansoni* and *Plasmodium berghei* on day 5 after injection of 10^6 *P. berghei*-parasitized red blood cells (pRBC). *Schistosoma mansoni*-*P. berghei*-co-infected C57BL/6 mice (Sm+PbA; $n = 18$) were analysed together with *P. berghei*-mono-infected (PbA; $n = 16$), *S. mansoni*-mono-infected (Sm; $n = 4$), and uninfected control mice ($n = 7$). Symbols indicate means and 95% confidence intervals. Data were Box-Cox-transformed and compared using bi-factorial analysis of variance followed by Tukey's Honestly Significant Difference (HSD) test. Asterisks indicate significant differences between the groups ($*P < 0.05$).

Fig. 5. Survival after co-infection with *Schistosoma mansoni* and *Plasmodium berghei*. *Schistosoma mansoni*-*P. berghei*-co-infected C57BL/6 mice (Sm+PbA; $n = 18$) were examined together with *P. berghei*-mono-infected mice (PbA; $n = 16$). Data were analysed using a Wilcoxon test. After 6 to 8 days of infection with *P. berghei*, the majority of the malaria-infected animals died, and there was no difference in survival between *S. mansoni*-*P. berghei*-co-infected and *P. berghei*-mono-infected animals ($P = 0.28$).

Fig. 6. Observed frequencies of cerebral score values (CSV) in the four experimental groups. Values ranging from 0 to 2 indicate the degree of brain histopathology (0 = none; 1 = moderate; 2 = severe). The mosaic plot presents the percentage occurrence of these score values, which add up to 100% within each group. Column width indicates sample size. Right column shows overall frequencies for animals of all experimental groups. (*Schistosoma mansoni*-*Plasmodium berghei*-co-infected (Sm+PbA): $n = 12$; *P. berghei*-mono-infected (PbA): $n = 9$; *S. mansoni*-mono-infected (Sm): $n = 5$; Control: $n = 7$).

Fig. 7. Histomorphology of brain sections of *Plasmodium berghei* ANKA-infected C57BL/6 mice on day 6 after challenge. (A) Brain section of a *Schistosoma mansoni*-*P. berghei*-co-infected mouse showing a healthy uninfected blood vessel. (B and C) Brain sections of a *P. berghei*-mono-infected animal showing (B) mononuclear cell accumulation and sequestration and (C) a microhaemorrhage (arrow). Sections stained with H&E. Magnification, x 400.

Fig. 8. Parasitemia in mice dying and surviving during the cerebral malaria (CM) vulnerability period and its influence on body temperature. As there was no statistical difference in survival between *Plasmodium berghei*-mono-infected (PbA) and *Schistosoma mansoni*-*P. berghei*-co-infected mice, all *Plasmodium*-infected mice were grouped together. (A) Time courses of parasitemia of individual mice are presented as solid lines. Lines ending between days 5 and 8 indicate death of the animal on the same or the following day. Most dying mice had parasitemias above 6% (dashed line) on days 6 and 7, whereas most surviving mice had parasitemias below 6% during this period. (B). On day 5 parasitemia was negatively correlated with body temperature. Figure shows scatter plot matrix and 95% density ellipse.

Correlation was tested using the Spearman rank correlation ($r^2 = -0.57$; $P < 0.001$; $n = 34$). RBC, red blood cell.

Fig. 9. Cytokine responses of IFN- γ , TNF- α , IL-5, IL-10 and IL-13 in mice co-infected with *Schistosoma mansoni* and *Plasmodium berghei* ANKA strain. C57BL/6 mice with an 8-to-9-week-old *S. mansoni*-infection were injected with 10^6 *P. berghei*-parasitized red blood cells (Sm+PbA) and were analysed together with *P. berghei*-mono-infected (PbA) mice. Data were logarithmically transformed and compared using ANOVA. Comparisons between the *S. mansoni*-*P. berghei*-co-infected and the *P. berghei*-mono-infected group were performed with Bonferroni-Holm's P value adjustment, with significance levels of $*P < 0.002$. The data shown are back-transformed and represent means and 95% confidence intervals. Horizontal lines indicate the detection limit. (Sm+PbA: $n = 4$, PbA: $n = 4$).

Fig. 10. Chemokine response of IP-10, monocyte chemoattractant protein-1 (MCP-1), monokine induced by IFN- γ (MIG) and macrophage inflammatory protein-1 (MIP-1 α) after co-infection with *Plasmodium berghei* (PbA) and *Schistosoma mansoni* (Sm). Data were analysed and presented as described in Fig. 9.

Effect of helminth coinfection on severe malarial disease?

Coinfection model of schistosomiasis and cerebral malaria (CM):

Survival

Coinfection with *S. mansoni* does not prevent *P. berghei*-induced early death

Brain histopathology:

Coinfection with *S. mansoni* protects against CM-associated brain histopathology

Figure 1

Figure 3

Figure 4

Figure 5

Figure 6

Figure 8

Figure 9

Figure 10