博士論文（要約）

Hemophagocytosis in visceral leishmaniasis

(内臓型リーシュマニア症における血球貪食)

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Contents

General introduction

Visceral leishmaniasis (VL), also known as kala-azar, is caused by parasitic protozoa of the genus *Leishmania*. Endemic countries of VL include India, Bangladesh, Nepal, Brazil, Ethiopia and Sudan. It is estimated that there are 300,000 new cases of VL and 20,000 deaths annually (WHO, 2012). In sand flies, the insect vector for *Leishmania*, the parasites develop as promastigotes in the midgut. Once transmitted to mammalian hosts through blood feeding by sand flies, these parasites proliferate as amastigotes within macrophages in the spleen, liver and bone marrow. VL is characterized by clinical manifestations such as fever, weight loss, hepatosplenomegaly and anemia (Fig. 1).

According to a systematic analysis of the literature on anemia in VL, the prevalence of anemia among VL patients is quite high despite of different geographical backgrounds and the severity of anemia is categorized into moderate to severe anemia (1) (Fig. 2). In general, the causes of anemia can be classified into two categories, reduced normal RBC production and increased RBC destruction (2). In VL patients, various mechanisms are proposed in both two categories of reduced RBC production and increased RBC destruction. Since bone marrow is one of the major sites for

Leishmania parasites proliferation, it is possible that erythropoiesis in bone marrow of VL patients is interfered after infection. Several reports describe abnormal bone marrow structures in VL patients (3, 4). Other reports have mentioned that RBCs from VL patients are microcytic and hypochromic (5, 6), implying defective erythropoiesis by iron deficiency. On the other hand, some groups consider RBCs from VL patients are destructed by hemolysis resulted from the change of membrane or adhere of autoantibody to RBC (7-9). Thus, the pathogenesis of anemia during VL remains controversial partly because there are experimental and ethical limitations in studying the pathogenesis using human subjects.

Therefore, appropriate animal models for studying VL pathology are needed to elucidate the mechanisms of anemia during the disease, while there had not been a mouse model of VL exhibiting anemia. There are only a few reports on anemia in rodent models of *Leishmania* infection. In mice, there was only one report on anemia which is caused by *L. tropica* infection (10), but none by *L. donovani* complex. *L. donovani*-induced anemia was reported only in hamster models (11-13). Although hamsters are regarded as a better model for VL pathology than mice, they have fewer advantages in immunological studies such as availability of reagents (e.g., antibodies) and labor in animal work (e.g., housing, generation of transgenic animals).

As a part of my master thesis, I have developed a mouse model of VL by *L. donovani* exhibiting anemia for the first time (14). Mice chronically infected with *L. donovani* had low hematocrit, hemoglobin and erythrocyte counts while having up-regulated erythropoiesis, suggesting hemolytic events due to infection. Through the study, it is proposed that hemophagocytosis is one of the hemolytic events associated with anemia in the infected mice. The spleen is the major place for hemophagocytosis; there, multinucleated giant cells heavily infected with amastigotes are markedly observed and are the major cell type phagocytosing erythrocytes. These results suggest that heavy infection of macrophages with *Leishmania* parasites triggers phagocytosis of erythrocytes resulting in anemia during murine VL. Because hemophagocytosis has been reported in human VL cases, reproduction of the pathology in mice may facilitate an understanding of the mechanisms leading to anemia during VL. Thus, my master thesis work contributed to advancing the understanding on the mechanism of anemia during VL.

However, the mechanisms of hemophagocytosis or the physiological function of hemophagocytosis still need to be further examined in order to connect the study on anemia/hemophagocytosis to development of novel interventions for VL. Questions to be addressed include signals required for induction of hemophagocytosis through direct

parasite infection and from other accessory cells, molecular mechanisms in macrophages for induced self-eating of erythrocytes, and influence of hemophagocytosis to *Leishmania* survival. Induction of hemophagocytosis by *Leishmania* infection means that a macrophage becomes the place for harboring both parasites and erythrocytes simultaneously. This situation is not necessarily true in other infection-associated hemophagocytosis. For example, infection of *Trypanosoma brucei*, the related kinetoplastid parasite, can also cause anemia (15), whereas the parasites proliferate extracellularly in the blood of mammalian hosts with a clear contrast to intracellular parasitism of *Leishmania*. These indicate that molecular mechanisms of hemophagocytosis and its role to infecting pathogens can be different among infectious diseases causing hemophagocytosis.

In my Ph.D. thesis, the contribution of hemophagocytes to anemia during VL, molecular mechanisms of hemophagocytosis during VL and the role of hemophagocytosis in *Leishmania* survival have been addressed using an experimental model. Chapter 1 has focused on detailed characterization of hemophagocytosis during experimental VL and demonstrated that direct infection by *L. donovani* is the key for macrophage hyper-activation. In chapter 2, molecular mechanisms for *Leishmania*-induced hemophagocytosis have been focused, resulting in identification of

a candidate molecule responsible for the phenomenon. Chapter 3 has addressed the influence of erythrocyte uptake by infected macrophages demonstrating the positive impact of hemophagocytosis to parasite survival. I believe that results in this thesis will serve as the first step of elucidation of hemophagocytosis during VL which contribute to a better intervention in controlling the human diseases.

Please note that the presented thesis includes contents previously reported as a part of my master thesis in order to make the design and significance of this study clearer. Figures previously reported in my master thesis are described as Supplementary Figures (e.g., Fig. S1).

Chapter 1:

Macrophage hyper-activation by *Leishmania donovani* leading to hemophagocytosis during experimental visceral leishmaniasis

Abstract

Anemia often occurs in infectious diseases, whereas the mechanisms of infection-associated anemia may be variable depending on the diseases. In VL, the mechanisms of anemia remained still elusive due to lack of appropriate animal model. In my master thesis, a mouse model of VL exhibiting anemia was developed, and using this model it was demonstrated that hemophagocytosis in the spleen of infected mice is a possible cause of anemia during VL. In this chapter, more detailed analyses on anemia and hemophagocytosis during VL were performed. At 24 weeks of *L. donovani* infection, BALB/cA mice exhibited splenomegaly, hepatomegaly and anemia. There were no apparent signs of defective erythropoiesis in the infected mice but rather indication of up-regulated hemolysis by serum indirect bilirubin. Histological analyses on the spleen of infected mice demonstrated macrophages phagocytosing erythrocytes.

Autoantibodies to erythrocytes or damages on erythrocyte membrane were not detected, implying those are not related the induced hemophagocytosis. The spleen was the major place for hemophagocytosis when compared with the liver and bone marrow, the other major tissues for parasitization; there, multinucleated giant cells heavily infected with amastigotes were markedly observed and were the major cell type phagocytosing erythrocytes. In addition, hemophagocytosis in the spleen and anemia of the infected mice were improved by treatment of the mice with an antileishmanial drug, suggesting that direct infection by parasites is one of the key factors causing hyper-activation of host macrophages to engulf blood cells. Taken together, these results suggest that heavy infection of macrophages with *Leishmania* parasites in the spleen triggers phagocytosis of erythrocytes resulting in anemia during experimental VL.

1.1. Introduction

Anemia is one of typical symptoms during VL. However, the mechanisms of anemia in VL patients are still unclear. In fact, various mechanisms are proposed to cause either reduced RBC production or increased RBC destruction during human VL. For example, increased bleeding, abnormal structure of bone marrow or iron deficiency anemia, alteration of RBC membrane, bound of IgG to RBC or hemophagocytosis were speculated as causes for anemia during VL. Study on human case is limited ethically and physically, and to identify causes which contribute to the pathogenesis actually, animal model is needed.

There are only a few reports on anemia in rodent models of *Leishmania* infection. In mice, there was only one report on anemia which is caused by *L. tropica* infection (10), but none by *L. donovani* complex. *L. donovani*-induced anemia was reported only in hamster models (11-13). Although hamsters are regarded as a better model for VL pathology than mice, they have fewer advantages in immunological studies such as availability of reagents (e.g., antibodies) and labor in animal work (e.g., housing, generation of transgenic animals). In the master's dissertation, we have reported anemia in mice infected with *L. donovani* first time (14) and analyzed the

possible cause of anemia and hemophagocytosis was suspected to contribute to anemia. However, other possible cause of anemia was not fully examined. In this study, other possible cause including decreased erythropoiesis in bone marrow and iron status was examined. In addition, experimental infections using another strain of parasites or mice were performed to examine other factor needed for the generation of anemia.

The presented results demonstrated the importance of hemophagocytosis into anemia during VL and that hemophagocytic activity induced by *L. donovani* infection resulted from macrophage hyper-activation.

1. 2. Materials and methods

1. 2.1. Mice and parasites

Male BALB/cA mice were purchased from Japan Clea, Tokyo, Japan. All mice were maintained under specific pathogen-free conditions. The mice were used for experiments at the age of 6-8 weeks. This animal experiment was reviewed and approved by an institutional animal committee at the Graduate School of Agricultural and Life Sciences, The University of Tokyo (Approval No. P14-930, P16-254 and P16-275).

Promastigotes of *Leishmania donovani* (MHOM/NP/03/D10, gifted from National BioResource Project at Nagasaki University (16); MHOM/SU/62/2S-25-C2, gifted from Dr. Katakura at Hokkaido University (17)) were cultured in medium TC199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS; Thermo Fisher Scientific, Pittsburgh, US) and 25 mM HEPES buffer (MP Biomedicals, France) at 25ºC.

1. 2.2. Experimental infection, hematological analyses and autopsy

L. donovani promastigotes in a late log or stationary phase were washed with phosphate-buffered saline (PBS: Nissui) by centrifugation at 1,600 ×*g* for 10 min, and

were resuspended with PBS at the concentration of 1×10^8 cells/ml. Mice were infected with 1×10^7 *L. donovani* promastigotes by intravenous injection into the tail vein. After 12 and 24 weeks of infection, blood was collected from orbital sinus of mice under anesthesia with isoflurane (Pfizer Japan Inc., Tokyo, Japan) by using heparinized capillary tubes (TERUMO, Tokyo, Japan). Hematocrit was determined by centrifuging the tubes at 15,000 ×*g* for 10 min. Hemoglobin was measured following Zander's procedure (18). The number of blood cells was counted by microscopic examination and mean corpuscular volume was calculated. For analysis on polychromatic erythrocytes, thin blood smears were prepared by using the heparinized blood, followed by fixation with methanol (WAKO, Osaka, Japan) for 5 min and staining with 5% Giemsa solution (Merck KGaA, Parmstadt, Germany) for 25 min. The ratio of polychromatic erythrocytes to total erythrocytes was calculated through microscopic observation of the stained smears at 200× magnification.

After the blood collection for hematological analyses, cardiac puncture was performed on those mice under anesthesia with isoflurane to collect the whole blood. The mice were then sacrificed by cervical dislocation to collect the spleen, liver and bone marrow. Serum was collected from the blood after centrifuging at 5,000 ×*g* for 10 min, and was analyzed for erythropoietin level by using Mouse Erythropoietin

Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). Serum iron (Fe) and total iron binding capacity (TIBC) were measured by Nitroso-PSAP direct assay, bilirubin by enzyme method, total protein (TP) by Biuret method and albumin (19) by BCG method using automatic analyzer, Hitachi 7180 (Hitachi, Tokyo, Japan). Unsaturated iron binding capacity (UIBC) and globulin (GLB) were calculated. Stamp smears of the spleen and liver were fixed for 5 min in methanol and stained for 25 min with 5% Giemsa solution. Amastigotes were counted by microscopic observation of the stained smear at 1,000× magnification, and Leishman-Donovan Units (LDU) was enumerated as the number of amastigotes per 1,000 host nuclei × the tissue weight in grams according to a previous study (20).

1. 2.3. Administration of antileishmanial drug to infected mice

Liposomal amphotericin B (L-AmB) was purchased from Dainippon Sumitomo Pharma, Osaka, Japan. L-AmB was dissolved at 4 mg/ml in deionized water and diluted at 400 μg/ml with 0.9% (w/v) NaCl solution. At 24 weeks of infection, the mice received a single daily dose (200 μg) of the L-AmB intraperitoneally for 5 days. Mice were euthanized at 10 days after the completion of treatment to examine parasite burden and hematological status.

1.2.4. Hematoxylin and eosin (HE) staining and quantitative analyses of hemophagocytes

The tissues collected at the time of sacrifice were fixed with buffered 20% formalin (Sumitani Shoten Co., Ltd, Japan) and embedded in paraffin. The paraffin-embedded tissues were sectioned at 4 μm thickness. The tissue sections were dewaxed and stained with Mayer's hematoxylin solution (WAKO) for 90 sec and rinsed in running tap water for 1 hour. Next, the sections were stained with eosin solution (MUTO PURE CHEMICALS CO., Ltd., Tokyo, Japan) for 2 min. In the HE-stained section of the spleen, the number of infected macrophages, hemophagocytes and multinucleated giant cells in the total splenic macrophages were counted in 5 random microscopic fields at 1,000× magnification. Also, around 100 hemophagocytes per section were individually analyzed for the number of host nuclei as well as that of *L. donovani* amastigotes infected by microscopic observation at 1,000× magnification. In this study, a macrophage was defined as a large cell (~20 μm in size, except for multinucleated giant cells) with large cytoplasm and round non-polymorphic nucleus. Definitions for hemophagocytes and multinucleated cells were given as those engulfing red blood cells inside their phagosomal compartments and those with multiple round nuclei, respectively.

1. 2.5. Erythrocyte osmotic fragility test

Osmotic fragility of erythrocytes was examined according to Stijulemans's protocol (21) with modifications. Solutions containing 0.7, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, 0.3 and 0.2% of NaCl (WAKO) were prepared and 200 μl of each solution were applied to V bottom 96 well plates (Thermo). Two microliters of heparinized blood from either naive or 24 week-infected mice were added to each well followed by gentle pipetting. After incubation at room temperature for 2 h, 100 μl supernatant from each well with erythrocyte sedimentation was transferred to a new 96 well flat bottom plate and the absorbance at 550 nm was measured. Degree of hemolysis for each well was calculated according to the absorbance of wells for erythrocytes treated with deionized water as 100% hemolysis and that for PBS treatment as 0% hemolysis. Hemolysis rate was plotted against the concentration of NaCl in the medium and the NaCl concentrations in which 50% of RBCs were lysed were determined.

1. 2.6. Direct anti-IgG agglutination test

Heparinized blood from either naïve or 24 week-infected mice was washed with DMEM twice by centrifugation at 200 ×g for 10 min, followed by resuspension with PBS to 25% hematocrit. Five microliters of the suspension were applied to each well of a V bottom 96 well plate containing 100 μl of PBS and were mixed well. Then

50 μl of goat anti-mouse IgG antibody (1/1,000 dilution in PBS, Thermo) were added to each well. Erythrocyte agglutination was determined based on the presence of clump by visual examination. As controls, blood from a naïve mouse was pretreated with indicated amounts of anti-mouse RBC monoclonal antibody (clone 34-3C, Hycult Biotech, Uden, Netherland) at room temperature for 1 h, before probing with anti-mouse IgG antibody.

1. 2.7. Statistical analyses

Statistical comparisons of means between naive and infected mice were performed by two-way ANOVA followed by Bonferroni's multiple comparison test, one-way ANOVA followed by Tukey's test or unpaired *t* test with GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, USA). Difference between groups was considered as statistically significant when *P* value was less than 0.05.

1. 3. Results

1. 3.1. Mice infected with *L. donovani* exhibited splenomegaly and anemia

L. donovani infection induced hepatosplenomegaly in BALB/cA mice. The spleen and liver of the infected mice became significantly larger in size over time than those of uninfected mice. Mean weight per body weight ± SD of the spleen and liver from those infected mice were 0.72 ± 0.18% and 2.96 ± 0.16% at 12 weeks post infection (p.i.), 6.95 ± 0.25% and 6.81 ± 0.11% at 24 weeks p.i, respectively (Fig. S1A and C). In contrast, those of the uninfected mice (age-matched to the 24 week-infected mice) were 0.27 ± 0.02% and 5.40 ± 0.20%, respectively. Parasite burden in both tissues also showed increase from 12 to 24 weeks p.i. Mean ± SD of LDU for the spleen were 58.9 ± 37.0 at 12 weeks p.i. and 796 ± 159 at 24 weeks p.i., for the liver were 551 ± 282 at 12 weeks p.i. and 2,291 ± 279 at 24 weeks p.i. (Fig. S1B and D).

Microscopic observation of HE-stained spleen at 24 weeks-infected mice revealed pathological changes compared with that of the uninfected. The enlarged spleens from the infected mice were coupled with expansion of both red pulp and white pulp, whereas both red pulp and white pulp were structurally retained. The red pulp showed more significant expansion than white pulp, and was filled with increased

number of macrophages/histiocytes representing a characteristic of diffuse histiocytosis (data not shown).

In addition to hepatosplenomegaly, the 24 week-infected mice represented anemia with lower hematocrit, hemoglobin and red blood cell counts (38.7 ± 2.16%, 13.9 ± 1.49 g/dl and 6.73 x 10^6/μl, respectively) than the naive mice (45.2 ± 1.48%, 17.3 ± 0.83 g/dl and 8.53 x 10^6/μl, respectively) (Fig. S1E to G). At the 12 weeks post infection, no significant decrease in the above hematological parameters was found (Fig. S1E).

1. 3. 2. There were no signs of aplastic or iron deficiency anemia in *L. donovani*-infected mice±

Those infected mice had higher levels of serum erythropoietin compared with the naïve mice (1,589 ± 712 pg/ml vs. 94.3 ± 100 pg/ml) (Fig. S2A) and higher frequency of polychromatic erythrocytes in peripheral blood than the naïve mice (4.70 ± 1.76% vs. 1.39 ± 0.30%, respectively) (Fig. S2B and C).

Next, bone marrows of 24 week-infected mice were examined if there are some histopathological disorders including aplastic bone marrow or fibrosis by microscopic observation. However, no significant histological change was seen in bone marrow after Ld infection (Fig. 3A and B). In order to see the association between anemia and iron

deficiency during experimental VL, mean corpuscular volume, serum Fe, unsaturated iron binding capacity (UIBC) and total iron binding capacity (TIBC) were examined. Those of 24 week-infected mice (57.9 ± 5.40 fl, 150.4 ± 17.3 μg/dl, 278.0 ± 27.4 μg/dl and 497.5 ± 38.7 μg/d, respectively) (Fig. 3C-F) were not lower than naïve mice (53.2 ± 3.55 fl and 203.6 ± 16.4 μg/dl, 231.2 ± 23.1 μg/dl and 425.2 ± 27.5 μg/dl, respectively).

To examine if malnutrition by infection affect anemia, serum total protein (TP) was measured. Serum TP was rather increased in infected mice compared to naïve mice (6.36 ± 0.59 g/dl vs 4.84 ± 0.17 g/dl) (Fig. 3G). This increased TP was resulted from serum globulin increase (Fig. 3H), while albumin in serum was not changed (Fig. 3I).

1. 3.3. Serum indirect bilirubin in *L. donovani*-infected mice was increased.

Biochemical examination showed that serum indirect bilirubin in *L. donovani*-infected mice was higher (0.11 ± 0.01 mg/dl) (Fig. 4A) than those of naïve mice (0.05 ± 0.01 mg/dl). In contrast, serum direct bilirubin in *L. donovani*-infected mice and naïve mice were comparable (0.00 ± 0.00 mg/dl vs. 0.00 ± 0.01 mg/dl) (Fig. 4B). As a result, serum total bilirubin was higher in *L. donovani*-infected mice (0.11 ± 0.02 mg/dl) than those of naïve mice (0.05 ± 0.01 mg/dl) (Fig. 4C).

1. 3.3. The osmotic fragility of RBCs was not increased during *L. donovani* infection

Since up-regulated hemolysis was suspected in infected mice by the increased serum indirect bilirubin, the osmotic fragility test on RBCs was performed to examine whether the membrane fragility increased during *L. donovani* infection. In both infected and uninfected mice, as the concentration of NaCl decreased, the rate of hemolysis increased (Fig. S3A). There was no significant difference in concentration corresponding with 50% hemolysis between infected (0.48 ± 0.02%) and uninfected mice (0.45 ± 0.01%) (Fig. S3B).

1. 3.4. No IgG was detected on RBCs from infected mice

Direct anti-IgG agglutination test was performed to examine the presence of IgG molecules bound to RBCs and possibly lead to IgG-associated hemolysis. Neither infected nor uninfected mice showed positive results (Fig. S4, lower panels). The first several wells of positive control (RBCs binding with twice-fold serial dilutions of anti-RBC IgG) showed positive results. The smallest amount of IgG per RBC detectable in this assay was 5 ng (Fig. S4, upper panel), which corresponds with 800 molecules of IgG per RBC.

1. 3. 5. Hemophagocytosis in 24 week-infected spleen

Next, the spleen of the 24 weeks-infected mice was examined for any other hemolytic event by microscopic observation of the HE-stained section. It was observed

that erythrocytes were internalized in phagosomal compartments of the splenic macrophages in red pulp (Fig. S5). At 24 weeks, hemophagocytes accounted for 28.6% of the total splenic macrophages in the infected mice. In contrast, such macrophages were not observed in the spleen of uninfected mice. The liver and bone marrow of the 24 week-infected mice were also examined for the presence of hemophagocytes. Although amastigotes were detected in those tissues, there were less frequency of hemophagocytes in the bone marrow than the spleen and no detectable levels of hemophagocytes in the liver (Fig. S6). At 12 weeks post infection, hemophagocytes were seen less frequently; 9.41% of the total splenic macrophages.

1. 3. 6. Heavy infection and giant cell phenotype as characteristics of hemophagocytes

The infection status of hemophagocytes was evaluated to examine if *Leishmania* infection influences hemophagocytosis by macrophages. Histological analyses on the spleen from the 24 week-infected mice revealed that all of the hemophagocytes were infected with amastigotes although infected macrophages accounted for 50.2% in the total splenic macrophages (Fig. S7). Furthermore, hemophagocytosis was more prominent in heavily infected macrophages. When infection status was categorized based on the number of parasite for each macrophage as none (0 amastigote), low (1-10 amastigotes), moderate (11-20 amastigotes) and high

(more than 20 amastigotes), the percentages of splenic macrophages with low, moderate and high infection were comparable (16.0 ± 1.7%, 19.4 ± 2.4% and 14.7 ± 1.5%, respectively) (Fig. S7). In contrast, the majority of hemophagocytes were categorized in the high infection group (66.5 ± 6.2%), followed by moderate (26.2 ± 7.9%) and low (11.7 ± 3.7%) (Fig. S7).

Besides the heavy infection, multinucleated giant cell (MGC) phenotype was prominent in those hemophagocytes (Fig. S8A). The multinucleated macrophages accounted for 15.0 ± 6.2% of the total splenic macrophages (Fig. S8B). There were no such cells observed in the spleen of uninfected mice. Although a few multinucleated macrophages were found in the liver or bone marrow of the infected mice, the ratio in those tissues was lower than that in spleen. The multinuclear phenotype was more prominent in hemophagocytes; 60.4 ± 5.8% of hemophagocytes in the spleen of the infected mice were multinucleated (Fig. S8B).

1. 3.7. Antileishmanial drug improved anemia and hemophagocytosis

In order to examine if clearance of *L. donovani* can reverse hemophagocytosis and anemia during experimental VL, the infected mice were treated by L-AmB. After the completion of the treatment, parasite burden dramatically decreased; the mean LDU for the spleen and liver decreased by 95.3% and 99.2% from those of pretreated mice

(Fig. S9B and D). The spleen and liver become smaller than before the treatment; 64.4% and 49.8% recovery, respectively (Fig. S9A and C), considering difference between values from infected and age-matched naïve mice as 100%. In addition, hematological parameters of the treated mice recovered. The recovery rates of hematocrit, hemoglobin and the number of RBC calculated were 98.5%, 56.0% and 73.9%, respectively (Fig. S9E to G), while high frequency of polychromatic erythrocytes decrease by 82.5% (Fig. S9H). Furthermore, hemophagocytosis in the spleen become less frequently; from 26.3% to 12.0% (Fig. S9I).

AmB-treated mice also showed recovered iron (Fig. 5A to C) and bilirubin status (Fig. 5D to F), while kidney disease marker (Uric acid, creatinine, blood urea nitrogen and glucose) were increased before treatment (Fig. 5G to J).

1. 3. 8. 本項の内容は、学術雑誌論文として出版する計画があるため公表できない。3 年以内に出版予定。

1. 3. *L. donovani* 2S-infection caused anemia and hemophagocytosis in the spleen of BALB/cA mice

L. donovani 2S-infected mice were examined for parasite burden, hematological parameter and pathology as performed with *L. donovani* D10-infected mice. The 2S-infected mice had similar extent of parasite burden in the spleen to the

D10-infected mice (the number of amastigote/1000 host nuclei: 510 ± 224) (Fig. 7C) and slightly less extent of splenomegaly; 2.96 ± 0.41% (Fig. 7A). On the contrary, parasite burden in the liver of the 2S-infected mice was low and the number of amastigotes per 1,000 host nuclei was 15 ± 10 (Fig. 7D). In those infected mice, hemophagocytosis and anemia occur (Fig. 7E to H) as with D10-infected mice.

1. 4. Discussion

In the present chapter, the causes of anemia during VL were explored using a mouse VL model. Although bone marrow histology and iron status were examined for possibility of decreased erythropoiesis, there were no significant histopathological changes in bone marrow of infected mice including fibrosis or aplastic bone marrow (Fig. 3A and B). Serum iron level or MCV of infected mice were comparable to that of naïve mice (Fig. 3 C to F) suggesting that iron deficiency did not happen during the infection. In contrast, the factors indicating induced erythropoiesis including the number of polychromatic erythrocytes and erythropoietin in peripheral blood increased in the infected mice (Fig. S2A, B and C) indicating compensatory erythropoiesis was rather up-regulated in the infected mice showing anemic status. These results suggest hemolytic events occur during experimental VL.

One of the causes leading to hemolysis is physical damage to RBCs. Therefore, RBCs from the infected mice were examined if they were damaged. Peripheral erythrocytes from the 24-week infected mice showed no apparent damage when examined by microscopy, and there was not apparent difference in osmotic fragility from the uninfected mice (Fig. S3). The other major cause of hemolysis is autoantibody

(22). There are some reports on emergence of anti-erythrocyte antibodies in human VL patients (8, 9). Therefore, direct agglutination test by using anti-IgG was performed to detect autoantibodies on erythrocytes. No agglutination was found when peripheral blood from the infected mice was examined (Fig. S4). Although the detection sensitivity of the presented assay was not high, a previous report described that autoimmune-associated hemolytic anemia patients showed positive results on direct agglutination test while few VL patients showed positive results and implied that the number of IgG on RBCs from VL was as not high as autoimmune hemolytic anemia patients (9).

On the other hand, histopathological observation revealed that phagocytosis of RBCs by macrophages was up-regulated in the spleen of the infected mice. Because erythropoiesis was not dampened and other hemolytic events did not occur in the infected mice, hemophagocytosis in splenic macrophages was suspected to contribute to anemia the most in experimental VL. The finding of prominent hemophagocytosis by heavily infected macrophages also lowers the likelihood that damages/changes to erythrocytes contribute to this phenomenon, and rather suggests that hemophagocytosis in VL mainly resulted from hyper-activation of macrophages by *L. donovani* infection. Identification of MGCs as the major hemophagocytes (Fig. S8) also supports the idea

that abnormality in not erythrocytes but macrophages is an important factor for hemophagocytosis during VL.

There are some reports on animal models for infection-associated hemophagocytosis by *Salmonella* (23), EB-virus (24) and *Trypanosoma brucei* (25). Different from *Leishmania* parasites, however, *T. brucei* presents extracellular parasitism in the mammalian hosts. Therefore, infection-associated macrophage activation for hemophagocytosis is not due to direct infection of those cells by *T. brucei*. In contrast, it was demonstrated that direct infection of amastigotes is an important factor to make macrophages hemophagocytic during *Leishmania* infection. This is supported by the finding that heavily infected macrophages were more phagocytic than those with no or low infection (Fig. S7). Pilonieta *et al.* have reported that in the case of *Salmonella* infection the bacteria can be found more precisely in hemophagocytes as seen in *L. donovani* infection (23). The authors speculated that *Salmonella* in hemophagocytes derive nutrient iron from commandeered erythrocytes. This speculation may be applied to *Leishmania* parasites because they do not have heme synthetic pathway (26). To ensure that parasitism is needed for generation of hemophagocytosis in VL, infected mice were treated with antileishmanial drug. In the parasite-cleared spleen, the frequency of hemophagocytes was reduced suggesting

parasitism is an important factor for generation of hemophagocytosis during VL.

There are several types of MGCs including Langhans giant cell and foreign body giant cell. Cytokines and T cells play key roles in MGC development; IFN-γ is a key factor for development Langhans giant cells (27), whereas IL-4 and IL-13 seems important for foreign body giant cell (28, 29). Involvement of cytokines and lymphocytes for hemophagocytosis has been reported for various infectious diseases. IFN-γ and $CD8^+$ T cells are central in hemophagocytosis during lymphocytic choriomeningitic virus infection (30). Also, IFN-γ-deficient mice failed to manifest hemophagocytosis during *T. brucei* infection (25). Both IFN-γ and IL-4 can cause anemia/hemophagocytosis through different pathways (31, 32). *L. donovani*-infected mice had increased splenic levels of both IFN-γ and IL-4 than naïve mice (Khatanbaatar, Ph. D. thesis, 2014, The Univ. of Tokyo). Therefore, *L. donovani*-infected macrophages seem to receive extra signals like cytokines to become hemophagocytic.

Although anemia is accompanied with VL patients at high frequency, there have been few VL mouse model exhibiting anemia. One of the possible reasons is infection period; most of experimental infections with *L. donovani* are performed within 8 weeks (33, 34). However as shown in Fig. S1 , anemia occur on VL infected mice at late stage of infection, and that finding is correspond with the character of VL; which is

chronic infection and take several months to present symptoms. Other possible reason is the strain of parasite used in the presented study. In this study, *L. donovani* D10 strain, which is different from such strains as LV9 and LV82 used in the other groups (11, 35), was used first as a causative agent for experimental VL. Although both LV9 and LV82 strains cause chronic infection into the spleen, those parasites are cleared from the liver at late stage of infection unlike D10 keeping high infection in the liver even at 24 weeks after infection (36). To examine if hemophagocytosis/anemia are D10-specific or not, another strain of *L. donovani*, 2S were examined in addition to D10. *L. donovani* 2S caused sustained chronic infection in the spleen and transient infection in the liver (Fig. 7), which resembles the infection kinetics of LV9 and LV82 rather than D10. However, the 2S-infected mice showed hemophagocytosis and anemia as seen in D10-infected mice (Fig. 7). This result supports that infected splenic macrophages are important for hemophagocytosis despite of infection duration in the liver, and also suggested that regardless of parasite strain, hemophagocytosis/anemia can be caused by with *L. donovani* infection.

There are reports on hemophagocytosis in human VL patients (37-42). In those reports, hemophagocytes engulfing red blood cells were observed in the bone marrow of VL patients (37-42). Those hemophagocytes can be cleared after treatment with

antileishmanial drugs such as amphotericin B and sodium stibogluconate (37, 39-42). VL patients representing hemophagocytosis are often diagnosed as HPS since such the typical symptoms of VL as fever, splenomegaly and lymphadenopathy are also common in those of HPS (38-40). This misdiagnosis sometimes delays the treatment for VL (38, 40). The fact that anemia and hemophagocytosis, the two clinical manifestations during human VL, can be achieved in a mouse model is encouraging to understand the immunopathological mechanisms. In this study, antileishmanial drug improved the both anemia and hemophagocytosis (Fig. S9). These results correspond with human VL case report (37, 39-42) . Moreover, from the results using the mouse VL model, the spleen is found to be a major site for hemophagocytosis rather than bone marrow during VL (Fig. S6). Based on this finding, the HE-stained spleen sections from VL patients obtained at necropsy (collected by Dr. Dos-Santos, Oswaldo Cruz Foundation, Salvador, Brazil) were observed for hemophagocytosis. As expected from the previous results using mouse model, hemophagocytes were found in the spleen of patients with severe VL. These results imply that hemophagocytosis occur more frequently than reported and could play an important role in anemia during VL. In the future, by exploring the correlation between the frequency of hemophagocytosis and hematological parameter such as hemoglobin will lead to elucidation of the pathogenesis of anemia during human

VL.

Chapter 2:

Down-regulation of SIRP-α in hemophagocytes by *Leishmania donovani* infection.

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。3 年以内に出版予定。

Chapter 3:

Hemophagocytosis by *Leishmania donovani*-infected macrophages contributes to parasite survival.

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。

3 年以内に出版予定。

General discussion

During infection with pathogens, host immune responses are indispensable for protection against the pathogens, but often bring adverse effects called immunopathology. Some examples are described as follow. As is well known, symptoms in common cold or the septic shock, which is the fatal medical condition, is resulted from systemic response to infection (82). One of the inflammation-related protein, myeloid-related protein (MRP) 14 is known to contribute to this septic shock and MRP14-KO mice showed higher survival rate compared to WT mice in LPS-induced shock (104). Although MRP14 is important for activating TLR4 response to infection (105), by promoting accumulation of inflammatory cells in the liver and up-regulating pro-inflammatory molecules and NO, cause the hepatic injury to mice infected with *Plasmodium berghei* (106). Also in VL, strong humoral responses is activated to cope with parasites while increased B-cell and plasma cells become a cause of splenomegaly in infected mice with *L. donovani* (36). Understanding the mechanisms of pathological immune responses will contribute to case managements and better outcomes of not only infectious diseases but also other immunological disorders

including cancer, autoimmune diseases and allergy. This is one of the reasons why I have studied immunopathology of VL with the focus on anemia.

Anemia often occurs in infectious diseases, whereas the mechanisms of infection-associated anemia may be variable depending on the diseases (15, 107-109). In VL, the mechanisms of anemia remained still elusive due to lack of appropriate animal models. I have developed for the first time a mouse model of VL exhibiting anemia as a part of my master thesis , which has led to the finding that hemophagocytosis in the spleen of is a possible cause of anemia during VL (14). However, detailed mechanisms of hemophagocytosis remained unclear. This was the motivation for my Ph.D. thesis to explore more detailed mechanisms of hemophagocytosis and its contribution to anemia during VL.

In chapter 1, the possible causes for anemia were explored using experimental models of VL, and hemophagocytosis by splenic macrophages was suspected to contribute to anemia during VL. An essential condition for macrophages to become hemophagocytes in vivo seemed to be ‘being (heavily) infected with parasites’. In fact, treatment of the infected mice showing hemophagocytosis and anemia with an antileishmanial drug reversed both of the conditions (Fig. S9). In order to demonstrate the requirement more directly, in vitro models of hemophagocytosis were explored in

chapter 2. It was revealed also for the first time that infection with *Leishmania* parasites is sufficient for turning macrophages into hemophagocytes (Fig. S10). Importantly, the phagocytic activity of the infected macrophages was not non-specific up-regulation of phagocytosis, and was rather biased toward RBCs when compared with polystyrene beads (Fig. 8). These results suggest that parasite infection causes disruption of intact self-recognition of normal RBCs by macrophages. Then, what kind of molecules can be associated with such the untuned self-recognition? I have focused on SIRPα as a negative regulator of engulfment of self-cells. The molecule is a receptor for CD47 which is identified as a marker of 'self' on RBCs (110), and ligation of CD47 and SIRPα gives 'don't-eat-me' signals to macrophages resulting in inhibition of RBC engulfment by macrophages. It was a very intriguing finding that *Leishmania* infection led to down-regulation of SIRPα in macrophages (Fig. 9). To my knowledge, *Leishmania* is the first pathogen revealed to modify the expression of SIRPα in macrophages. This finding is very important because it serves as strong evidence that pathogens can cause collapse self-recognition in mammalian hosts. In many infectious diseases including infection with EB virus (111) and *Streptococcus pyogenes* (112), as a result of up-regulated production of cross-reactive antibodies to antigens of pathogens which is able to recognize the self-antigens, host cells or tissues could be damaged.

Similarly, by infection and subsequent inflammatory response, self-antigens could be modified and recognized as not self by host cells (113).

Although these findings that *Leishmania*-induced hemophagocytosis are novel and intriguing, they bring another question; what is a reason for the parasites to change characters of the infected macrophages in that way? Hemophagocytes in experimental VL seemed to be hyper-activated as represented by their multinuclear phenotype (Fig. S8) but at the same time may serve as niche for *Leishmania* as characterized by heavy infection in hemophagocytes (Fig. S7). Is hemophagocytosis by infected macrophages beneficial or harmful to the parasites? To get an answer for this question, in chapter 3, the influence of existence of RBCs in macrophages on survival of *Leishmania* was explored. Results of in vitro experiments demonstrated that RBCs benefit parasite survival in macrophages (Fig. 12). In addition, one of the antioxidant molecules, *Hmox1* mRNA level was also higher in RBC-supplemented cells (Fig. 16), suggesting RBCs in infected hemophagocytes result in cell lysis followed by hemoglobin digestion and heme detoxication. Also in vivo, RBCs seemed to be degraded within hemophagocytes, at least to cell lysis and hemoglobin digestion as represented by Fig. 13. From these results, RBCs engulfed by infected macrophages were considered to be digested and change environment in macrophages to *Leishmania*-friendly.

Intracellular parasites deprived molecules (e.g. ATPs, amino acids, metal) from their host cells for their proliferation. Among these parasites, there is not so many species that not only take nutrients from their host cells but also change host cells to one's liking. One of the rare examples is *Plasmodium falciparum*. In addition to providing nutrients, RBCs infected with *P. falciparum* is changed to stick to endothelial cells, by that alteration, infected cells could evade from transportation to the spleen and make rosette formation with recruited uninfected cells for further proliferation (114). This modification of host cells by *P. falciparum* is carried out through expression of parasites-derived proteins by infected RBCs. On the other hand, alteration of macrophages infected with *Leishmania* parasites is more unusual because parasites utilize the self-recognition system of hosts by manipulating expression of host's own protein. In this respect, *Leishmania* are biologically unique pathogens.

Together, through the presented study, it was suggested that hemophagocytosis in VL has influence on symptoms of the disease through direct mechanism (i.e., anemia) or through indirect mechanisms by offering *Leishmania* parasites an incubator for growth. Further studies on this topic may contribute to not only understanding of symptoms other than anemia during VL but also elucidation of hemophagocytosis due to other causes. Moreover, in the era that antileishmanial chemotherapy is the main

stream of treatment for VL, this study will offer the alternative choice for disease management by revealing molecular mechanisms of immunopathology for targets of symptom relief drugs.

Tables

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Figure legends

Figure 1. Visceral leishmaniasis

Figure 2. Anemia in human visceral leishmaniasis

The frequency (A) and the severity (B) of anemia in VL patients are researched in literature on case reports. Mean (*circle*), median *(square*), or range (*bar*) of Hb levels in VL patients (*square*), treated VL patients (*red*), or controls (*blue*) are shown. *Green* symbols represent VL patients with confirmed HIV infection. *Error bars* represent SD

Figure S1. Anemia caused by *L. donovani* infection

Mice were infected with $1x10^7$ *L. donovani* promastigotes. After 12 and 24 weeks, the infected mice as well as age-matched naïve mice were sacrificed to examine organ weights and parasite burden of the spleen (A and B) and the liver (C and D), hematocrit (E), hemoglobin (F) and peripheral blood red blood cell counts (G). (F) and (G) represented results from 24 week-post infected mice and age-matched naive mice. The open circles or bars represent naïve mice and closed symbols or bars represent infected

mice. The mean and SD of at least 3 mice in each group are shown. These are representative of three independent experiments with similar results. *P* values by two-way ANOVA followed by Bonferroni's multiple comparison test (for E) or unpaired *t* test (for F and G) are shown. ns, not significant.

Figure S2. Up-regulated erythropoiesis in *L. donovani*-infected mice

Serum erythropoietin levels (A) and ratio of polychromatic erythrocytes in peripheral blood (C) of 24 week-post infected mice and age-matched naïve mice are shown. The open bars represent naïve mice and closed bars represent infected mice. The mean and SD of at least 4 mice in each group are shown. These are representative of three independent experiments with similar results. *P* values by two-way ANOVA followed by unpaired *t* test (for A and C) are shown. ns, not significant. Figure S2B shows representative microscopic images of Giemsa-stained smears of peripheral blood from infected mice. Arrows indicate polychromatic erythrocytes. Scale bar, 20 μm.

Figure 3. No signs of aplastic or iron deficiency anemia in *L. donovani*-infected mice

Representative images of the bone marrow from naïve mice (A) and 24 week-infected mice (B) are shown. Scale bar, 20 μm. MCV in peripheral blood (C), Fe in serum (D),

UIBC (E), TIBC (F), TP (G), GLB (H) and ALB (I) in serum of mice are shown. The open bars represent naïve mice and closed bars represent infected mice. The mean and SD of at least 4 mice in each group are shown. Ns; not significant, *; *P<0.05*, ***; *P<0.0005.*

Figure 4. Increased serum indirect bilirubin in *L. donovani*-infected mice

Indirect bilirubin (A), direct bilirubin (B) and total bilirubin (C) in serum of mice are shown. The open bars represent naïve mice and closed bars represent infected mice. The mean and SD of at least 4 mice in each group are shown. Ns; not significant, ***; *P<0.0005, ***; P<0.0001*.

Figure S3. Osmotic fragility of erythrocytes from *L. donovani*-infected mice

Osmotic fragility profile of RBCs from naïve mice (open circles, n = 5) or infected mice (closed circles, n = 5). Mean and SD of % hemolysis for naïve mice or infected mice (A) and the concentration of NaCl corresponding with 50% hemolysis (B) are shown. This experiment was conducted once.

Figure S4. Undetectable IgG binding on erythrocytes from *L. donovani*-infected mice

by direct agglutination test

Individual wells shown in the lower panel correspond to individual mice in both groups (n = 5 for each group). As controls, blood from a naïve mouse was pretreated with indicated amounts of anti-mouse RBC antibody before probing with anti-mouse IgG antibody (upper panel). This experiment was repeated twice.

Figure S5. Hemophagocytosis in the spleen of *L. donovani*-infected mice.

(A, B) Low magnification images of HE-stained sections of the spleen from naïve (A) or *L. donovani*-infected mice (B). Hemophagocytes engulfing multiple erythrocytes (arrows) were found in the spleen of the infected mice, whereas such cells were not found in that of the naïve mice. (C) High magnification image of HE-stained section of the spleen from the infected mice. *L. donovani* amastigotes (arrowheads) were often found in hemophagocytes. Scale bars, 20 μm.

Figure S6. Histology of the liver and bone marrow from *L. donovani*-infected mice

Representative images of the liver (A) and bone marrow (B) of *L. donovani*-infected mice at 24 weeks post infection are shown. Arrows indicate macrophages harboring *L. donovani* amastigotes. Scale bar, 20 μm.

Figure S7. Heavy infection of hemophagocytes by *Leishmania* parasites.

The number of amastigotes per macrophages/hemophagocytes in the spleen of *L. donovani*-infected mice was analyzed by microscopy of HE-stained tissue sections. Host cells were categorized based on the number of parasites per cell as none (0 amastigote), low (1-10 amastigotes), moderate (11-20 amastigotes) and high (more than 20 amastigotes). The graph shows percentages of cells with no, low, moderate and high infection in either total macrophages or hemophagocytes only. This is representative of two experiments with similar results.

Figure S8. Multinuclear feature of hemophagocytes in the spleen of *L. donovani*-infected mice.

(A) A representative image of MGCs engulfing erythrocytes observed in the spleen of *L. donovani*-infected mice. Scale bar, 20 μm. (B) Ratio of mononuclear and multinuclear cells in either total macrophages or hemophagocytes only. This is representative of two experiments with similar results.

Figure S9. Improved anemia and hemophagocytosis by antileishmanial drug

Mice were infected with $1x10^7$ *L. donovani* promastigotes. After 24 weeks, the infected mice were administered with antileishmanial drug, L-AmB for 5 days and sacrificed 10 days after the completement of treatment to examine organ weights and parasite burden of the spleen (A and B) and the liver (C and D), hematocrit (E), hemoglobin (F), peripheral blood red blood cell counts (G), ratio of polychromatic erythrocytes in peripheral blood (H) and percentage of hemophagocytes in total splenic macrophages (I). The open bars represent naïve mice, closed circles or bars represent infected mice and grey circles or bars represent L-AmB-treated mice (AmB). The mean and SD of 5 mice in each group are shown. This experiment was conducted once. *P* values by one-way ANOVA followed by Tukey's test (for A, C, E to I) or unpaired *t* test (for B and D) are shown. ns, not significant.

Figure 5. Improved hemolytic status by antileishmanial drug

Biochemical analysis of AmB-treated mice. Fe (A), UIBC (B), TIBC (C), indirect bilirubin (D), direct bilirubin (E), total bilirubin (F), UA (G), Cre (H), Bun (I) and Glu (J) in serum of mice are shown. The open bars represent naïve mice, closed circles or bars represent infected mice and grey circles or bars represent L-AmB-treated mice (AmB). The mean and SD of 5 mice in each group are shown.

Figure 6. 本項の内容は、学術雑誌論文として出版する計画があるため公表できない。3 年以内に出版予定。

Figure 7. Anemia and hemophagocytosis caused by *L. donovani* 2S-infection

Mice were infected with $1x10^7$ *L. donovani* D10 or 2S promastigotes. After 24 weeks, the infected mice as well as age-matched naïve mice were sacrificed to examine organ weights and parasite burden of the spleen (A and C) and the liver (B and D), hematocrit (F), hemoglobin (G) and peripheral blood red blood cell counts (H). (F) to (H) represented results from 24 week-post infected mice with *L. donovani* 2S and age-matched naive mice. The open or bars represent naïve mice and closed bars represent infected mice, gray bars represent 2S-infected mice. The mean and SD of at least 3 mice in each group are shown. These are representative of two independent experiments with similar results. *P* values by unpaired *t* test are shown. ns, not significant; *:*P*<0.05, **;*P*<0.01, ****;*P*<0.001. Figure 7E shows the representative picture of spleen from 2S-infected mice. Arrows indicate hemophagocytes. Bar; 20 um.

Figure8-17.本項の内容は、学術雑誌論文として出版する計画があるため公表できない。3 年以内に出版予定。

Figures

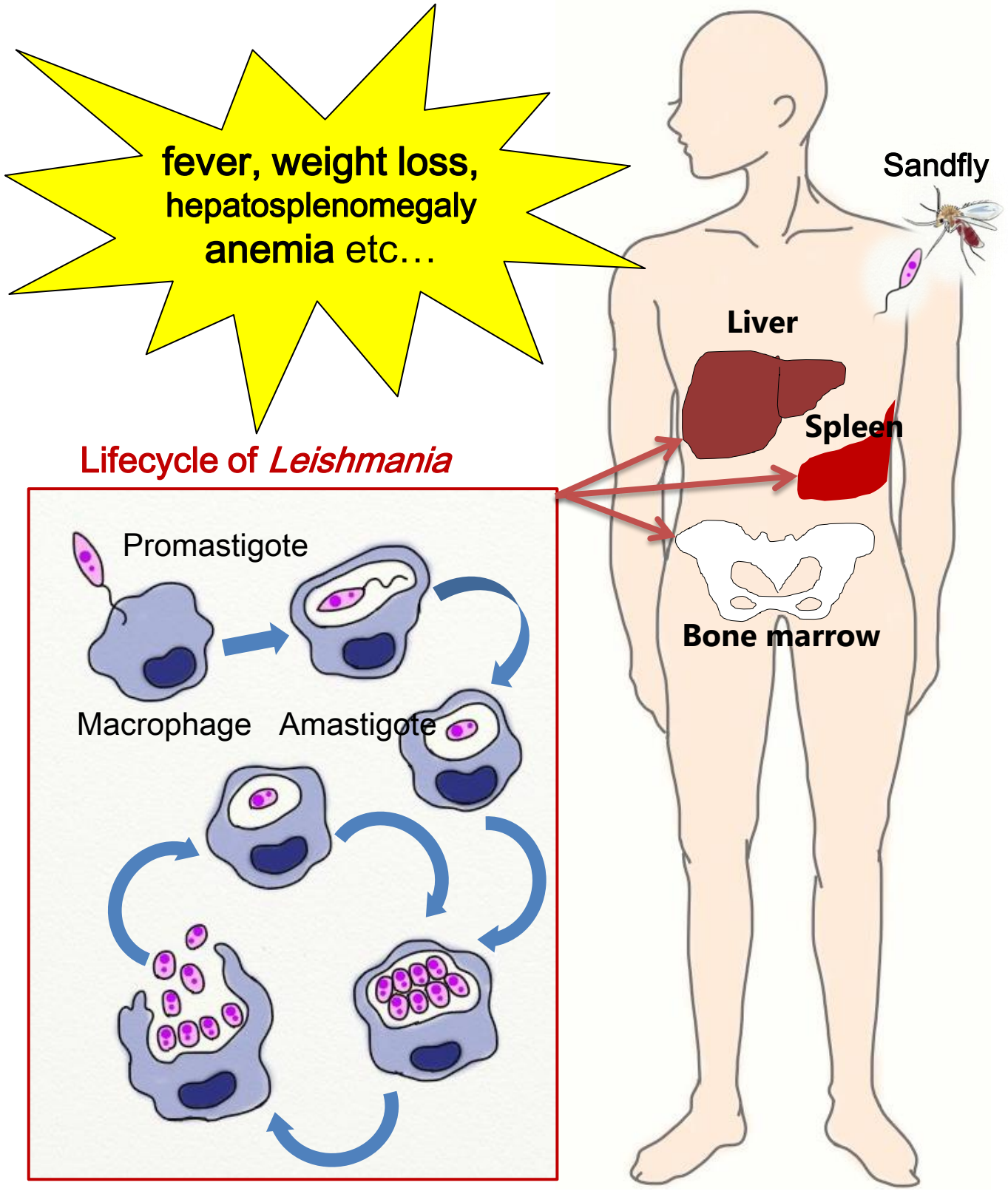
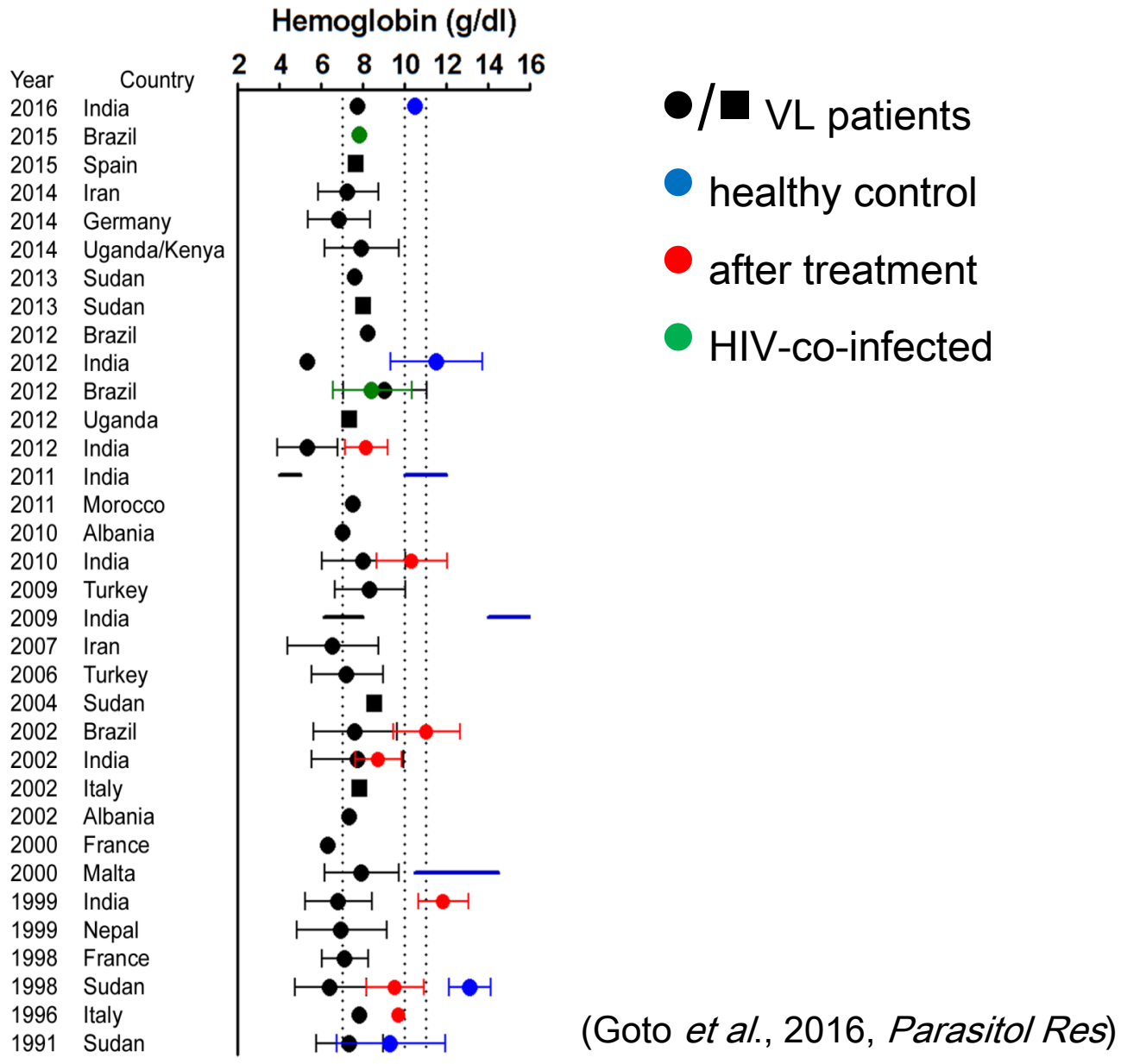


Figure 1. Visceral leishmaniasis

A

Frequency	No. of patients	Reference
100%	n=23	Marwaha, 1991, *Trop Geogr Med*
100%	n=94	Al-Jurayyan, 1995, *Pediatr Radiol*
87%	n=143	Cartwright, 1948, *Blood*

B



(Goto *et al.*, 2016, *Parasitol Res*)

Figure 2. Anemia in human visceral leishmaniasis

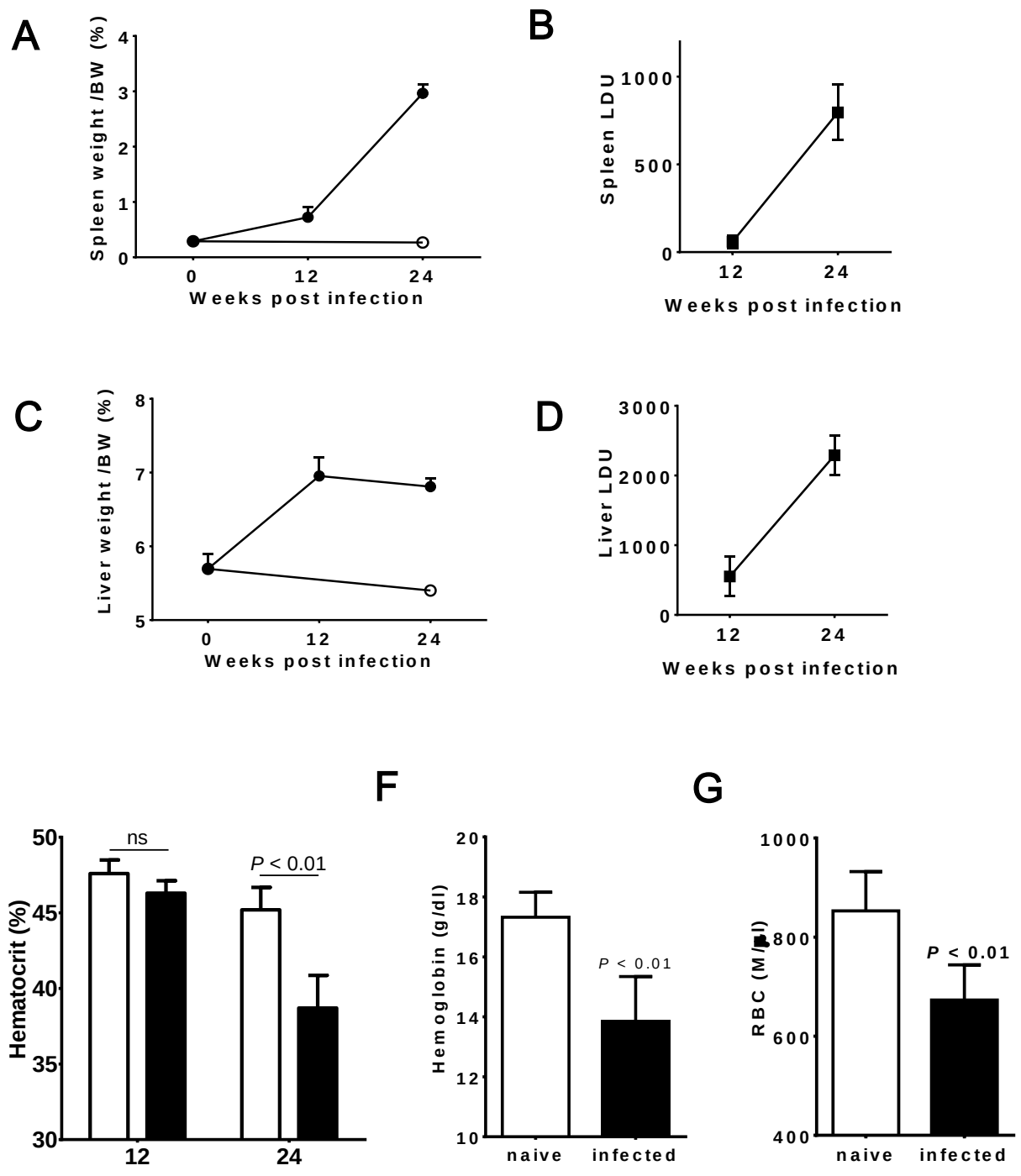


Figure S1. Anemia caused by *L. donovani* infection

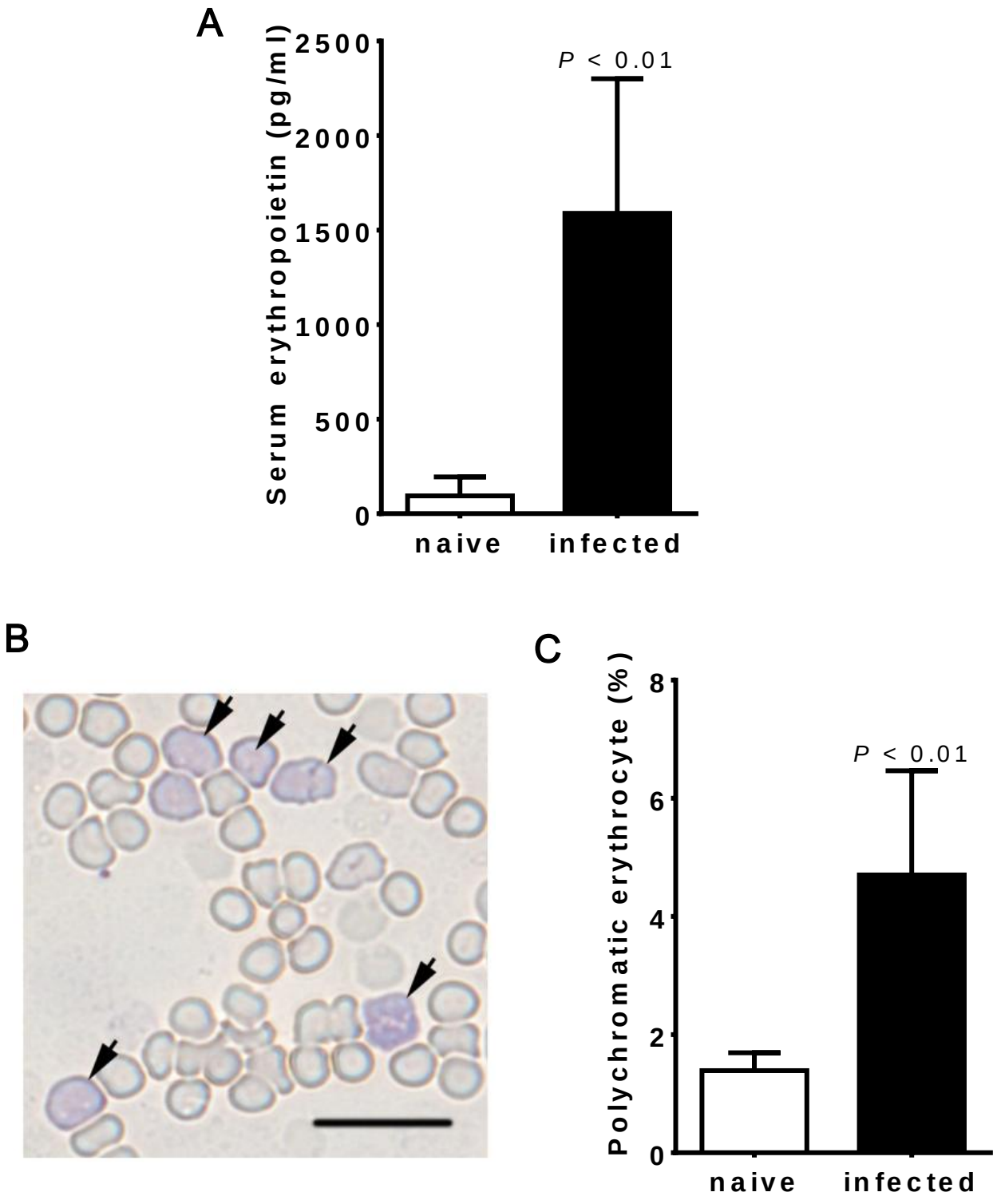


Figure S2. Up-regulated erythropoiesis in *L. donovani*-infected mice

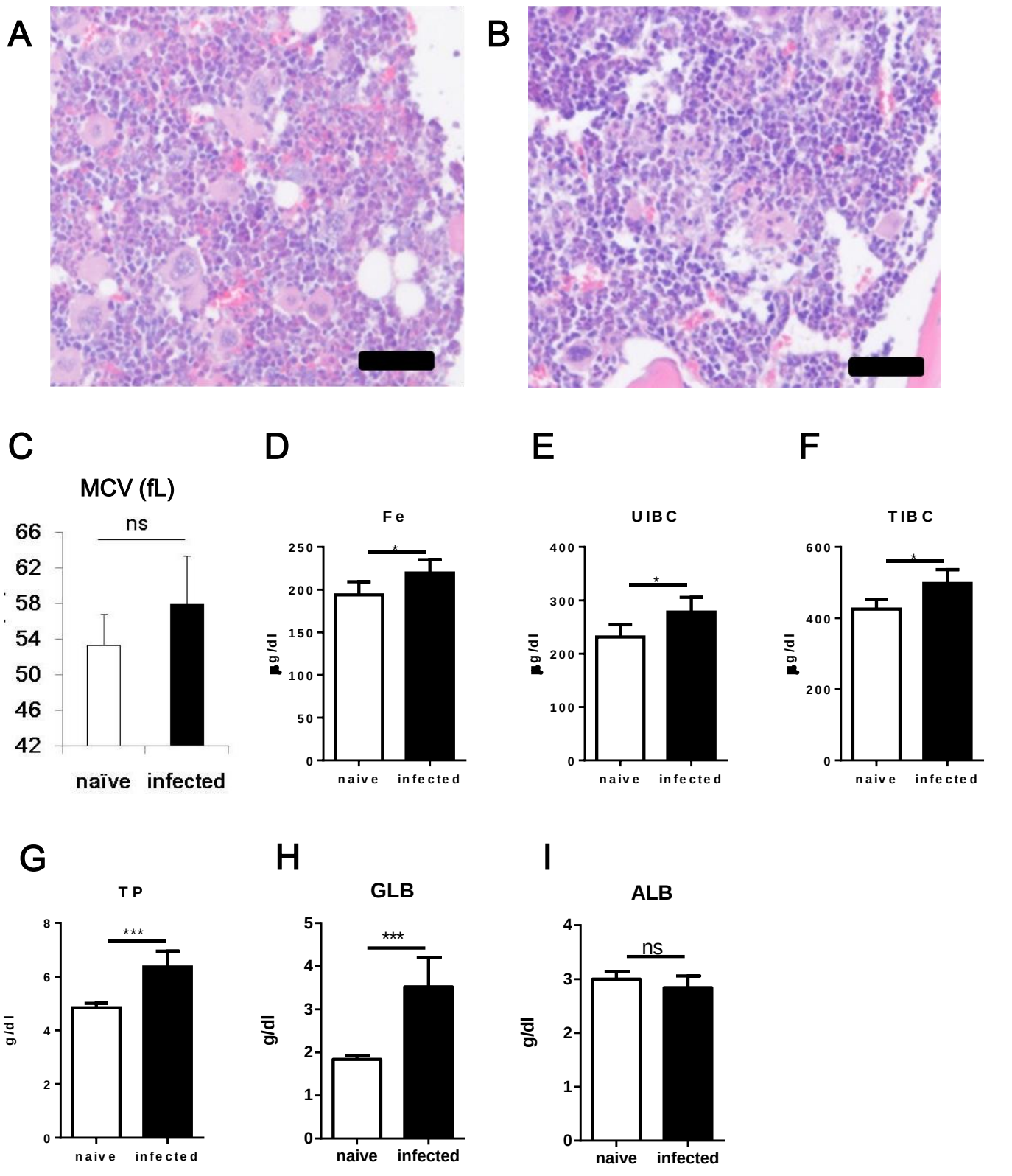


Figure 3. No signs of aplastic or iron deficiency anemia in *L. donovani*-infected mice

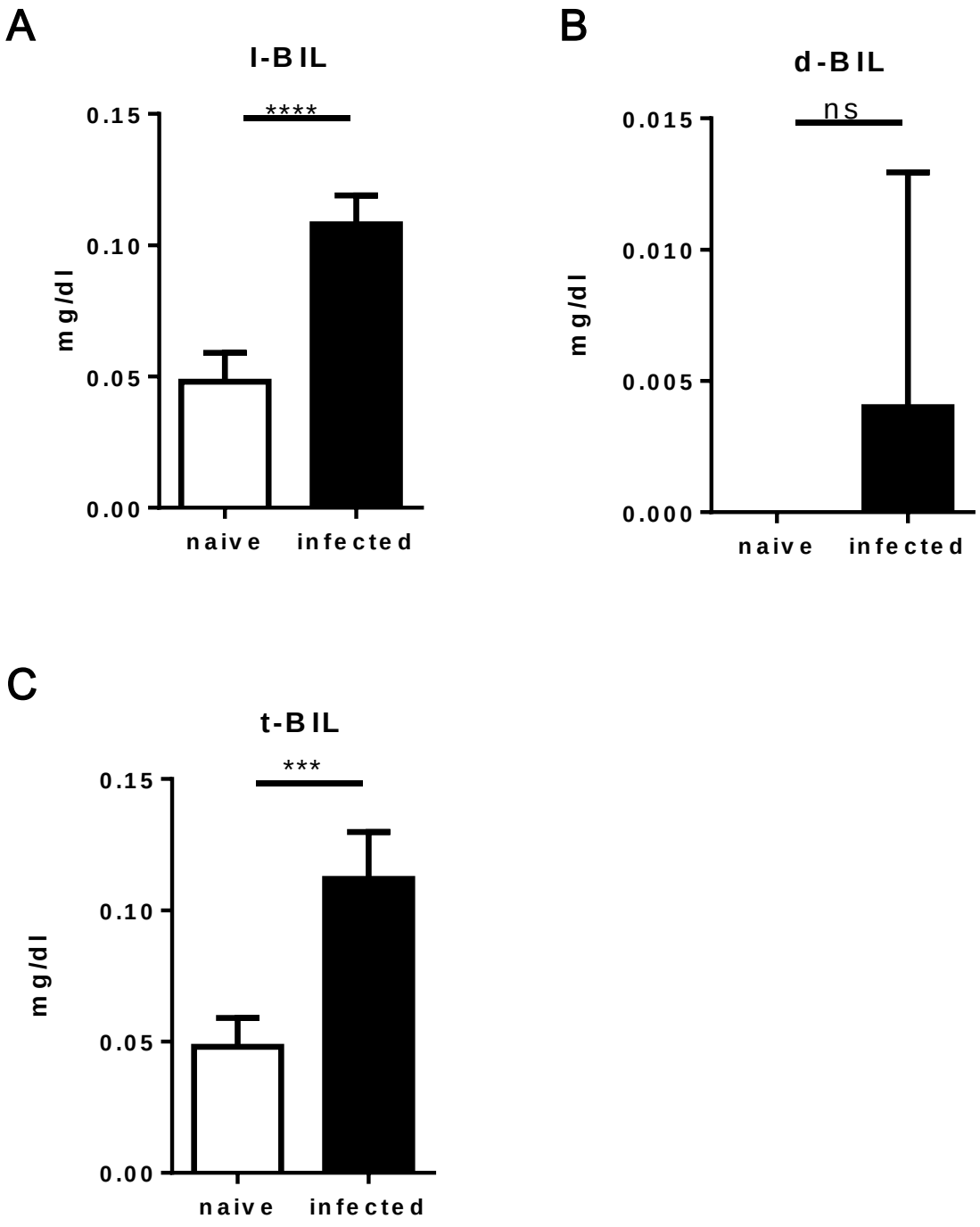


Figure 4. Increased serum indirect bilirubin in *L. donovani*-infected mice

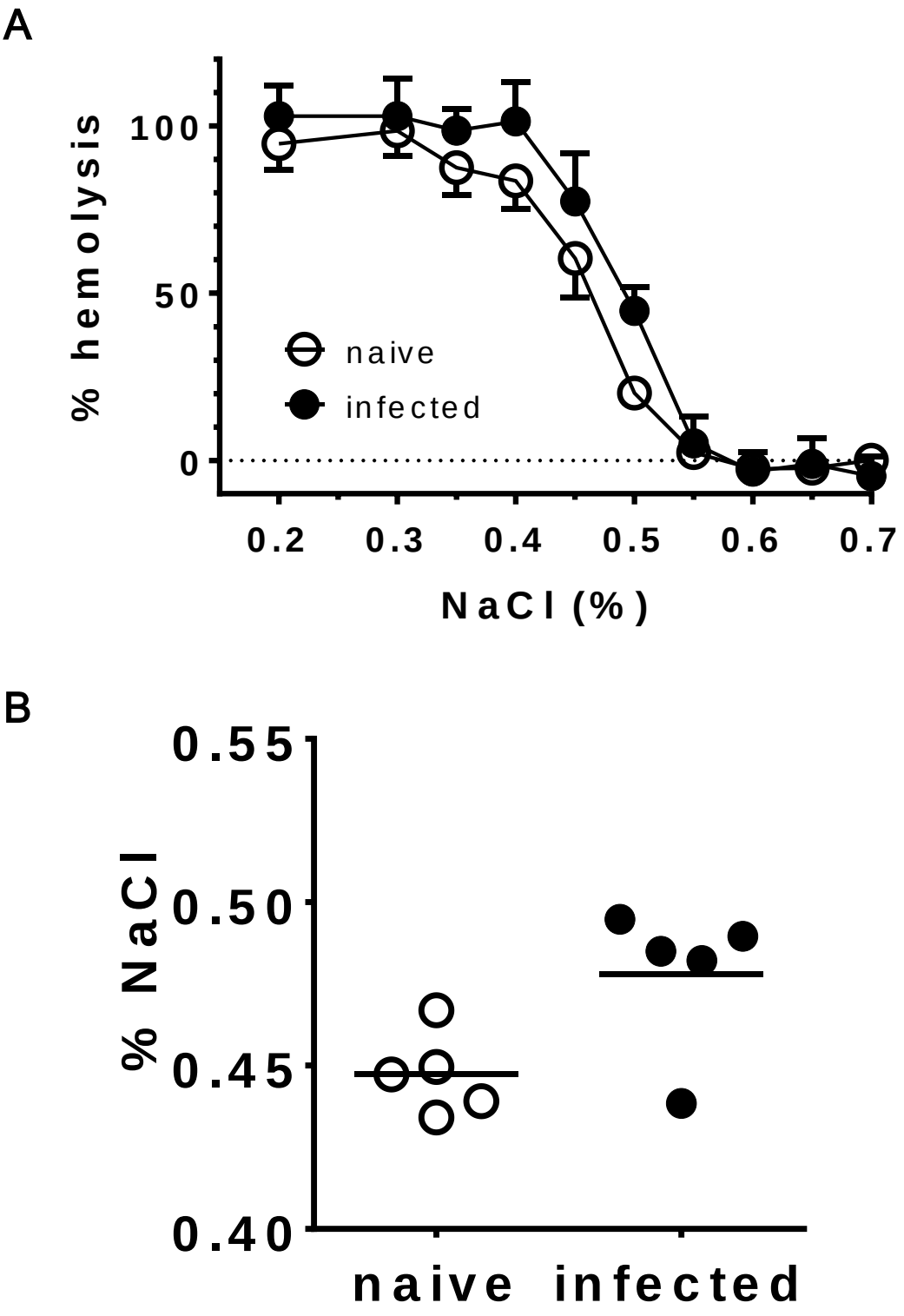


Figure S3. Osmotic fragility of erythrocytes from *L. donovani*-infected mice

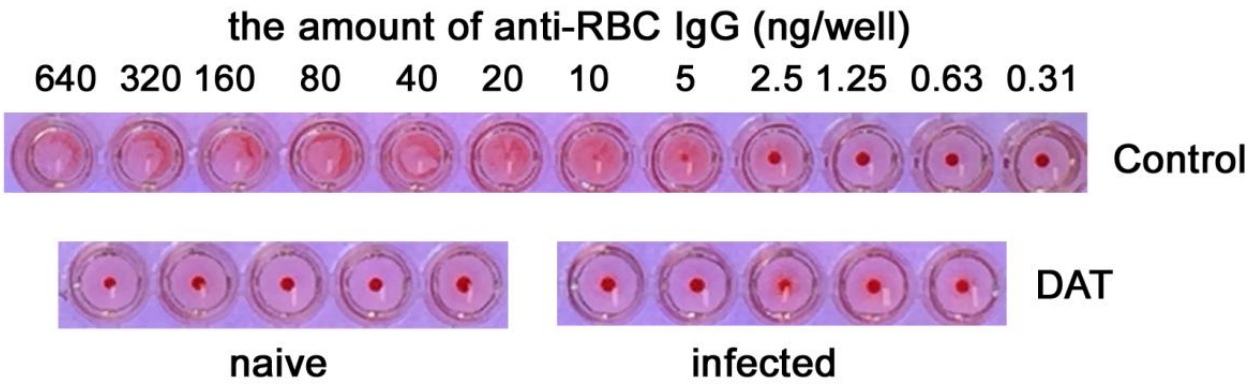


Figure S4. Undetectable IgG binding on erythrocytes from *L. donovani*-infected mice by direct agglutination test

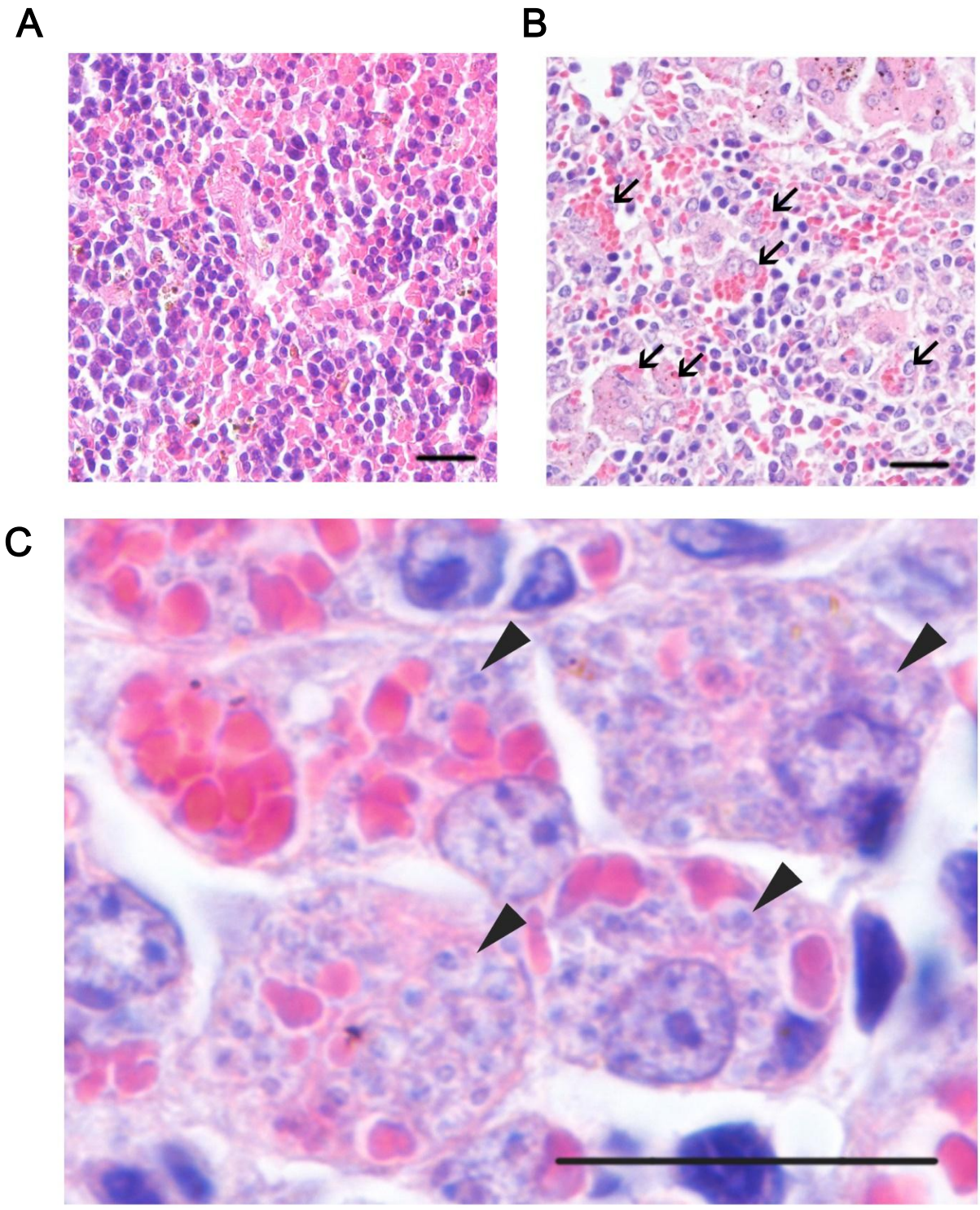


Figure S5. Hemophagocytosis in the spleen of *L. donovani*-infected mice

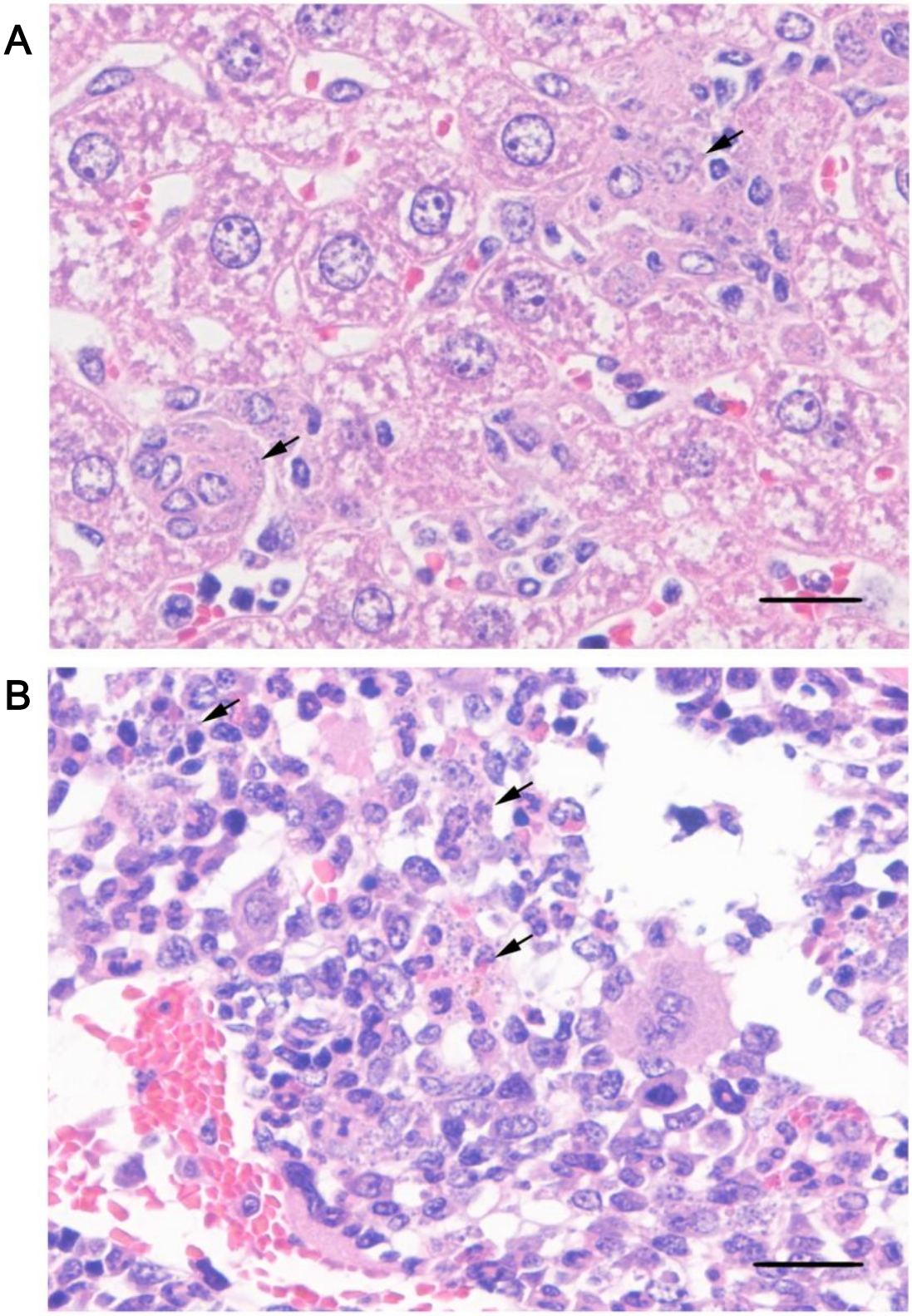


Figure S6. Histology of the liver and bone marrow from *L. donovani*-infected mice

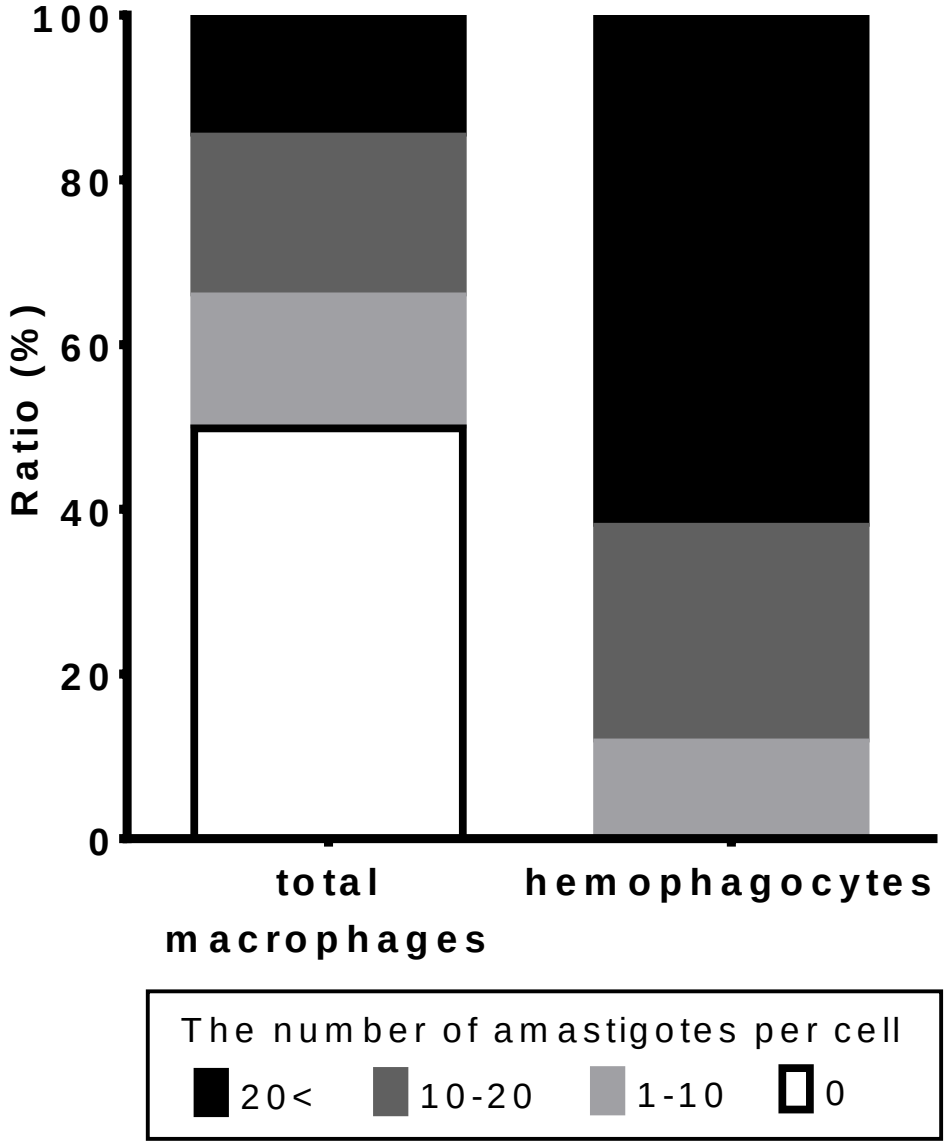


Figure S7. Heavy infection of hemophagocytes by *Leishmania* parasites.

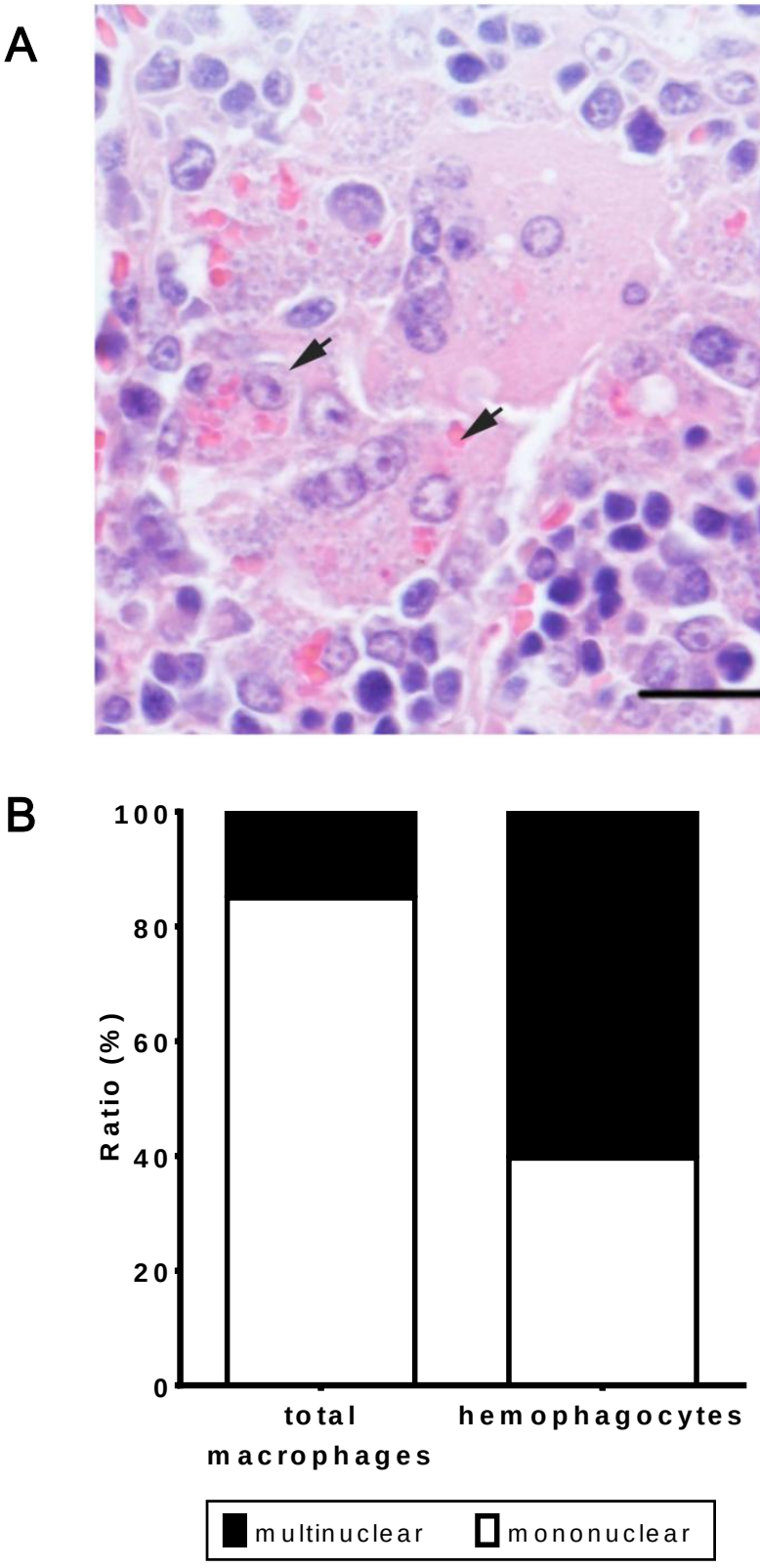


Figure S8. Multinuclear feature of hemophagocytes in the spleen of *L. donovani*-infected mice

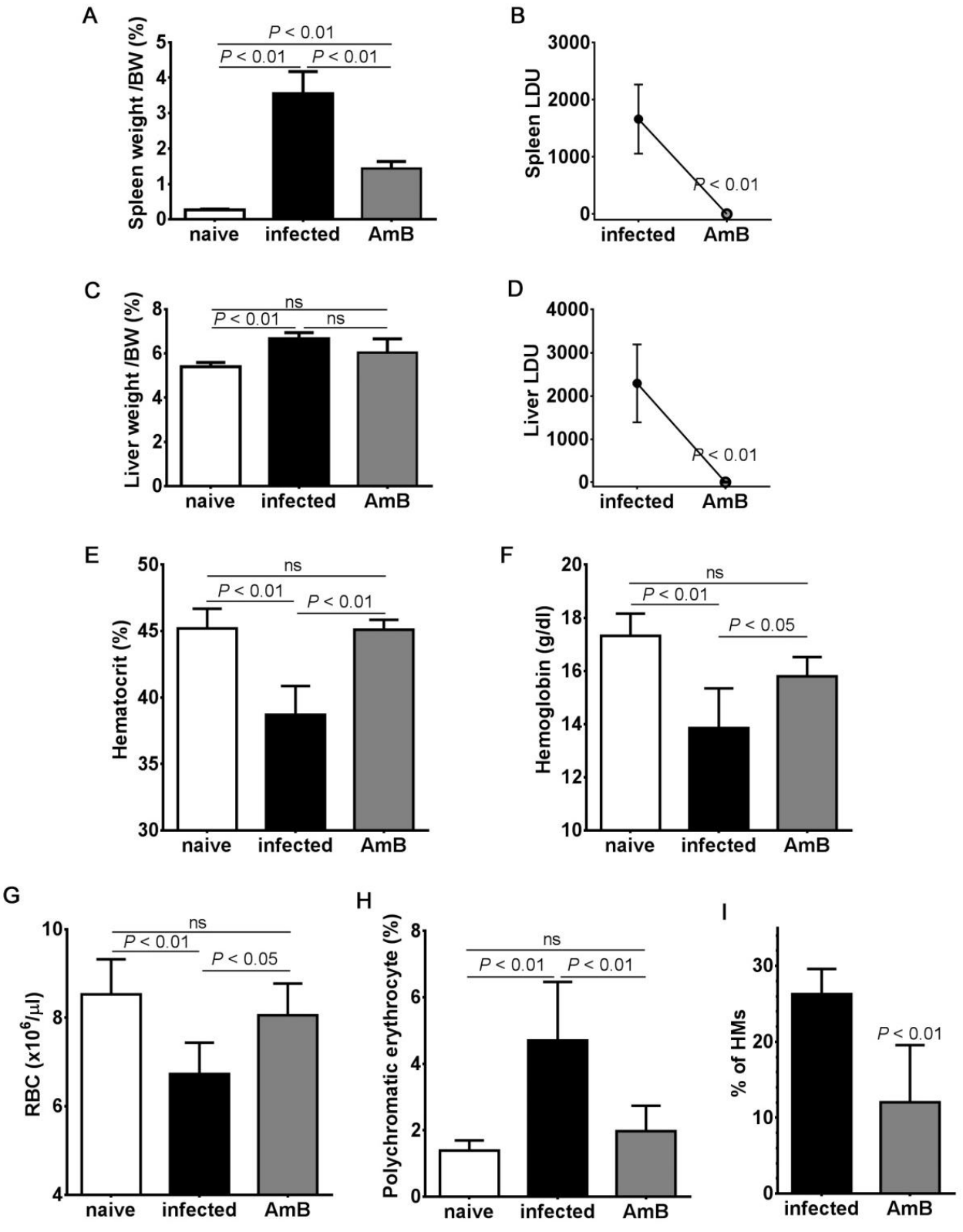
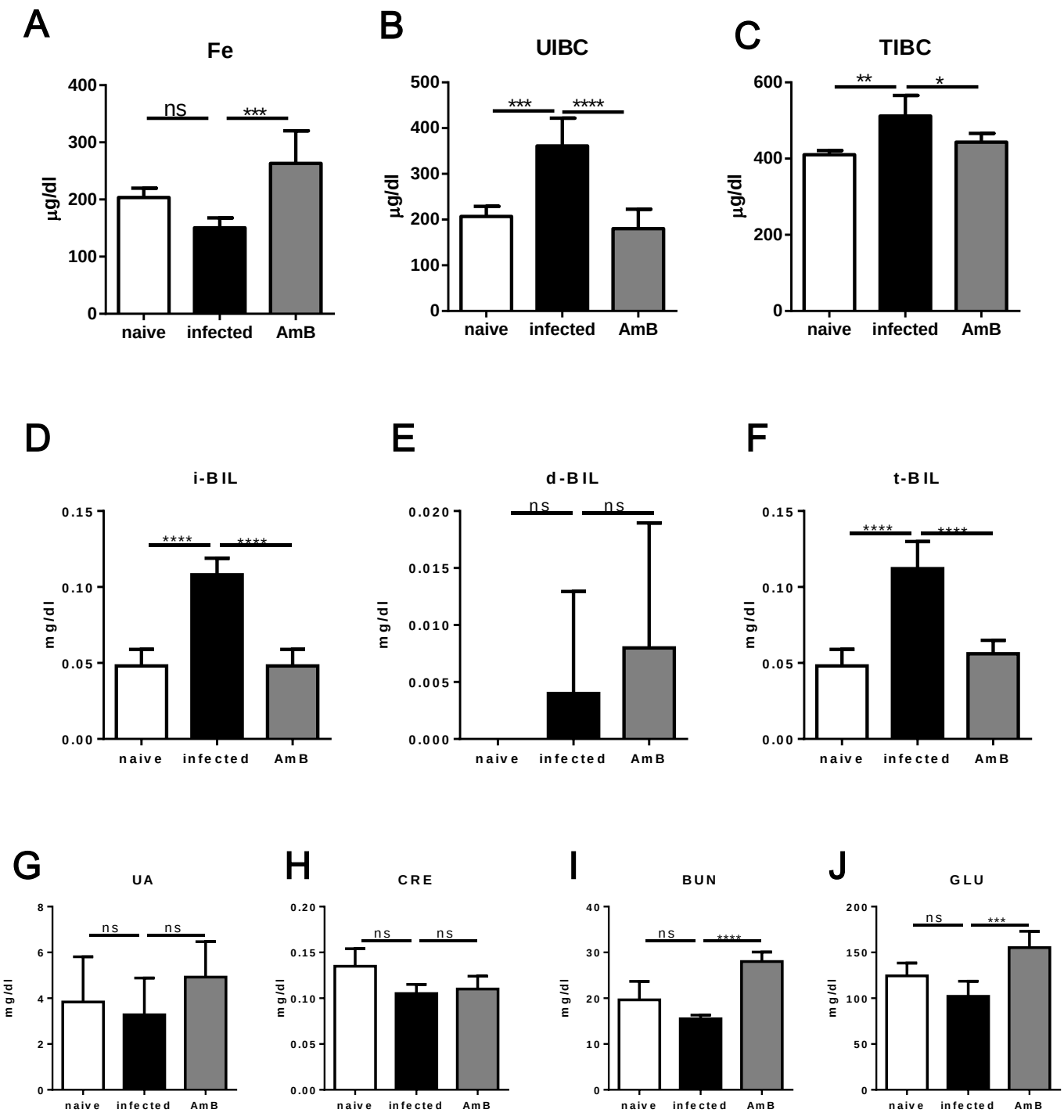


Figure S9. Improved anemia and Hemophagocytosis by antileishmanial drug



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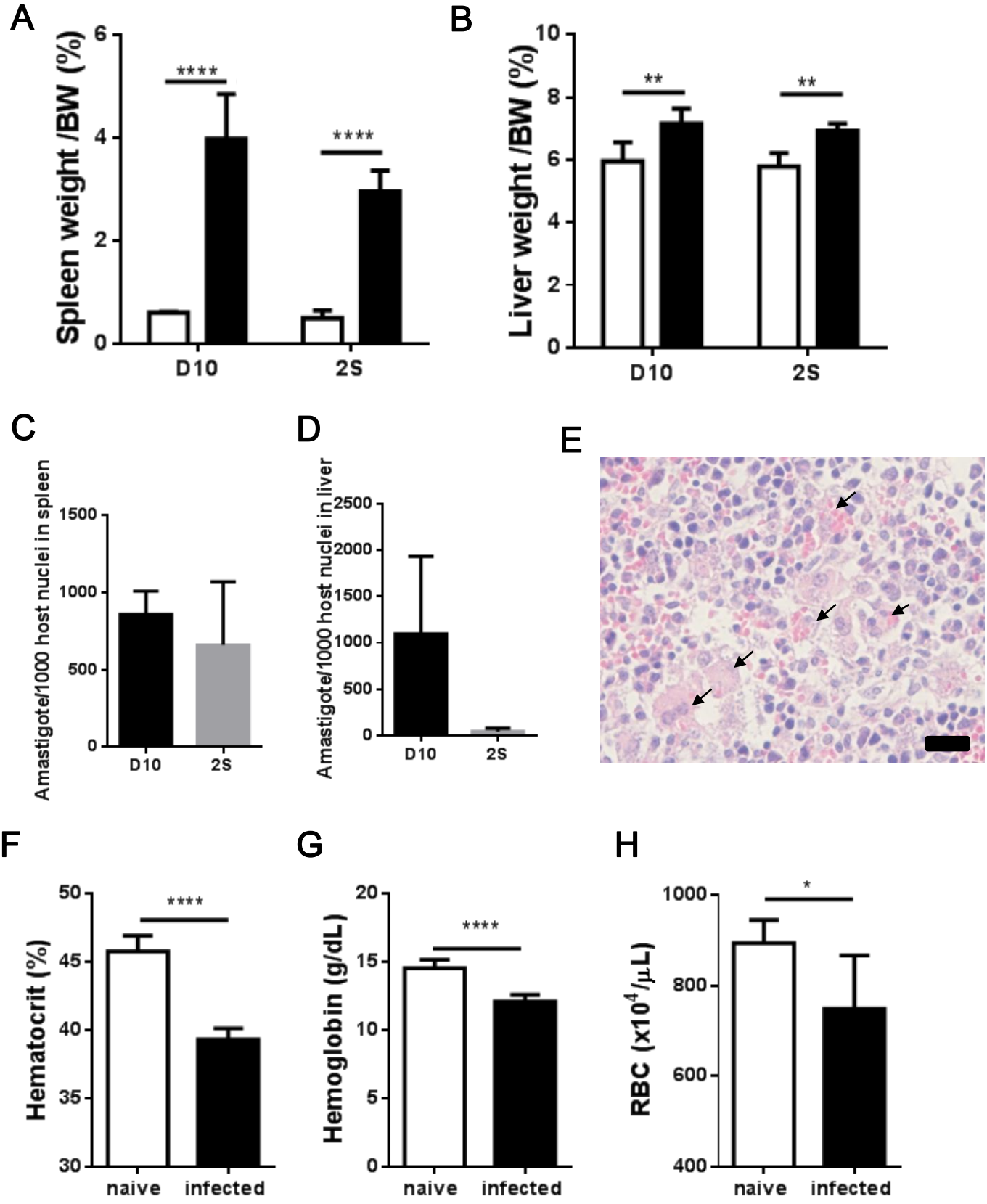

Figure 7. Anemia and hemophagocytosis caused by *L. donovani* 2S-infection

Figure 8-17.
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References

1. Goto Y, Cheng J, Omachi S, Morimoto A. Prevalence, severity, and pathogeneses of anemia in visceral leishmaniasis. Parasitol Res. 2017 Feb;116(2):457-64.

2. Ogawa S IY, Sofue H, Shiozawa M, Imawari M, Ota k, Ozawa T, Goto H, Chiba T, Hanabusa T, Ban S, Fujita T, Mimori H and Yamamoto K, editor. Internal Medicine. Seventh ed2009.

3. Al-Ghazaly J, Al-Selwi AH, Abdullah M, Al-Jahafi AK, Al-Dubai W, Al-Hashdi A. Pattern of haematological diseases diagnosed by bone marrow examination in Yemen: a developing country experience. Clin Lab Haematol. 2006 Dec;28(6):376-81.

4. Dhingra KK, Gupta P, Saroha V, Setia N, Khurana N, Singh T. Morphological findings in bone marrow biopsy and aspirate smears of visceral Kala Azar: a review. Indian J Pathol Microbiol. 2010 Jan-Mar;53(1):96-100.

5. Elnour IB, Akinbami FO, Shakeel A, Venugopalan P. Visceral leishmaniasis in Omani children: a review. Ann Trop Paediatr. 2001 Jun;21(2):159-63.

6. Cachia EA, Fenech FF. A Review of Kala-Azar in Malta from 1947 to 1962. Trans R Soc Trop Med Hyg. 1964 May;58:234-41.

7. Samanta S, Ghoshal A, Bhattacharya K, Saha B, Walden P, Mandal C. Sialoglycosylation of RBC in visceral leishmaniasis leads to enhanced oxidative stress, calpain-induced fragmentation of spectrin and hemolysis. PLoS One. 2012;7(7):e42361.

8. Pontes De Carvalho LC, Badaro R, Carvalho EM, Lannes-Vieira J, Vinhaes L, Orge G, et al. Nature and incidence of erythrocyte-bound IgG and some aspects of the physiopathogenesis of anaemia in American visceral leishmaniasis. Clin Exp Immunol. 1986 Jun;64(3):495-502.

9. Vilela RB, Bordin JO, Chiba AK, Castelo A, Barbosa MC. RBC-associated IgG in patients with visceral leishmaniasis (kala-azar): a prospective analysis. Transfusion. 2002 Nov;42(11):1442-7.

10. Djoko-Tamnou J, Leclerc C, Modabber F, Chedid L. Studies on visceral *Leishmania tropica* infection in BALB/c mice. I. Clinical features and cellular changes. Clin Exp Immunol. 1981 Dec;46(3):493-8.

11. Lafuse WP, Story R, Mahylis J, Gupta G, Varikuti S, Steinkamp H, et al. *Leishmania donovani* infection induces anemia in hamsters by differentially altering erythropoiesis in bone marrow and spleen. PLoS One. 2013;8(3):e59509.

12. Agu WE, Farrell JP, Soulsby EJ. Pathogenesis of anaemia in hamsters infected with Leishmania donovani. Z Parasitenkd. 1982;68(1):27-32.

13. Napier LE, Sharma LR. The Anaemia of the Leishmania-Infected Hamster. Ind Med Gaz. 1933 Dec;68(12):690.

14. Morimoto A, Omachi S, Osada Y, Chambers JK, Uchida K, Sanjoba C, et al. Hemophagocytosis in Experimental Visceral Leishmaniasis by Leishmania donovani. PLoS Negl Trop Dis. 2016 Mar;10(3):e0004505.

15. Szempruch AJ, Sykes SE, Kieft R, Dennison L, Becker AC, Gartrell A, et al. Extracellular Vesicles from Trypanosoma brucei Mediate Virulence Factor Transfer and Cause Host Anemia. Cell. 2016 Jan 14;164(1-2):246-57.

16. Pandey K, Yanagi T, Pandey BD, Mallik AK, Sherchand JB, Kanbara H. Characterization of *Leishmania* isolates from Nepalese patients with visceral leishmaniasis. Parasitol Res. 2007 May;100(6):1361-9.

17. Katakura K. An experimental challenge model of visceral leishmaniasis by Leishmania donovani promastigotes in mice. Parasitol Int. 2016 Oct;65(5 Pt B):603-6.

18. Zander R, Lang W, Wolf HU. Alkaline haematin D-575, a new tool for the determination of haemoglobin as an alternative to the cyanhaemiglobin method. I. Description of the method. Clin Chim Acta. 1984 Jan 16;136(1):83-93.

19. Alberts B JA, Lewis J, Raff M, Roberts K,, editor. Molecular Biology of THE CELL. fourth ed2004.

20. Huff CG, Nolf LO, Porter RJ, Read CP, Richards AG, Riker AJ, et al. An approach toward a course in the principles of parasitism. J Parasitol. 1958 Feb;44(1):28-45.

21. Stijlemans B, Cnops J, Naniima P, Vaast A, Bockstal V, De Baetselier P, et al.

Development of a pHrodo-based assay for the assessment of in vitro and in vivo erythrophagocytosis during experimental trypanosomosis. PLoS Negl Trop Dis. 2015 Mar;9(3):e0003561.

22. Kumakura S, Ishikura H, Kondo M, Murakawa Y, Masuda J, Kobayashi S. Autoimmune-associated hemophagocytic syndrome. Mod Rheumatol. 2004;14(3):205-15.

23. Pilonieta MC, Moreland SM, English CN, Detweiler CS. *Salmonella enterica* infection stimulates macrophages to hemophagocytose. MBio. 2014;5(6):e02211.

24. Brown DE, McCoy MW, Pilonieta MC, Nix RN, Detweiler CS. Chronic murine typhoid fever is a natural model of secondary hemophagocytic lymphohistiocytosis. PLoS One. 2010;5(2):e9441.

25. Cnops J, De Trez C, Stijlemans B, Keirsse J, Kauffmann F, Barkhuizen M, et al. NK-, NKT- and CD8-Derived IFNgamma Drives Myeloid Cell Activation and Erythrophagocytosis, Resulting in Trypanosomosis-Associated Acute Anemia. PLoS Pathog. 2015 Jun;11(6):e1004964.

26. Chang CS, Chang KP. Heme requirement and acquisition by extracellular and intracellular stages of *Leishmania mexicana amazonensis*. Mol Biochem Parasitol. 1985 Sep;16(3):267-76.

27. Most J, Neumayer HP, Dierich MP. Cytokine-induced generation of multinucleated giant cells in vitro requires interferon-gamma and expression of LFA-1. Eur J Immunol. 1990 Aug;20(8):1661-7.

28. McNally AK, Anderson JM. Interleukin-4 induces foreign body giant cells from human monocytes/macrophages. Differential lymphokine regulation of macrophage fusion leads to morphological variants of multinucleated giant cells. Am J Pathol. 1995 Nov;147(5):1487-99.

29. DeFife KM, Jenney CR, McNally AK, Colton E, Anderson JM. Interleukin-13 induces human monocyte/macrophage fusion and macrophage mannose receptor expression. J Immunol. 1997 Apr 1;158(7):3385-90.

30. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. Blood. 2004 Aug 1;104(3):735-43.

31. Zoller EE, Lykens JE, Terrell CE, Aliberti J, Filipovich AH, Henson PM, et al. Hemophagocytosis causes a consumptive anemia of inflammation. J Exp Med. 2011 Jun 6;208(6):1203-14.

32. Milner JD, Orekov T, Ward JM, Cheng L, Torres-Velez F, Junttila I, et al. Sustained IL-4 exposure leads to a novel pathway for hemophagocytosis, inflammation, and tissue macrophage accumulation. Blood. 2010 Oct 7;116(14):2476-83.

33. Reed SG, Roters SB, Inverso JA, Jones TC, Goidl EA. Immune responses to T-dependent and T-independent antigens during visceral leishmaniasis in mice: evidence for altered T-cell regulation of immune responses to non-parasite antigens. Cell Immunol. 1985 Nov;96(1):12-25.

34. Stager S, Alexander J, Carter KC, Brombacher F, Kaye PM. Both interleukin-4 (IL-4) and IL-4 receptor alpha signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. Infect Immun. 2003 Aug;71(8):4804-7.

35. Engwerda CR, Ato M, Cotterell SE, Mynott TL, Tschannerl A, Gorak-Stolinska PM, et al. A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during Leishmania donovani infection. Am J Pathol. 2002 Aug;161(2):429-37.

36. Omachi S, Fujii W, Azuma N, Morimoto A, Sanjoba C, Matsumoto Y, et al. B-cell activating factor deficiency suppresses splenomegaly during Leishmania donovani infection. Biochem Biophys Res Commun. 2017 Aug 5;489(4):528-33.

37. Fathalla M, Hashim J, Alkindy H, Wali Y. Cerebrospinal fluid involvement in a case of visceral leishmaniasis associated with hemophagocytic lymphohistiocytosis. Sultan Qaboos Univ Med J. 2007 Dec;7(3):253-6.

38. Kilani B, Ammari L, Kanoun F, Ben Chaabane T, Abdellatif S, Chaker E. Hemophagocytic syndrome associated with visceral leishmaniasis. Int J Infect Dis. 2006 Jan;10(1):85-6.

39. Ozyurek E, Ozcay F, Yilmaz B, Ozbek N. Hemophagocytic lymphohistiocytosis associated with visceral leishmaniasis: a case report. Pediatr Hematol Oncol. 2005 Jul-Aug;22(5):409-14.

40. Gagnaire MH, Galambrun C, Stephan JL. Hemophagocytic syndrome: A misleading complication of visceral leishmaniasis in children--a series of 12 cases.

Pediatrics. 2000 Oct;106(4):E58.

41. Marom D, Offer I, Tamary H, Jaffe CL, Garty BZ. Hemophagocytic lymphohistiocytosis associated with visceral leishmaniasis. Pediatr Hematol Oncol. 2001 Jan-Feb;18(1):65-70.

42. Bode SF, Bogdan C, Beutel K, Behnisch W, Greiner J, Henning S, et al. Hemophagocytic lymphohistiocytosis in imported pediatric visceral leishmaniasis in a nonendemic area. J Pediatr. 2014 Jul;165(1):147-53 e1.

43. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2007 Feb;48(2):124-31.

44. Tabata R, Tabata C, Terada M, Nagai T. Hemophagocytic syndrome in elderly patients with underlying autoimmune diseases. Clin Rheumatol. 2009 Apr;28(4):461-4.

45. Unal S, Cetin M, Kutlay NY, Elmas SA, Gumruk F, Tukun A, et al. Hemophagocytosis associated with leukemia: a striking association with juvenile myelomonocytic leukemia. Ann Hematol. 2010 Apr;89(4):359-64.

46. Molad Y, Stark P, Prokocimer M, Joshua H, Pinkhas J, Sidi Y. Hemophagocytosis by small cell lung carcinoma. Am J Hematol. 1991 Feb;36(2):154-6.

47. Tsoi WC, Feng CS. Hemophagocytosis by rhabdomyosarcoma cells in bone marrow. Am J Hematol. 1997 Apr;54(4):340-2.

48. Vykuntaraju KN, Avinash T, Saini J, Appaji L. Secondary Hemophagocytic Lymphohistiocytosis Associated with Epstein-Barr Virus. Indian J Pediatr. 2015 Feb 25.

49. La Gruta NL, Kedzierska K, Stambas J, Doherty PC. A question of self-preservation: immunopathology in influenza virus infection. Immunol Cell Biol. 2007 Feb-Mar;85(2):85-92.

50. Mallory FB. A Histological Study of Typhoid Fever. J Exp Med. 1898 Nov 1;3(6):611-38.

51. Balasubramanian S, Kaarthigeyan K, Aparna V, Srinivas S. Tuberculosis associated hemophagocytic syndrome in infancy. Indian Pediatr. 2008 Jul;45(7):593-5.

52. Slovut DP, Benedetti E, Matas AJ. Babesiosis and hemophagocytic syndrome

in an asplenic renal transplant recipient. Transplantation. 1996 Aug 27;62(4):537-9.

53. Granert C, Elinder G, Ost A, Henter JI. Kala-azar in a one-year-old Swedish child. Diagnostic difficulties because of active hemophagocytosis. Acta Paediatr. 1993 Sep;82(9):794-6.

54. Nix RN, Altschuler SE, Henson PM, Detweiler CS. Hemophagocytic macrophages harbor *Salmonella enterica* during persistent infection. PLoS Pathog. 2007 Dec;3(12):e193.

55. Hayashi K, Ohara N, Teramoto N, Onoda S, Chen HL, Oka T, et al. An animal model for human EBV-associated hemophagocytic syndrome: herpesvirus papio frequently induces fatal lymphoproliferative disorders with hemophagocytic syndrome in rabbits. Am J Pathol. 2001 Apr;158(4):1533-42.

56. Sato K, Misawa N, Nie C, Satou Y, Iwakiri D, Matsuoka M, et al. A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. Blood. 2011 May 26;117(21):5663-73.

57. Fujiwara F, Hibi S, Imashuku S. Hypercytokinemia in hemophagocytic syndrome. Am J Pediatr Hematol Oncol. 1993 Feb;15(1):92-8.

58. Silva-Herzog E, Detweiler CS. Intracellular microbes and haemophagocytosis. Cell Microbiol. 2008 Nov;10(11):2151-8.

59. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999 Dec 3;286(5446):1957-9.

60. Goransdotter Ericson K, Fadeel B, Nilsson-Ardnor S, Soderhall C, Samuelsson A, Janka G, et al. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. Am J Hum Genet. 2001 Mar;68(3):590-7.

61. Molleran Lee S, Villanueva J, Sumegi J, Zhang K, Kogawa K, Davis J, et al. Characterisation of diverse PRF1 mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. J Med Genet. 2004 Feb;41(2):137-44.

62. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial

hemophagocytic lymphohistiocytosis (FHL3). Cell. 2003 Nov 14;115(4):461-73.

63. zur Stadt U, Schmidt S, Kasper B, Beutel K, Diler AS, Henter JI, et al. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. Hum Mol Genet. 2005 Mar 15;14(6):827-34.

64. Okuno T, Goto Y, Matsumoto Y, Otsuka H. Applications of recombinant Leishmania amazonensis expressing egfp or the beta-galactosidase gene for drug screening and histopathological analysis. Exp Anim. 2003 Apr;52(2):109-18.

65. Ravetch JV, Lanier LL. Immune inhibitory receptors. Science. 2000 Oct 6;290(5489):84-9.

66. Adams S, van der Laan LJ, Vernon-Wilson E, Renardel de Lavalette C, Dopp EA, Dijkstra CD, et al. Signal-regulatory protein is selectively expressed by myeloid and neuronal cells. J Immunol. 1998 Aug 15;161(4):1853-9.

67. Ashwin H, Seifert K, Forrester S, Brown N, MacDonald S, James S, et al. Tissue and host species-specific transcriptional changes in models of experimental visceral leishmaniasis [version 1; referees: 3 approved, 1 approved with reservations]. Wellcome Open Research. 2018;3(135).

68. Elward K, Gasque P. "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. Mol Immunol. 2003 Sep;40(2-4):85-94.

69. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell. 2009 Jul 23;138(2):271-85.

70. Ishikawa-Sekigami T, Kaneko Y, Okazawa H, Tomizawa T, Okajo J, Saito Y, et al. SHPS-1 promotes the survival of circulating erythrocytes through inhibition of phagocytosis by splenic macrophages. Blood. 2006 Jan 1;107(1):341-8.

71. Okazawa H, Motegi S, Ohyama N, Ohnishi H, Tomizawa T, Kaneko Y, et al. Negative regulation of phagocytosis in macrophages by the CD47-SHPS-1 system. J Immunol. 2005 Feb 15;174(4):2004-11.

72. Inagaki K, Yamao T, Noguchi T, Matozaki T, Fukunaga K, Takada T, et al. SHPS-1 regulates integrin-mediated cytoskeletal reorganization and cell motility.

EMBO J. 2000 Dec 15;19(24):6721-31.

73. Kong XN, Yan HX, Chen L, Dong LW, Yang W, Liu Q, et al. LPS-induced down-regulation of signal regulatory protein {alpha} contributes to innate immune activation in macrophages. J Exp Med. 2007 Oct 29;204(11):2719-31.

74. Londino JD, Gulick D, Isenberg JS, Mallampalli RK. Cleavage of Signal Regulatory Protein alpha (SIRPalpha) Enhances Inflammatory Signaling. J Biol Chem. 2015 Dec 25;290(52):31113-25.

75. Shirakabe K, Omura T, Shibagaki Y, Mihara E, Homma K, Kato Y, et al. Mechanistic insights into ectodomain shedding: susceptibility of CADM1 adhesion molecule is determined by alternative splicing and O-glycosylation. Sci Rep. 2017 Apr 10;7:46174.

76. Malemud CJ. Matrix metalloproteinases: role in skeletal development and growth plate disorders. Front Biosci. 2006 May 1;11:1702-15.

77. Schif-Zuck S, Gross N, Assi S, Rostoker R, Serhan CN, Ariel A. Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by resolvins and glucocorticoids. Eur J Immunol. 2011 Feb;41(2):366-79.

78. Nilsson A, Vesterlund L, Oldenborg PA. Macrophage expression of LRP1, a receptor for apoptotic cells and unopsonized erythrocytes, can be regulated by glucocorticoids. Biochem Biophys Res Commun. 2012 Jan 27;417(4):1304-9.

79. Kraal G, Rep M, Janse M. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. An immunohistochemical study. Scand J Immunol. 1987 Dec;26(6):653-61.

80. Lang T, Ave P, Huerre M, Milon G, Antoine JC. Macrophage subsets harbouring Leishmania donovani in spleens of infected BALB/c mice: localization and characterization. Cell Microbiol. 2000 Oct;2(5):415-30.

81. Kropf P, Freudenberg MA, Modolell M, Price HP, Herath S, Antoniazi S, et al. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite Leishmania major. Infect Immun. 2004 Apr;72(4):1920-8.

82. Alberts B JA, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of The Cell. 2004.

83. Hand WL, King-Thompson NL. Effect of erythrocyte ingestion on macrophage antibacterial function. Infect Immun. 1983 Jun;40(3):917-23.

84. Slater AF. Malaria pigment. Exp Parasitol. 1992 May;74(3):362-5.

85. Das NK, Biswas S, Solanki S, Mukhopadhyay CK. Leishmania donovani depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. Cell Microbiol. 2009 Jan;11(1):83-94.

86. Flannery AR, Huynh C, Mittra B, Mortara RA, Andrews NW. LFR1 ferric iron reductase of Leishmania amazonensis is essential for the generation of infective parasite forms. J Biol Chem. 2011 Jul 1;286(26):23266-79.

87. Huynh C, Sacks DL, Andrews NW. A Leishmania amazonensis ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. J Exp Med. 2006 Oct 2;203(10):2363-75.

88. Huynh C, Yuan X, Miguel DC, Renberg RL, Protchenko O, Philpott CC, et al. Heme uptake by Leishmania amazonensis is mediated by the transmembrane protein LHR1. PLoS Pathog. 2012;8(7):e1002795.

89. Campos-Salinas J, Cabello-Donayre M, Garcia-Hernandez R, Perez-Victoria I, Castanys S, Gamarro F, et al. A new ATP-binding cassette protein is involved in intracellular haem trafficking in Leishmania. Mol Microbiol. 2011 Mar;79(6):1430-44.

90. Paramchuk WJ, Ismail SO, Bhatia A, Gedamu L. Cloning, characterization and overexpression of two iron superoxide dismutase cDNAs from Leishmania chagasi: role in pathogenesis. Mol Biochem Parasitol. 1997 Dec 1;90(1):203-21.

91. Plewes KA, Barr SD, Gedamu L. Iron superoxide dismutases targeted to the glycosomes of Leishmania chagasi are important for survival. Infect Immun. 2003 Oct;71(10):5910-20.

92. Ghosh S, Goswami S, Adhya S. Role of superoxide dismutase in survival of Leishmania within the macrophage. Biochem J. 2003 Feb 1;369(Pt 3):447-52.

93. Genetu A, Gadisa E, Aseffa A, Barr S, Lakew M, Jirata D, et al. Leishmania aethiopica: strain identification and characterization of superoxide dismutase-B genes. Exp Parasitol. 2006 Aug;113(4):221-6.

94. Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, et

al. The genome of the kinetoplastid parasite, Leishmania major. Science. 2005 Jul 15;309(5733):436-42.

95. Gobert AP, Verriere T, Asim M, Barry DP, Piazuelo MB, de Sablet T, et al. Heme oxygenase-1 dysregulates macrophage polarization and the immune response to Helicobacter pylori. J Immunol. 2014 Sep 15;193(6):3013-22.

96. Liu G, Friggeri A, Yang Y, Park YJ, Tsuruta Y, Abraham E. miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. Proc Natl Acad Sci U S A. 2009 Sep 15;106(37):15819-24.

97. Department of Pathology KUSoMDoDP, Keio University Hospital, editor. How to make pathological specimens1968.

98. Mulaw T, Tariku A, Tsegaye AT, Abebe Z. Effect of iron-folic acid supplementation on change of hemoglobin among visceral Leishmaniasis patients in northwest Ethiopia: a retrospective follow up study. BMC Hematol. 2018;18:29.

99. Pham NK, Mouriz J, Kima PE. Leishmania pifanoi amastigotes avoid macrophage production of superoxide by inducing heme degradation. Infect Immun. 2005 Dec;73(12):8322-33.

100. Luz NF, Andrade BB, Feijo DF, Araujo-Santos T, Carvalho GQ, Andrade D, et al. Heme oxygenase-1 promotes the persistence of Leishmania chagasi infection. J Immunol. 2012 May 1;188(9):4460-7.

101. Almeida BFM, Silva KLO, Venturin GL, Chiku VM, Leal AAC, Bosco AM, et al. Induction of haem oxygenase-1 increases infection of dog macrophages by L. infantum. Parasite Immunol. 2017 Dec;39(12).

102. Quintela-Carvalho G, Luz NF, Celes FS, Zanette DL, Andrade D, Menezes D, et al. Heme Drives Oxidative Stress-Associated Cell Death in Human Neutrophils Infected with Leishmania infantum. Front Immunol. 2017;8:1620.

103. Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphanio S, et al. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nat Med. 2007 Jun;13(6):703-10.

104. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al.

Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007 Sep;13(9):1042-9.

105. Tsai SY, Segovia JA, Chang TH, Morris IR, Berton MT, Tessier PA, et al. DAMP molecule S100A9 acts as a molecular pattern to enhance inflammation during influenza A virus infection: role of DDX21-TRIF-TLR4-MyD88 pathway. PLoS Pathog. 2014 Jan;10(1):e1003848.

106. Mizobuchi H, Fujii W, Isokawa S, Ishizuka K, Wang Y, Watanabe S, et al. Exacerbation of hepatic injury during rodent malaria by myeloid-related protein 14. PLoS One. 2018;13(6):e0199111.

107. White NJ. Anaemia and malaria. Malar J. 2018 Oct 19;17(1):371.

108. Coombs RR. Cytomegalic inclusion-body disease associated with acquired autoimmune haemolytic anaemia. Br Med J. 1968 Jun 22;2(5607):743-4.

109. Michels K, Nemeth E, Ganz T, Mehrad B. Hepcidin and Host Defense against Infectious Diseases. PLoS Pathog. 2015 Aug;11(8):e1004998.

110. Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of CD47 as a marker of self on red blood cells. Science. 2000 Jun 16;288(5473):2051-4.

111. Sabbatini A, Bombardieri S, Migliorini P. Autoantibodies from patients with systemic lupus erythematosus bind a shared sequence of SmD and Epstein-Barr virus-encoded nuclear antigen EBNA I. Eur J Immunol. 1993 May;23(5):1146-52.

112. Fenderson PG, Fischetti VA, Cunningham MW. Tropomyosin shares immunologic epitopes with group A streptococcal M proteins. J Immunol. 1989 Apr 1;142(7):2475-81.

113. Karlsen AE, Dyrberg T. Molecular mimicry between non-self, modified self and self in autoimmunity. Semin Immunol. 1998 Feb;10(1):25-34.

114. Bull PC, Abdi AI. The role of PfEMP1 as targets of naturally acquired immunity to childhood malaria: prospects for a vaccine. Parasitology. 2016 Feb;143(2):171-86.