PNAS
www.pnas.org

Supplementary Information for

CRIg plays an essential role in intravascular clearance of bloodborne parasites by interacting with complement

Gongguan Liu, Yong Fu, Mohammed Yosri, Yanli Chen, Peng Sun, Jinjun Xu, Mingshun Zhang, Donglei Sun, Ashley B. Strickland, Zachary B. Mackey, and Meiqing Shi

Corresponding author: Meiqing Shi
Email: mshi@umd.edu

This PDF file includes:

Supplementary text
Figures S1 to S9
Legends for Movies S1 to S7

Other supplementary materials for this manuscript include the following:

Movies S1 to S7

www.pnas.org/cgi/doi/10.1073/pnas.1913443116

Supplementary Information Text

Materials and methods

Leukocyte isolation and flow cytometry

The liver was minced into small pieces and forced gently through a 70 μm cell strainer. The cells were suspended in RPMI-1640 medium containing 10% FCS and collected by centrifuging at 300xg. The pellet was resuspended in 10 mL 25% Percoll in HBSS and then centrifuged at 850xg for 30 min at 23ºC. The new pellet was resuspended in 2 mL ACK buffer and incubated at room temperature for 5 min, followed by washing with RPMI-1640 medium containing 10% FCS. Blood leukocytes were prepared via ACK buffer lysis and resuspended in RPMI-1640 medium containing 10% FCS.

Liver and blood leukocytes were incubated (15 min, 4°C) with purified anti-mouse CD16/CD32 (clone 2.4G2, Biolegend). To determine the uptake of parasites by KCs ($CD45^+F4/80^{hi}$), liver leukocytes were stained (30 minutes, 4°C) with APC/cy7-CD45 (clone 30-F11, Biolegend) and Alexa Fluor 647-F4/80 (clone BM8, Biolegend). Cells were washed twice with FACS washing buffer (1% BSA, 0.09% Sodium azide in PBS, pH=7.2-7.4), followed by fixation and permeabilization using Intracellular Fixation & Permeabilization Buffer Set (eBiosciences) according to manufacturer's instructions. Intracellular staining for TC13 antigen was performed by incubating the cells with polyclonal mouse anti-TC13 serum for 1 h at room temperature. After two washes with permeabilization buffer, cells were stained with Alexa Fluor 488 goat anti-mouse IgG/IgM (H+L) secondary antibody (ThermoFisher Scientific) for 30 minutes at room temperature, followed by a final wash in permeabilization buffer; To assess the frequency of the $Ly6C^{hi}$ ($CD45^+Ly6G^-CD11b^+Ly6C^{hi}$) and $Ly6C^{low}$ ($CD45^+Ly6G^-CD11b^+Ly6C^{low}$) monocytes in the blood, blood leukocytes were stained (30 minutes, 4°C) with APC/cy7-CD45 (clone 30-F11, Biolegend), Alexa Fluor 647-Ly6G (clone 1A8, Biolegend), BV510-CD11b (clone M1/70, Biolegend), and FITC-Ly6C (HK1.4, Biolegend), then washed twice with FACS washing buffer. Stained liver or blood cells were resuspended in staining buffer, tested by FACSCanto II, and analyzed using FlowJo software.

Intravital microscopy (IVM)

The Zeiss Axio Examiner Z1 system was used for the liver intravital imaging. Mice were anaesthetized by i.p. administration of a mixture of ketamine (200 mg/kg body weight) and xylazine (10 mg/kg body weight). Cannulation of the tail vein was performed for delivery of parasites or reagents. The mouse was then attached to the surgical stage by adhesive tape. The midline laparotomy was performed followed by a lateral incision along the costal margin to the midaxillary line. The abdominal skin was removed using scissors, and the abdominal muscle was removed using a cauterizer to avoid bleeding. The falciform ligament between the liver and the diaphragm was exposed and cut while holding the xiphoid process with a knot made of suture thread. The mouse was then moved to a customized acrylic imaging stage and the right lobe was gently exposed and covered with a trimmed cover slip. To prevent tissue dehydration, the exposed abdominal tissues were covered with saline-soaked gauze, and the liver was continuously moistened with a saline-soaked Kimwipe. An infrared heating lamp was used to maintain the body temperature of the mouse throughout the imaging.

To label Kupffer cells, mice were i.v. injected with Alexa Fluor 647- or PE-anti-F4/80 mAb (clone BM8, Biolegend) at 4 μg/mouse via the catheter 15 min prior to imaging. To label intravascular *T. congolense* TC13 parasites, infected mice were i.v. injected with Rhodamine 6G (1mM, 100 μL) via the catheter 15 min prior to imaging. For some experiments, mice were i.v. injected with $5x10^7$ *T. congolense* TC13 labeled in vitro by TRITC via the catheter immediately before imaging and injected with 1 mg of anti-TC13 IgM mAb 10 min after parasite delivery. Time-lapse videos were taken at 1- or 5- second intervals. For each experiment, at least 5 mice per group were used for in vivo visualization of the livers. For some experiments, only intravital images were taken. At least 10 microscopic fields were observed for each mouse.

Immunohistochemistry

Liver tissues were removed and frozen in OCT compound (Tissue-Tek). Frozen tissue blocks were cut on a cryostat microtome with a thickness of 7 μm; and sections were placed on coated glass slides (VWR). Tissue sections were fixed in cold acetone (-20°C) for 10 min. After fixation, the samples were blocked by incubation with 10% goat serum for 30 min at room temperature in a humidified chamber, followed by incubation with the primary antibody at 4°C overnight. Sections were then washed 5 times with 0.05% PBS/Tween-20 and incubated for 30 min with the fluorescence conjugated secondary antibodies. After three washes, tissues were preserved in mounting buffer (Southern Biotech) and sealed with nail polish (Electron

Microscopy Sciences). For each experiment, at least 4 mice per group were used. Two to three tissue sections were cut for each mouse. At least 10 microscopic fields were observed for each tissue section.

ELISAs for trypanosome-specific antibodies

Whole-trypanosome lysate was prepared by repeated freezing and thawing of freshly isolated *T. congolense* TC13 or dTomato-expressing *T. b. brucei* in the presence of 5 mM Tosyl-L-lysyl-chloromethane hydrochloride (TLCK, Sigma). The total protein content was determined by the Bradford Protein Assay kit (ThermoFisher Sciences) according to manufacturer's instructions. ELISA plates were coated overnight at 4°C with 50 μL of the lysate containing 25 μg/mL total protein, followed by two washes with 0.05% PBS/Tween-20. Nonspecific binding sites were blocked for 2 h at room temperature with 200 μL of 10% goat serum. Serial serum dilutions (100 μL/well) were incubated for 2 h at 37°C. After five washes, peroxidase-conjugated goat anti-mouse IgM and IgG isotype–specific antibodies (1:1000 dilution, 100 μL/well; Southern Biotech) were added to each well and the mixture was incubated for 2 h at room temperature. After washing, the color was developed by adding Tetramethylbenzidine (TMB, BD Pharmingen). After 30 minutes, the reaction was stopped with 50 μL of 1 N H_2SO_4, and optical densities were measured at 450 nm. Cut-off points were determined based on the values of serum samples from noninfected mice.

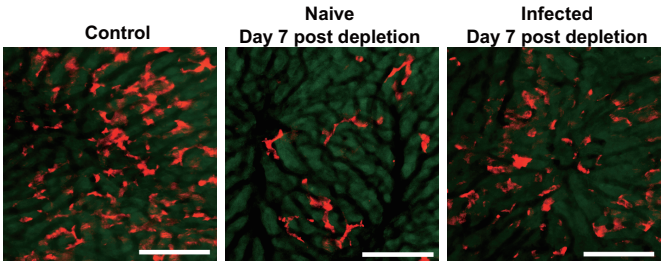

Fig. S1. Partial repopulation of Kupffer cells (KCs) after depletion

Intravital microscopy (IVM) was performed on the liver to visualize KCs (red). To label KCs, PE anti-F4/80 mAb was i.v. injected 15 min before imaging. Left panel: KCs of uninfected mice 24 h after i.v. administration of PBS liposomes (control); middle panel: KCs of uninfected mice 7 days after i.v. administration of clodronate liposomes; right panel: KCs of infected mice 7 days after i.v. administration of clodronate liposomes (The mice were i.p. infected with $1x10^3$ *T. congolense* TC13 24 h after administration of clodronate liposomes). Scale bars, 200 µm.

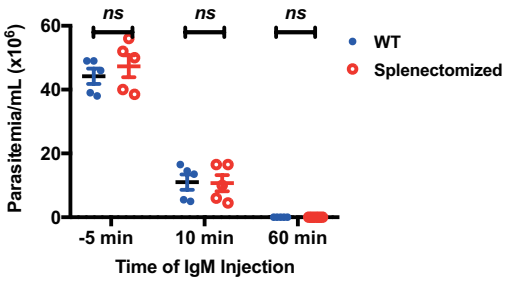

Fig. S2. Clearance of *T. congolense* in the blood of splenectomized mice

WT and splenectomized mice (n=5 per group) were i.v. infected with $5x10^7$ *T. congolense* TC13. Ten min later, the mice were i.v. injected with anti-TC13 IgM mAb. The parasitemia was determined -5, 10, and 60 min post mAb administration. Data are expressed as mean ± SEM. *ns*, not significant by Student's *t* test.

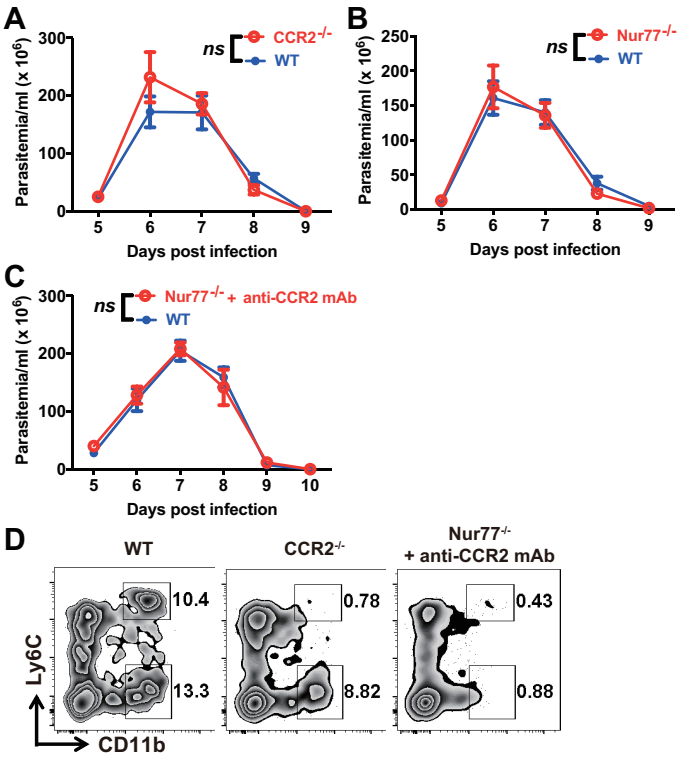

Fig. S3. Loss of Ly6C^{hi} and/or Ly6C^{low} monocytes does not affect parasitemia during *T. congolense* infection

WT and knockout mice were i.p. infected with 1x10^3 *T. congolense* TC13. Parasitemia was determined at various time points post infection. *(A)* WT and CCR2$^{-/-}$ mice (n=5 per group), *(B)* WT and Nur77$^{-/-}$ mice (n=5 per group), and *(C)* WT and Nur77$^{-/-}$ mice (n=3-5 per group) receiving anti-CCR2 mAb (i.v. delivery at 6 µg/mouse starting on day -1 and then every other day of infection) to deplete Ly6C^{hi} monocytes. *(D)* Representative flow cytometry plots showing loss of Ly6C^{hi} and Ly6C^{low} monocytes in the blood of Nur77$^{-/-}$ mice treated with anti-CCR2 mAb. Data are expressed as mean ± SEM of 2 independent experiments (*A and B*) or 1 experiment (*C*). *ns*, not significant by Student's *t* test.

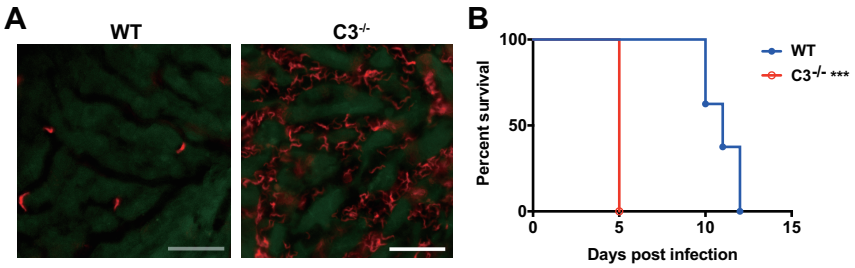

Fig. S4. Complement C3 is essential for parasite control during monomorphic *T. b. brucei* infection

(A) Representative IVM images showing the parasites (red) in liver sinusoids of WT and C3$^{-/-}$ mice 5 days post i.p. infection with 1x10^3 dTomato-expressing *T. b. brucei* Tb221. *(B)* The survival of WT and C3$^{-/-}$ mice (n=8 per group) i.p. infected with 1x10^3 dTomato-expressing *T. b. brucei* Tb221. Scale bars, 50 µm. Data are expressed as mean ± SEM of 2 independent experiments. ***p<0.001 by Log-rank test.

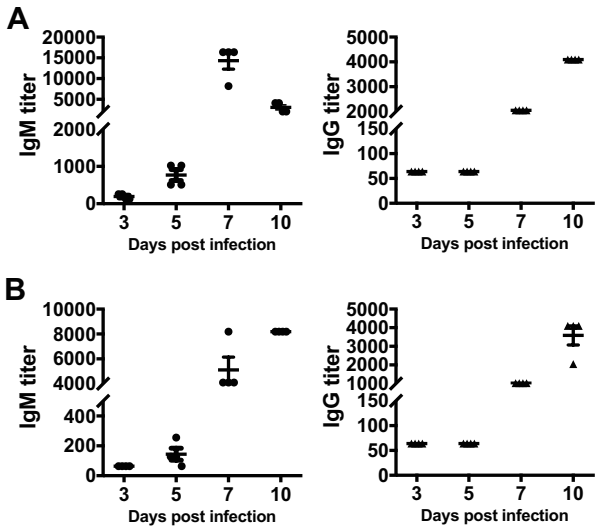

Fig. S5. Production of IgM and IgG antibodies during African trypanosome infection

WT mice (n=4 per group) were i.p. infected with $1x10^3$ dTomato-expressing *T. b. brucei* Tb221 or $1x10^3$ *T. congolense* TC13. Sera were collected 3, 5, 7, and 10 days post infection. Serum titers of IgM and IgG specific for Tb221 *(A)*, and TC13 *(B)* were measured by ELISA; cutoff points were determined based on O.D. values of samples from naive mice. Data are expressed as mean ± SEM.

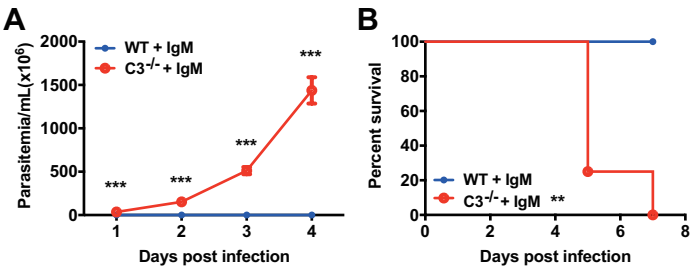

Fig. S6. IgM antibody administration leads to parasitemia control and prolongs survival in WT mice but not C3$^{-/-}$ mice during *T. congolense* infection

WT and C3$^{-/-}$ mice (n=4 per group) were i.v. infected with 5x10^7 *T. congolense* TC13, followed by i.v. administration of anti-TC13 IgM mAb 10 min later. *(A)* Parasitemia of infected mice. *(B)* The survival of infected mice. Data are expressed as mean ± SEM of 2 independent experiments. **p<0.01, ***p<0.001 by Student's *t* test or log-rank test.

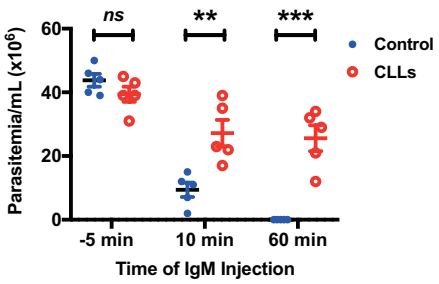

Fig. S7. Antibody-mediated parasite clearance is dependent on KCs

Splenectomized mice (n=5 per group) with or without treatment with clodronate liposomes (CLLs) to deplete KCs were i.v. infected with $5x10^7$ *T. congolense* TC13. Ten min later, the mice were i.v. injected with anti-TC13 IgM mAb. The parasitemia was determined -5, 10, and 60 min post mAb administration. Data are expressed as mean ± SEM. *ns*, not significant, **p<0.01, ***p<0.001 by Student's *t* test.

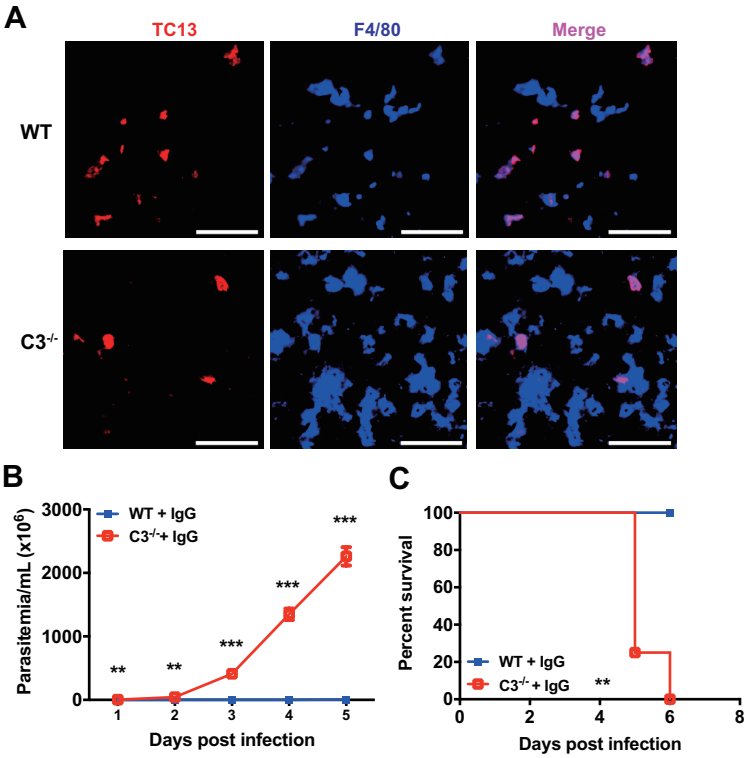

Fig. S8. Role of IgG antibody in parasitemia control during *T. congolense* infection

WT and C3$^{-/-}$ mice (n=4 per group) were i.v. infected with 5x10^7 *T. congolense* TC13, followed by i.v. administration of anti-TC13 IgG mAb 10 min later. *(A)* Immunohistological staining was performed on liver sections of infected mice 60 min post mAb administration to determine the parasite TC13 antigens (red) inside KCs (blue, labeled by anti-F4/80 mAb). *(B)* Parasitemia of the infected mice. *(C)* The survival of infected mice. Scale bars, 50 µm. Data are expressed as mean ± SEM of 2 independent experiments. **p<0.01, ***p<0.001 by Student's *t* test or log-rank test.

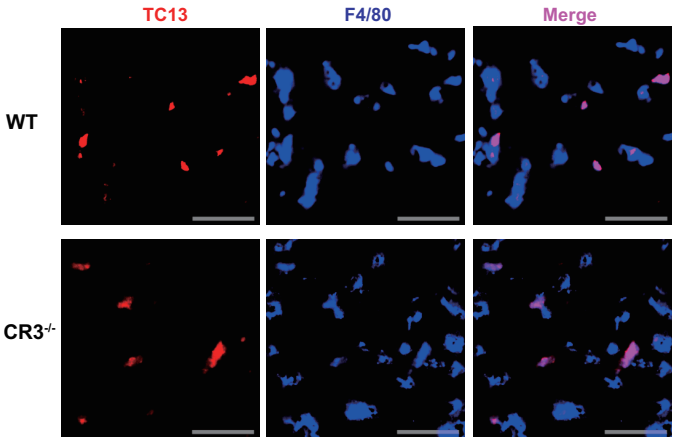

Fig. S9. CR3 is dispensable for IgM mediated parasite internalization

WT and CR3$^{-/-}$ mice were i.v. infected with 5x10^{7} *T. congolense* TC13, followed by i.v. administration of anti-TC13 IgM mAb 10 min later. Immunohistological staining was performed on liver sections of infected mice 60 min post mAb administration to determine the parasite TC13 antigens (red) in KCs (blue, labeled by anti-F4/80 mAb). Scale bars, 50 μm.

Movie S1 (separate file). Live cell imaging showing the dynamics of capture of dTomato-expressing *T. b. brucei* TB221 (red) by murine J774 macrophages (Green, labeled by CSFE) in the presence of mouse serum and anti-TB221 IgM mAb.

Movie S2 (separate file). Intravital imaging showing the kinetics of capture of dTomato-expressing *T. b. brucei* TB221 (red) in the liver sinusoids of mice 5 days post i.p. infection of $1x10^3$ parasites.

Movie S3 (separate file). Intravital imaging showing parasites within liver sinusoids of WT mice (left panel) and $C3^{-/-}$ mice (right panel) 7 days post i.p. infection of $1x10^3$ *T. congolense* TC13.

Movie S4 (separate file). Intravital imaging showing parasites (red) within liver sinusoids of WT mice (left panel) and $C3^{-/-}$ mice (right panel) 5 days post i.p. infection of $1x10^3$ dTomato-expressing *T. b. brucei* TB221.

Movie S5 (separate file). Intravital imaging showing the incidences of parasite capture in WT mice (left panel) and $C3^{-/-}$ mice (right panel) after i.v. administration of anti-TC13 IgM mAb. Ten min prior to the mAb administration, mice were i.v. infected with $5x10^7$ *T. congolense* TC13 labeled by TRITC in vitro.

Movie S6 (separate file). Intravital imaging showing parasites within liver sinusoids of WT mice (left panel), $CR3^{-/-}$ mice (middle panel), and $CRIg^{-/-}$ mice (right panel) 7 days post i.p. infection of $1x10^3$ *T. congolense* TC13.

Movie S7 (separate file). Time lapse video showing the in vivo complement deposition on live parasites. Mice were i.v. injected with clodronate liposomes to deplete KCs and 24 h later i.v. infected with $5x10^7$ *T. congolense* TC13 labeled by TRITC in vitro, followed by i.v. injection with or without anti-TC13 IgM mAb 10 min later. Twenty min after infection, blood was collected. Parasites were purified from the blood and then stained with anti-C3b/iC3b mAb. Left panel: Without IgM injection, C3b/iC3b (green) was not detected on *T. congolense* (red). Right panel: With IgM injection, C3b/iC3b (green) was detected on the parasites (red).