Sequential *Plasmodium chabaudi-Plasmodium berghei* infection provides a novel model of severe malarial anemia

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Running Title: NOVEL MURINE MODEL OF SEVERE MALARIAL ANEMIA

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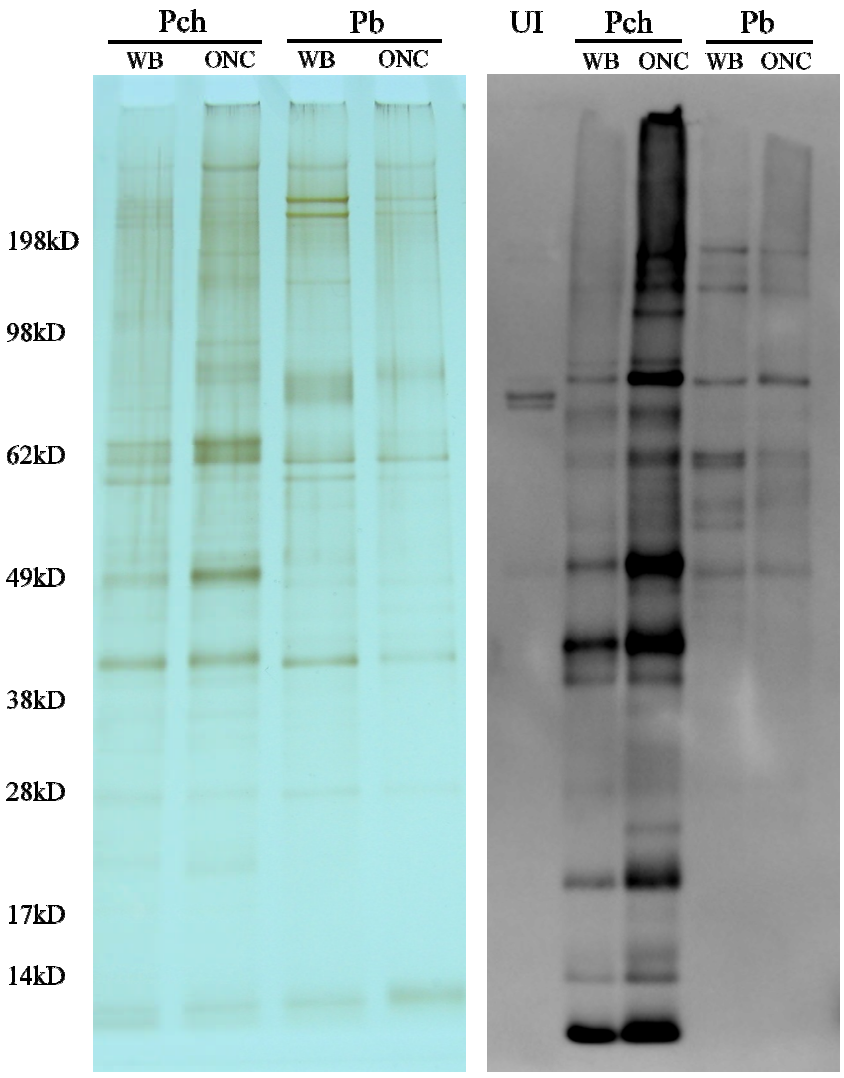
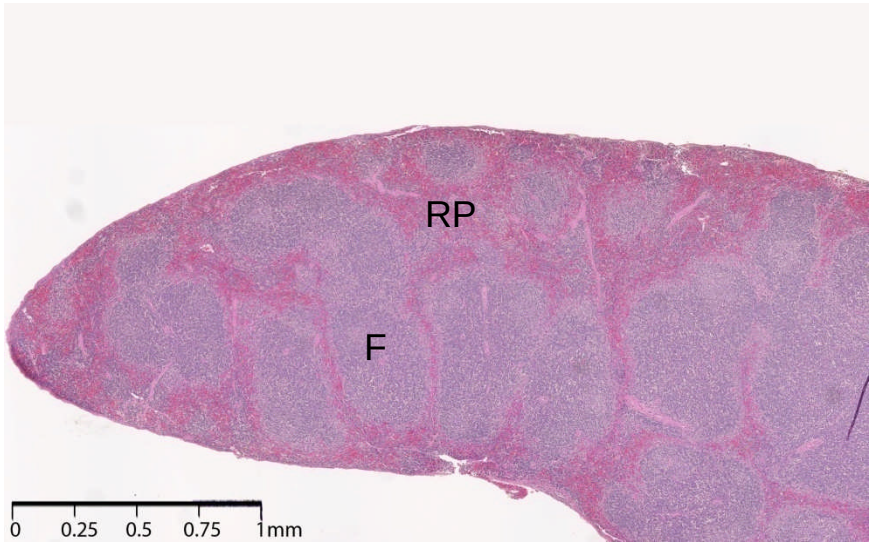


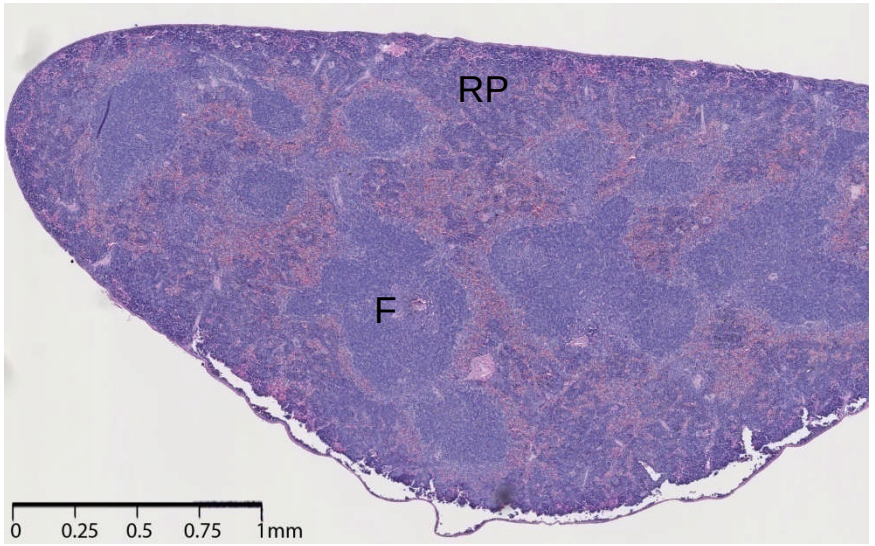
Figure 1. Verification of malaria antigen by western blot. 0.5 µg of protein from a whole blood lysate (WB) or an overnight culture lysate (ONC) of *P. chabaudi* (Pch) or *P. berghei* (Pb) infected mouse blood were loaded into each well of two 4-12% SDS acrylamide gels. The image on the left is a silver stain of one gel confirming the presence of proteins in the lysates. The proteins in the second gel were transferred to a nitrocellulose membrane. The membrane was blocked with 1% IgG-free BSA (Jackson ImmunoResearch, West Grove, PA) in tris-buffered saline (TBS) pH = 8.0 containing 0.05% Tween 20. Plasma from an immune mouse (*P. chabaudi/P. berghei* 20 days post-infection) was used as primary antibody at a dilution of 1:500. Horseradish peroxidase-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD) was used as secondary antibody at a dilution of 1:5000. Membranes were developed with the Pierce ECL western blotting substrate (Thermo Scientific) and visualized on the Fuji LAS-3000 with ImageReader software.

A

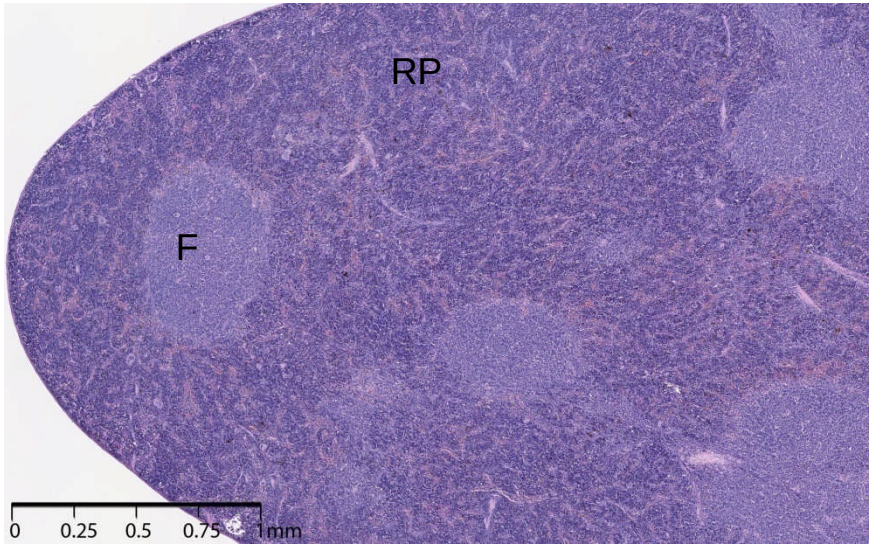
Uninfected/Naive



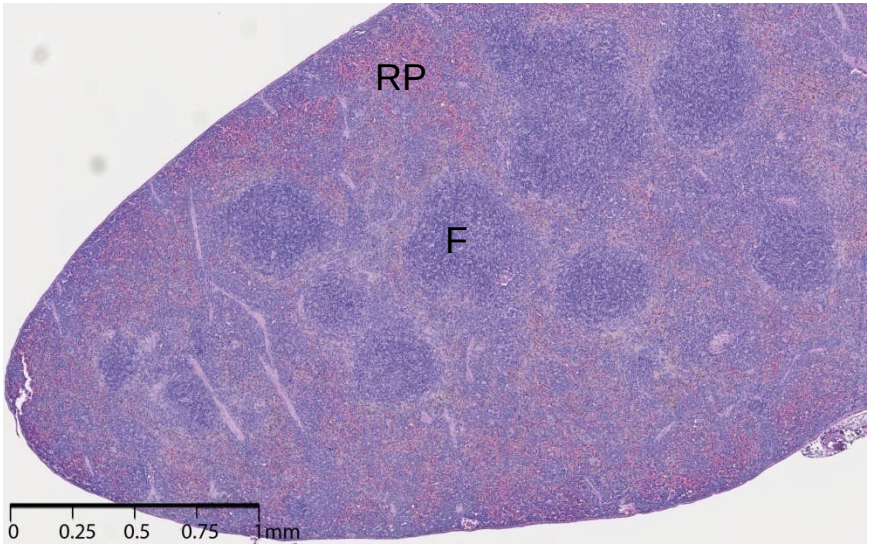
P. chabaudi/sham (Pch-sham)



P. chabaudi/P. berghei (Pch-Pb)

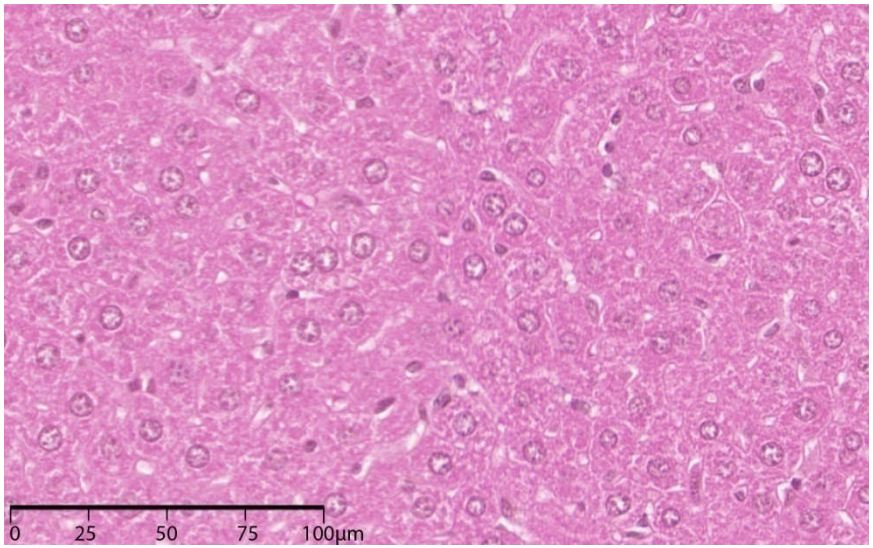


P. berghei (Pb)

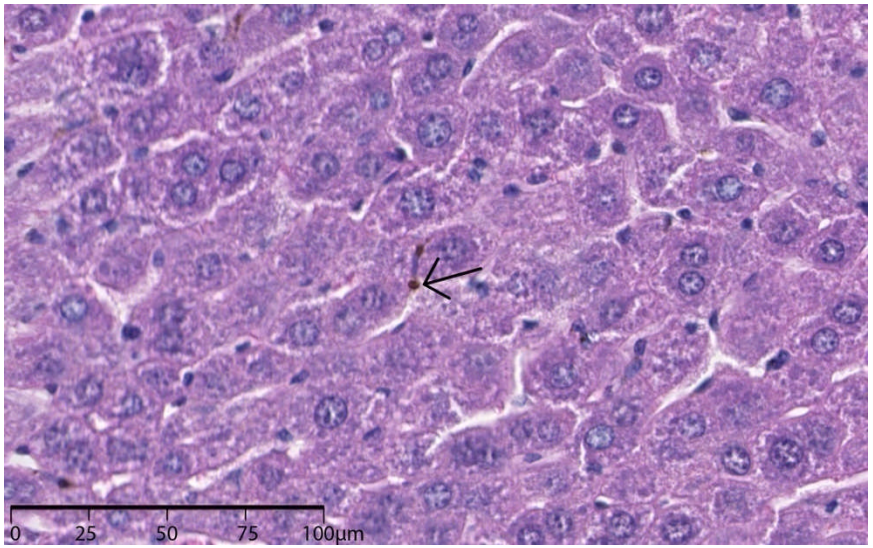


B

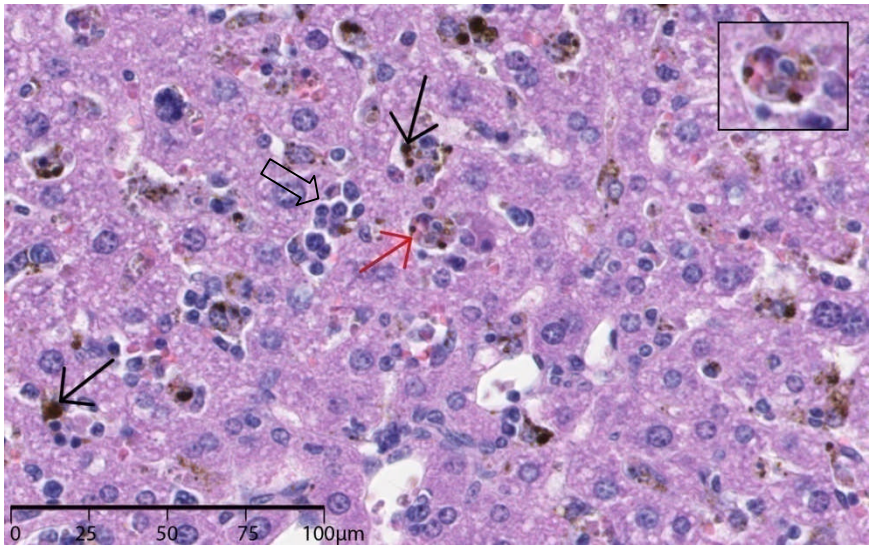
Uninfected



P. chabaudi/sham (Pch-sham)



P. chabaudi/P. berghei (Pch-Pb)



P. berghei (Pb)

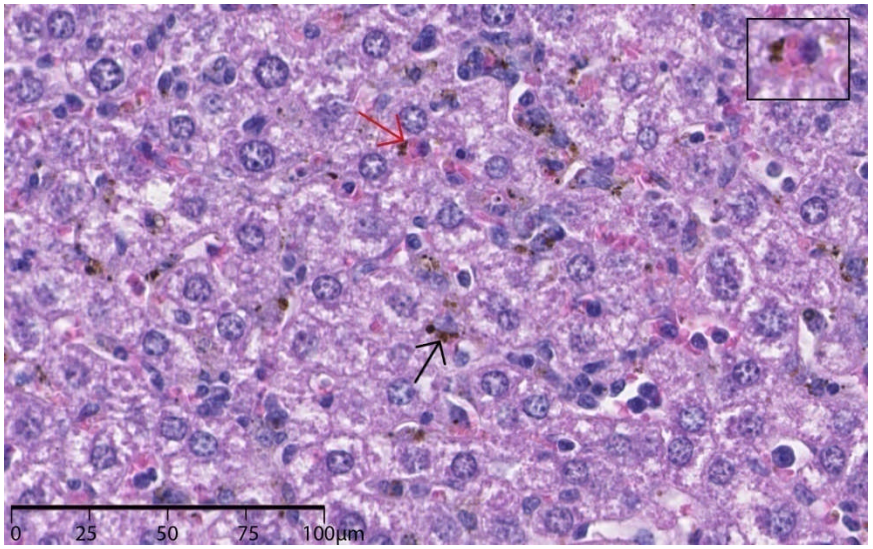


Figure 2

Figure 2. Hematoxylin and eosin stains of formalin-fixed paraffin-embedded liver and spleen. A) Spleens of infected animals showed expansion of lymphoid follicles (F). In addition, the red pulp (RP) in Pch-sham and Pch-Pb animals showed expansion of erythropoietic precursors which is most marked in the latter. B) The liver shows evidence of pigment within macrophages (black arrows) and erythrophagocytosis (red arrows and insets) in Pch-Pb and Pb mice. The liver of Pch-Pb animal also shows islands of erythropoiesis, open arrow.

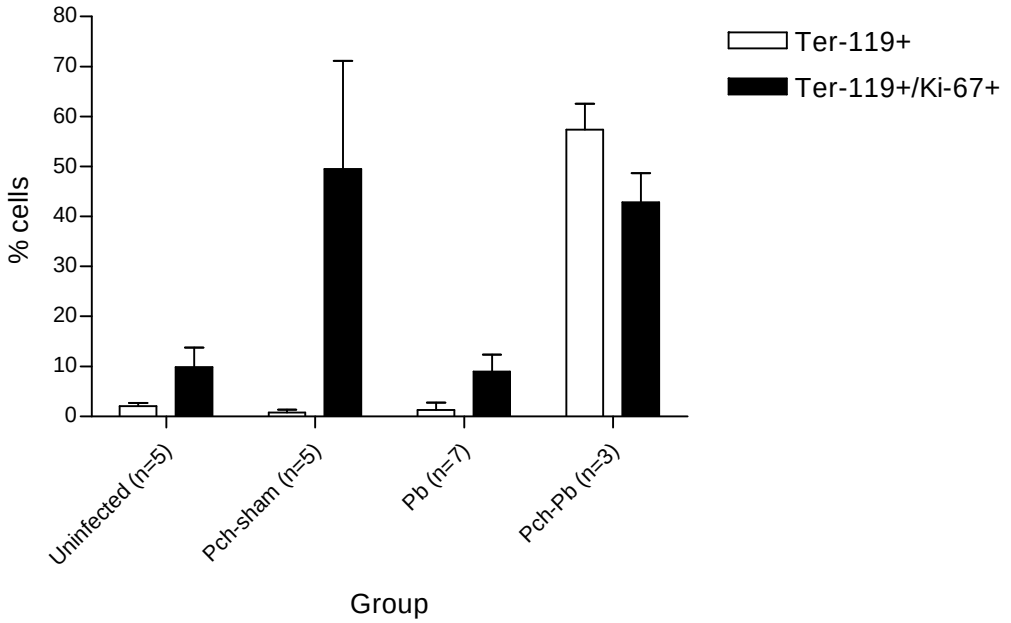
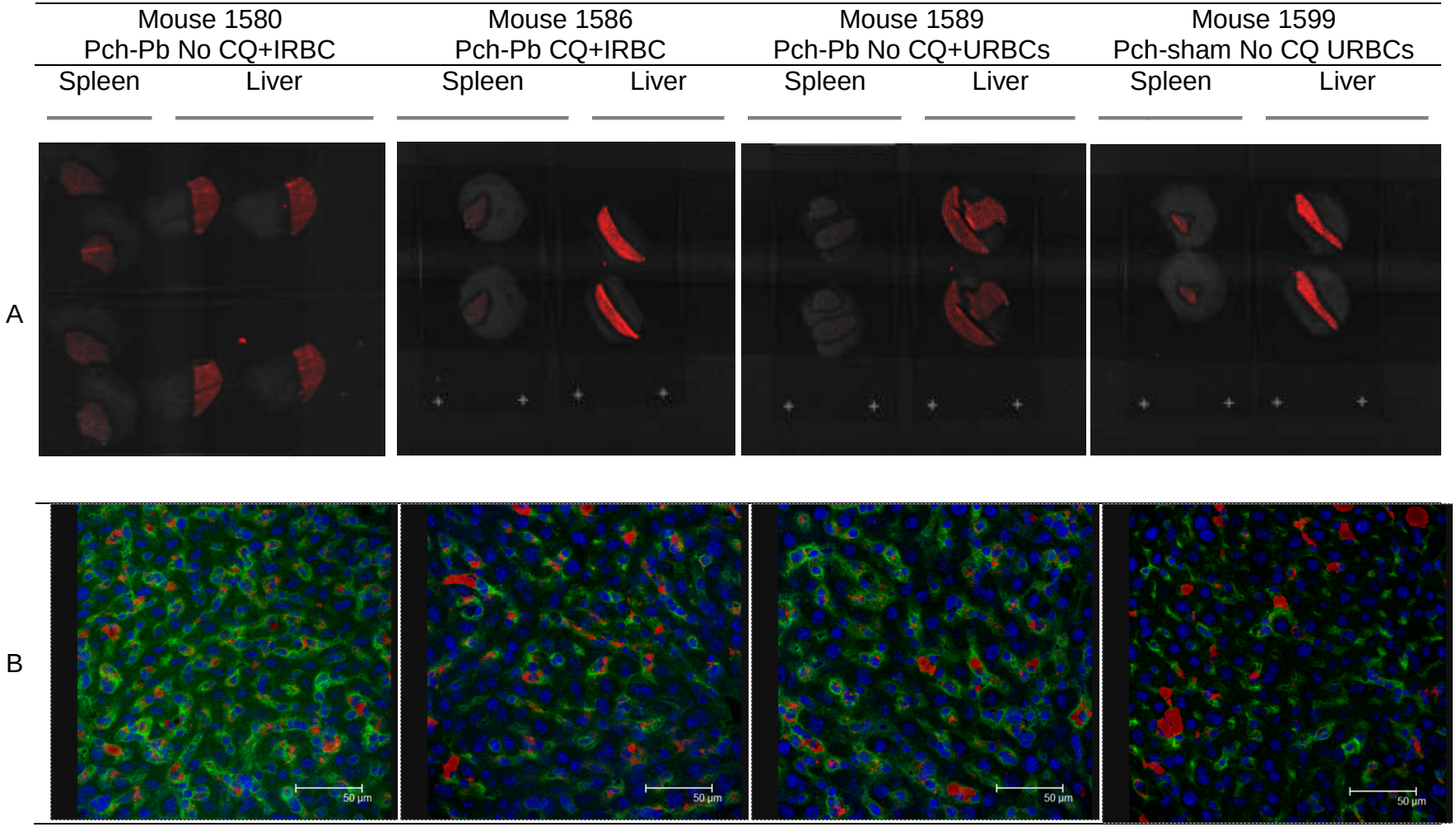
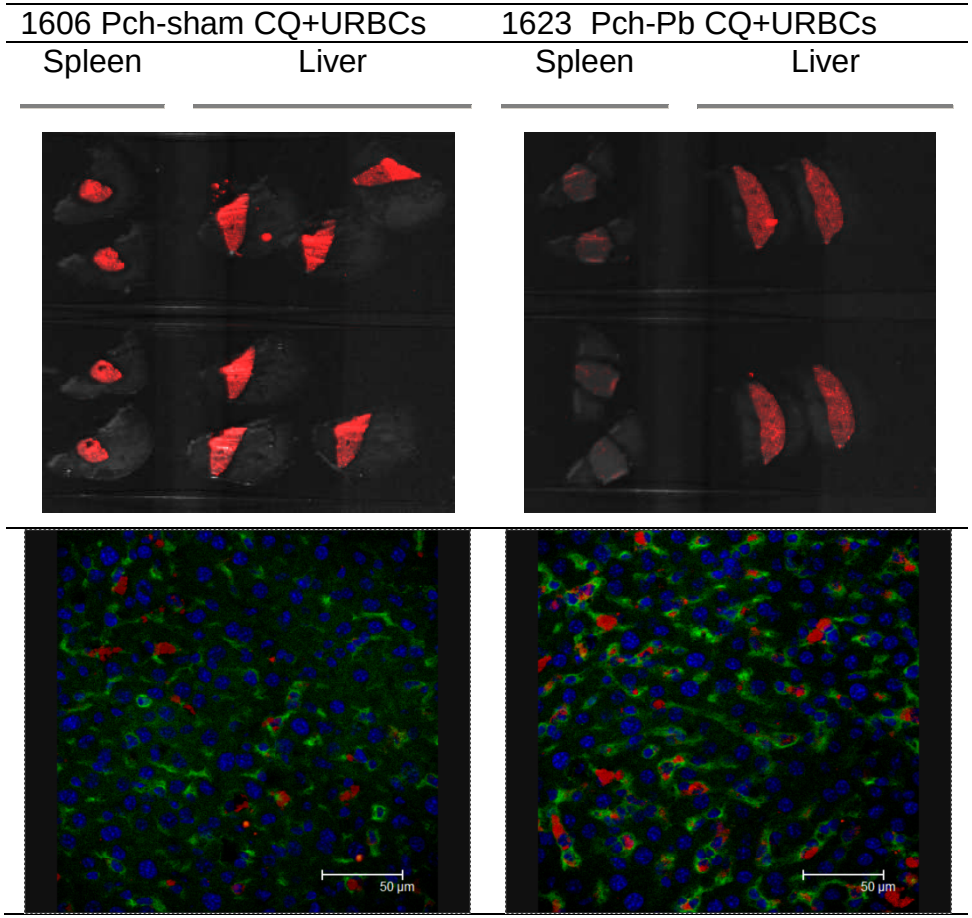
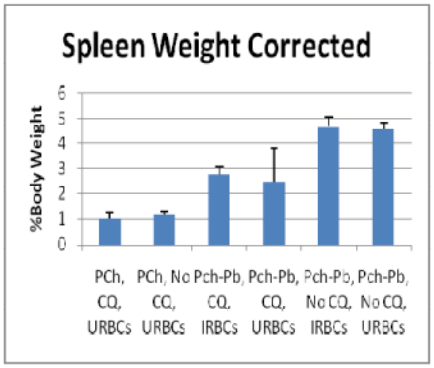


Figure 3. Spleens from Pch-Pb mice have a greater percentage of lysis resistant Ki-67-Ter-119. The white bars show the percent of lysis-resistant splenic cells that are TER-119+ (glycophorin). The black bars represent the percent TER-119+ cells that are Ki67+. Spleens obtained from animals at the time of euthanasia were placed into 5 mL cold PBS and crushed with a 3 mL syringe plunger. The resulting cell suspension was passed through a 40 µm cell strainer and centrifuged at 400g to pellet the cells. The cells were washed with 5 mL cold PBS and counted with a hemacytometer followed by lsysis in 1 mL RBC lysis buffer (Sigma-Aldrich). The cells were then resuspended in 5 mL of PBS and pelleted as above. Cell pellets were washed with 5 mL PBS and resuspended in 1 mL PBS containing 1% BSA. The cells were then counted again and preparations of 10^7 cells/mL were made for each spleen. For detection of Ki-67 and Ter-119, approximately 10^6 cells were fixed in 100 µL of 4% paraformaldehyde in PBS for 30 min at room temperature. Following fixation, the cells were washed once with PBS containing 2% BSA (FACS buffer) and stored in the same buffer overnight at 4 °C. The following day, the cells were permeabilized by incubation in 150 µL of PBS containing 2% BSA and 0.5% saponin at room temperature for 10 minutes. This was followed by incubation for 20 minutes at room temperature in a 1:50 dilution of FITC-labeled mouse anti-Ki67 (BD Biosciences) in permeabilization buffer, a wash in FACS buffer, and incubation for 30 minutes in a 1:50 dilution of PE-labeled rat anti-mouse TER-119 (BD Biosciences). Finally, the cells were resuspended in cold 1% paraformaldehyde in PBS. Acquisition was done on an LSRII flow cytometer using FACS Diva software.


Mouse 1580
Pch-Pb No CQ+IRBC
Mouse 1586
Pch-Pb CQ+IRBC
Mouse 1589
Pch-Pb No CQ+URBCs
Mouse 1599
Pch-sham No CQ URBCs
Spleen
Liver
Spleen
Liver
Spleen
Liver
Spleen
Liver
A
B
50 µm
50 µm
50 µm
50 µm





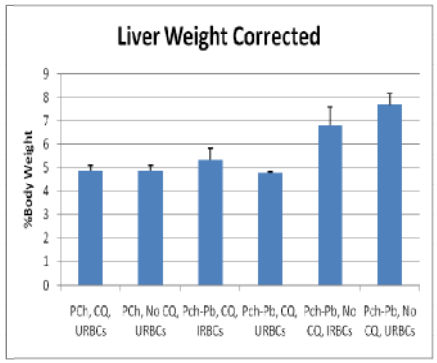


Figure 4. Representative global fluorescence images of liver and spleen (rows A and C) and liver immunofluorescence microscopy (Rows B and D) from animals injected with *ex vivo*-labeled RBCs in the experiment outlined in Figure 5 panels D-F of the main manuscript. On day 5 some groups were treated with chloroquine (CQ) or not, and some groups were injected with RBCs from Pch-Pb animals with a *P. berghei* parasitemia of 3% (IRBCs) or from uninfected animals (URBCs) on day 5 post *P. berghei* infection (Pch-Pb). The organs were harvested on day 9. Pch animals had recovered from *P. chabaudi* infection and had no detectable parasitemia by Giemsa-stained blood smears. Global fluorescence shows that fluorescence is concentrated in the liver in all the animals, suggesting that this is the site where most of the RBCs are trapped. Paradoxically, Pch-Pb animals show less liver fluorescence despite their higher degree of anemia perhaps due to a dilution of labeled RBCs with trapped unlabeled RBCs. All the livers showed varying degrees of

erythrophagocytosis. The spleens of Pch animals showed greater global fluorescence than other groups which was confirmed by immunofluorescence microscopy showing increased RBC trapping (data not shown).