Mechanisms of Effect of Iron Status and Iron Repletion on Bacterial and Malarial Infection – Implications for Transfusion of Older, Stored RBCs

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BACKGROUND: Iron is the most common dietary deficiency. Studies suggest that iron repletion is detrimental for children in malarial endemic areas.

STUDY DESIGN AND METHODS: A mouse model to study the interactions between iron status, iron interventions, and malarial and bacterial infections was developed. Mice were infected with *Salmonella typhimurium* and/or *Plasmodium yoelii* parasites. *Salmonella* infected mice were supplemented with iron sulfate or transfused fresh, stored, or no RBCs. Stored RBCs were prepared using C57BL/6 donor RBCs stored in AS-1 in DEHP-plasticized bags (Fenwal).

RESULTS: Iron deficiency worsens infection with *Salmonella*, (median survival 7 days vs. 16 days). Co-infection with rodent malaria leads to earlier death due to overwhelming bacterial sepsis in both iron-deficient (median survival 5 days vs. 7 days) and iron-replete (median survival 10 days vs. 16 days) mice. To test whether clearance of RBCs alone, rather than immunosuppression due to the malarial parasite, can cause this synergy, *Salmonella*-infected mice were transfused with no RBCs, fresh RBCs, or stored RBCs. The transfusion of 2-week old stored RBCs leads to rapid RBC clearance and earlier death due to bacterial sepsis (median survival 4 days vs. 28 days). Finally, mice orally supplemented with iron sulfate have a similar exacerbation of bacterial sepsis.

CONCLUSIONS: These findings suggest that rapid RBC clearance can exacerbate a bacterial infection, potentially by providing a source of “free” iron. This model will be used to optimize iron repletion strategies for use in malaria endemic areas and to study the adverse effects of transfusion of stored RBCs.

Keywords: malaria; salmonella; iron; non-transferrin bound iron; red blood cell

Introduction

Iron deficiency is associated with neurological manifestations, including cognitive and developmental delay in children.[1,2] The effects of host iron status and repletion strategies on malarial and bacterial infection in humans are critical, but unresolved, public health issues in regions where malaria is endemic and iron deficiency is common. The Pemba trial was a randomized, placebo-controlled trial of children aged 1-35 months in Pemba, Zanzibar.[3] Approximately 24,000 children were randomized to daily oral iron and folic acid supplementation; iron, folic acid, and zinc; or placebo. However, the Data and Safety Monitoring Board recommended that the trial with the iron-containing groups be stopped early, primarily because children receiving iron were 12% more likely to die or need hospital treatment for an adverse event. Overall, this study demonstrated that the incidence of malaria and other infectious diseases were significantly increased in children receiving iron and folic acid. Although the World Health Organization Consultation[4] identified plasma non-transferrin-bound iron (NTBI) as a probable cause of the adverse events in the Pemba trial, the mechanism is still unresolved.

Plasma NTBI levels in humans increase for up to 3.5 hours after administration of iron supplements in doses similar to those in the Pemba trial.[5] Similarly, transfusion of RBCs to neonates[6] and a mouse RBC transfusion model show that transfusions, particularly of older, stored RBCs lead to increased plasma NTBI levels.[7] Finally, by destruction of parasitized RBCs, malarial infection also leads to release of iron-containing molecules into the circulation and accumulation of hemozoin in phagocytes.[8] The rodent malarial parasite used in this study, *Plasmodium yoelii*, preferentially infects reticulocytes and produces a fulminant, but non-lethal, infection with high parasitemia, which is eventually cleared, but later recrudesces.[9]

In a rural African community, there is a 2.5% probability that a child will acquire invasive bacteremia in the first 5 years of life, and 26% of hospital deaths are associated with bacteremia.[10] Non-typhoidal *Salmonella* is one of the most frequently isolated bacterial species, representing 18% of isolates in children >60 days old in Africa.[11] Bacteremia, especially with non-typhoidal *Salmonella*, is strongly associated with severe anemia, although iron deficiency may be somewhat protective.[12] A consistent association between severe malaria and non-typhoidal *Salmonella* has also been observed.[13-15] The prevalence of co-infection varies by region;[16] however, non-typhoidal *Salmonella* is the most frequent,[13-15] or among the most frequent,[11] bacterial isolates from malarial infected children. Co-infection with non-typhoidal *Salmonella* is also associated with increased morbidity and mortality in humans and in animal models with severe malaria.[13,17,18] Non-typhoidal *Salmonella* is a common cause of meningitis in Malawian children <2 years of age, and the prevalence is highest during the few months after the peak malaria season.[19] To compound the problem, non-typhoidal *Salmonella* isolates are also highly resistant to the commonly available antibiotics in Africa.[18,20] Despite the strong association and synergy between non-typhoidal *Salmonella* and malaria, their relative contributions to the pathogenesis and increased mortality of co-infection is unknown.[17] Although these bacteria require iron for growth, the effect of iron status on co-infection with these bacteria and *Plasmodium* is not known. We developed a mouse model of iron deficiency to examine the complex interactions between iron status, iron repletion, and infection with malaria and non-typhoidal *Salmonella*.

Materials and Methods

Mice

Female C57BL/6 weanling mice, 21 days old, (Charles River) were randomized by cage (5 per cage) to receive the control, iron-replete diet containing 45 ppm iron (Harlan TD.80396 with supplemented iron) or the iron-deficient diet containing 2-6 ppm iron (Harlan TD.80396). Every 2 weeks thereafter, 50 μL of blood was collected by submandibular bleed for measuring baseline hematological parameters. After 70 days on this diet, 3 mice per group were sacrificed to assess total liver iron. In some experiments, mice were administered ferrous sulfate (1 mg/kg; Sigma) by oral gavage. Mice used for transfusion experiments were male C57BL/6 mice, 8-12 weeks of age (Charles River). Procedures were approved by Institutional Animal Care and Use Committees.

Mouse RBCs: collection, storage, and derivatives

C57BL/6 mice were bled aseptically by cardiac puncture into CPD (14% final concentration) obtained directly from primary collection packs (Fenwal). Whole blood collected from 20-40 mice was pooled and leukoreduced using a Neonatal High Efficiency Leukocyte Reduction Filter (Purecell Neo, Pall Corporation), centrifuged (3700g for 15 min), plasma removed, and RBCs resuspended with AS-1 (Fenwal) to a final hematocrit of 60%. The packed RBCs were stored in the DEHP-plasticized diversion pouch from the primary collection packs (Fenwal), at 4°C for up to 14 days. Residual leukocytes were enumerated by flow cytometry (LeucoCOUNT kit, BD Biosciences). On the day of transfusion, a 500 μL aliquot of stored RBCs was inoculated into a Peds Plus/F blood culture bottle (BD Diagnostic Systems) and loaded into the BACTEC™ Fx (BD Diagnostic Systems), a continuous monitoring blood culture system, for up to 5 days or until bacterial growth was detected.

Transfusion and 24-hr post-transfusion RBC recovery

Packed RBCs (350 μL; equivalent to 2 human units) were transfused through the retro-orbital plexus of Isoflurane-anesthetized mice. The proportion of transfused RBCs circulating at 24-hours post-transfusion (i.e. the 24-hr post-transfusion recovery (PTR)) was measured by a dual-labeling method, as described.[7]

Growth, propagation, and infection with *Plasmodium yoelii*

Several days prior to infection, 2 donor mice were infected intraperitoneally with 200 μL of *Plasmodium yoelii*, strain 17X, thawed from a frozen stock (prepared by mixing blood obtained by aseptic cardiac puncture of C57BL/6 mice (5-15% parasitemia) with an equal volume of Glycerolyte 57 solution (Fenwal), cooling at 4°C for 10 minutes to allow for equilibration, and freezing at -80°C). Parasitemia of donor mice was quantified by examining Wright-Giemsa stained peripheral blood smears until parasitemia reached 5-15% (i.e. in 2-4 days). Donor mice were euthanized and blood obtained by aseptic cardiac puncture. After quantifying the parasitemia of pooled blood, samples were diluted with normal saline to $5x10^5$ infected RBCs/mL and test mice were inoculated intraperitoneally with 200 μL of this stock ($1x10^5$ infected RBCs/mouse).

Growth, storage, and inoculation of S. typhimurium

The non-typhoidal *S. typhimurium*, strain LT2 (ATCC) was stored at -80°C in Nutrient Broth (8 g/L (BBL Microbiology Systems) and NaCl (5 g/L)) supplemented with 10% DMSO. Nutrient Broth (5 mL) was inoculated with frozen stock and grown for ~4 h at 37°C with shaking (to mid-log phase). Bacteria were harvested by centrifugation (4000g at 4°C), washed twice with PBS, suspended in 2 mL PBS, and absorbance measured at

600 nm. Preliminary studies using plate-dilution correlated absorbance level with colony forming units (CFUs). Mice were inoculated intraperitoneally with 10^3 CFUs in 200 μL of sterile saline. At death, cardiac puncture-obtained blood was serially diluted in sterile PBS and cultured on SS agar (BBL Microbiology Systems) to quantify bacteremia. All surviving mice were euthanized 28 days after infection.

Histology and immunohistochemistry

At necropsy, the organs were removed, fixed overnight with 10% neutral buffered formalin, and embedded in paraffin. Sections were stained with hematoxylin & eosin (H&E) or with a tissue Gram stain. Images were captured using an Olympus BX40 microscope (Center Valley, PA) and a SPOT INSIGHT digital camera (Diagnostic Instruments; Sterling Heights, MI).

Iron-related analytes

Hemoglobin was quantified by a modified Drabkin's assay[21] at a 1:251 dilution of stored RBCs to Drabkin's reagent (Ricca Chemical Company; Arlington, TX); optical density measured at 540 nm was compared to Count-a-part Cyanmethemoglobin Standards Set (Diagnostic Technology, Inc.). Total liver iron was quantified using a wet ashing procedure.[7] Ferritin was quantified using a mouse ferritin ELISA (Kamiya Biomedical Company) following the manufacturer's instructions.

Statistical analysis

Significance between two means was calculated using a two-tailed Mann-Whitney U test. Significance of mouse survival was calculated using a log-rank (Mantel-Cox) test. A *P* value of <0.05 was considered significant. Statistical analyses were performed using Prism 5 (GraphPad Software, Inc.).

Results

Mice fed an iron-deficient diet are anemic with decreased total liver iron.

After 70 days on the iron-deficient diet, mice were anemic (hemoglobin: 12.7 $\pm$ 3.8 g/dL vs. 18.6 $\pm$ 4.1 g/dL,, mean $\pm$ s.d.) and gained significantly less weight as compared to control animals fed the iron-replete diet (Fig.1a,b). In a representative group of 3 mice from each group, total liver iron and ferritin were decreased in mice fed the iron-deficient diet (Fig. 1c,d). Peripheral blood smears of iron deficient mice showed hypochromic RBCs, target cells, anisocytosis, and poikilocytosis, suggesting severe iron deficiency (Fig. 1e,f).

Iron-deficient mice had worse survival with bacterial infection and with co-infection with malaria.

Iron-deficient mice infected with *S. typhimurium* died more rapidly than their iron-replete counterparts (median survival 7 days vs. 16 days; Fig. 2a). Co-infection with malaria significantly worsened survival in both iron-deficient (median survival 5 days vs. 7 days) and iron-replete (median 10 days vs. 16 days) mice (Fig. 2a).

To model the conditions of the Pemba trial[3] and test whether iron supplementation exacerbates infection, mice received daily oral iron supplementation (ferric sulfate, 1 mg/kg) for 4 days prior to infection with *S. typhimurium*, supplementation continued for a total of 10 days. Iron supplementation reduced the median survival of iron-deficient and iron-replete mice by 1 day, however only in iron-replete mice did this reach statistical significance (Fig. 2b).

Infected mice die of severe bacterial sepsis.

At death, the level of bacteria in blood was higher in infected iron-deficient mice (Fig. 3a,b). In 4 of 5 iron-replete mice infected with *Salmonella* alone, no bacteria were detected by blood culture. All *Salmonella*-infected mice had gross hepatosplenomegaly; histologic examination revealed distorted hepatic and splenic architecture, with hepatitis and massive expansion of the splenic red pulp with inflammatory cells. Gram-negative bacilli were observed on tissue Gram stain (Fig. 3c). These findings suggest that the cause of death was organ dysfunction due to hepatic and splenic bacterial load and/or overwhelming sepsis.

Transfusion of older, stored RBCs, exacerbates *S. typhimurium* infection.

To test whether RBC clearance alone is sufficient to exacerbate *Salmonella* infection, mice (5 per group) were infected intraperitoneally with 1000 CFUs of *S. typhimurium* and transfused 350 μL of saline, fresh RBCs, or 14-day stored RBCs. RBCs stored for 14 days had a mean 24-hour PTR of 32% (SD 4.2%; n=5). Bacterially-infected mice transfused with 14-day stored RBCs survived for a median of 4 days, whereas mice transfused with fresh RBCs and non-transfused mice survived for a median of >28 days ($p<0.01$ Log-rank (Mantel-Cox) Test; Fig. 4a). At death, mice transfused older, stored RBCs were severely bacteremic (median $1x10^5$ bacteria/mL blood) as compared to non-transfused mice and those transfused with fresh RBCs (medians of 20 and 0 bacteria/mL blood, respectively). All mice had hepatosplenomegaly at necropsy with evidence of severe bacterial burden in the liver and spleen (not shown).

Discussion

Iron is an essential nutrient for eukaryotes and prokaryotes. In humans, iron deficiency impairs cell proliferation and immune function.[22] In the Pemba substudy, in which the initial iron status of participating children was known by zinc protoporphyrin and hemoglobin assessment, iron deficiency was associated with an increased adverse event rate.[3] The results presented here suggest that, similar to those in the Pemba substudy, iron deficiency is immunosuppressive and results in a worse outcome in both a model mono-bacterial infection and in co-infection with a model malarial parasite. Potential causes for this immunodeficiency that will be explored in future studies include: suppressed or altered cytokine responses in iron deficient immune cells[23] and reduced respiratory burst in iron-deficient macrophages and neutrophils.[24]

Iron restriction is an important host defense mechanism for pathogen killing. In situations of iron overload or hemolysis, the host's mechanisms for controlling iron flux can be overwhelmed and bacteremia may result.[22] The findings from the current study suggest that oral iron supplementation, transfusion of older stored RBCs, and co-infection with a non-lethal malarial parasite each result in exacerbation of bacterial sepsis and accelerated mortality. A packed human RBC unit contains ~250 mg of iron;[25] thus, clearance of up to 25% of a unit, as allowed by the FDA criteria, results in delivery of up to 60 mg of iron to the monocyte/macrophage system. It should also be recognized that 24-hr PTR is often lower than 75%,[26,27] especially in ill patients as opposed to healthy human volunteers.[27,28] Thus, although the 24-hr PTR in the current study was only 32%, some human transfusions result in this amount of RBC clearance,[26] and many patients are rapidly transfused with multiple units resulting in an additive iron load.

These results highlight the complex interaction between host's iron status and bacterial infections. The most common deferral for female blood donors is for a low hemoglobin.[29] This has led some to argue that female blood donors should be iron supplemented.[30-32] Although possibly valuable for maintaining the blood supply, these

iron-repletion strategies must be carefully considered given their effect on iron flux and potential for harm.

Salmonella is a common cause of invasive bacterial infection in children with sickle cell disease.[33] One potential explanation for this propensity for *Salmonella* infections is that proliferation of these bacteria is favored by the chronic hemolysis in sickle cell disease. Similarly, humans with hemochromatosis, an iron overload disorder associated with low intracellular macrophage iron, are predisposed to infections with *Vibrio vulnificus*.[34] Finally, plasma from mice transfused with older, stored RBCs have increased NTBI and support increased proliferation of *E. coli in vitro*.[7] These findings highlight how altering iron flux by various mechanisms can predispose the host to bacterial species that evolved to benefit from specific derangements in iron homeostasis. Thus, a transfused patient may manifest a worsened bacterial infection if a specific ferrophilic bacterial species was involved.

In conclusion, the current murine model of iron status, iron repletion, and bacterial infection provides evidence that iron status (either steady state or altered dynamically by oral iron repletion, co-infection with a malarial parasite, or transfusion of older, stored RBCs) has a significant impact on the outcome of a bacterial infection with a model organism. Most iron in humans is incorporated in RBCs and, although no human studies conclusively document that transfusions of older, stored RBCs are harmful, the current findings highlight the powerful role iron can play in bacterial infection. Future studies will extend these findings to other model pathogens and explore how these organisms use aberrant iron flux to their benefit.

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Figure legends

Figure 1. Weanling mice fed an iron-deficient diet for 70 days become severely iron deficient. Weanling female C57BL/6 mice were randomized to receive an iron-deficient diet or a control, iron-replete diet (n=60 per group). Results are presented as mean ± s.e.m. **(A)** Hemoglobin measured from a submandibular vein bleed every two weeks. **(B)** Mean weekly weight. **(C)** Ferritin measurement from plasma obtained by terminal cardiac puncture from 3 representative mice per group. **(D)** Total liver iron measured at necropsy of 3 representative mice per group. **(E)** Representative image of a peripheral blood smear from an iron-replete mouse. **(F)** Representative image of a peripheral blood smear from an iron-deficient mouse. Blood smears were Wright-Giemsa stained and imaged at an original magnification of 1000x.

Figure 2. Iron deficiency, co-infection with malaria, and oral iron repletion exacerbate infection with *Salmonella typhimurium*. Iron-deficient and iron-replete mice (n=5 per group) were inoculated intraperitoneally with either 1000 CFUs of *S. typhimurium*, strain LT2 or 100,000 *Plasmodium yoelii*, strain 17X parasites, or a combined infection at the same dose. **(A)** Kaplan-Meier survival curves of iron-deficient and iron-replete mice infected with the designated pathogens (n=5 per group). **(B)** Iron-deficient and iron-replete mice (n=5 per group) were either not repleted or repleted with 1 mg/kg of ferrous sulfate by oral gavage. All mice were inoculated intraperitoneally with 3000 CFUs of *S. typhimurium*, strain LT2 and Kaplan-Meier survival curves constructed. Significance was determined with a log-rank (Mantel Cox) test (*P<0.05, **P<0.01).

Figure 3. *Salmonella*-infected iron-deficient mice are more severely bacteremic than control iron-replete mice, and all mice have bacteria-associated organ pathology. (A) Iron-deficient and iron-replete mice (n=5 per group) were inoculated

intraperitoneally with either 1000 CFUs of *S. typhimurium*, strain LT2, or 100,000 *Plasmodium yoelii*, strain 17X parasites, or a combined infection at the same dose. Bacterial levels in blood in mice at death were measured by culturing serial dilutions of a 10 μL blood sample on SS agar and counting black colonies after a 24-hour incubation. Median and interquartile ranges shown. Note, 1 of 5 iron-deficient mice and 4 of 5 iron-replete mice infected with *Salmonella* alone and 2 of 5 iron-replete mice co-infected had no detectable growth on blood cultures. **(B)** In a separate experiment, iron-deficient and iron-replete mice (n=5 per group) were either not repleted or repleted with 1 mg/kg of ferrous sulfate by oral gavage. All mice were inoculated intraperitoneally with 3000 CFUs of *S. typhimurium*, strain LT2 and bacterial levels were measured at death, as above. Note, two iron-replete mice had no detectable bacterial growth and one iron-deficient mouse that was repleted with oral iron had no detectable bacterial growth in blood at death. **(C)** Representative images of histological sections of liver and spleen from iron-deficient and iron-replete mice infected with *S. typhimurium* with or without *P. yoelii* and with or without oral iron repletion, as above. Sections were stained with H&E or with a tissue Gram stain, as indicated. Arrows denote bacteria. Original magnification was 40x for spleen H&E, 400x for liver H&E, and 1000x for both Gram stains. Representative sections derived from 20 necropsies are shown.

Figure 4. Transfusion of older, stored RBCs reduces survival of *Salmonella*-infected mice. Male C57BL/6 mice (8-12 weeks of age) were inoculated intraperitoneally with 1000 CFUs of *S. typhimurium*, strain LT2 immediately followed by transfusion with 350 μL of saline, fresh RBCs, or 14-day stored RBCs. All surviving mice were euthanized 28 days after infection. **(A)** Kaplan-Meier survival curves of infected mice (n=5 per group). **(B)** Bacterial level in blood of mice at death was measured by

culturing serial dilutions of a 50 μL blood sample on SS agar, as above. Median and interquartile ranges shown. Note, blood cultures from one non-transfused mouse and 3 mice transfused with fresh RBCs had no detectable growth. Non-transfused mice had bacterial levels below 1 CFU/μL (thus, none showing on log-scale graph). *P<0.05, **P<0.01.

Figure 1

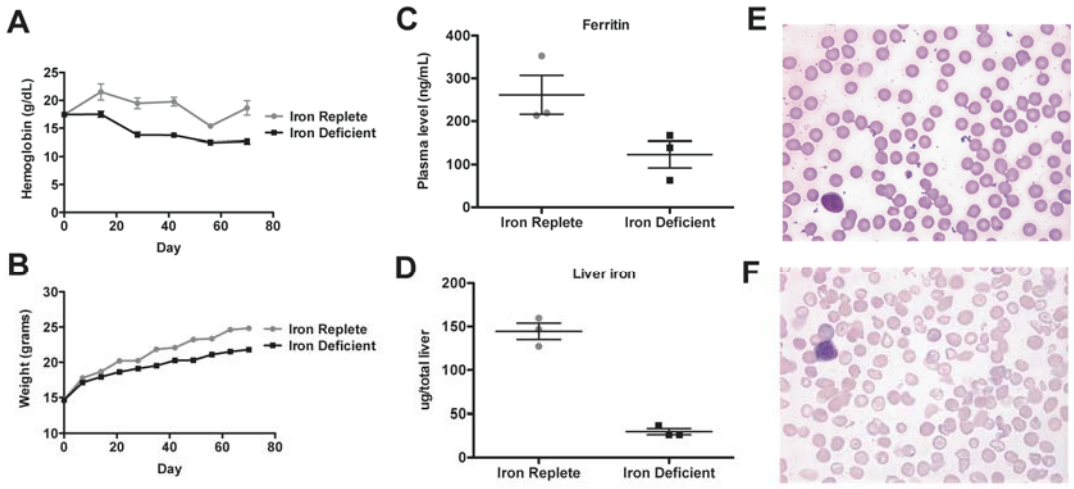
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Figure 2

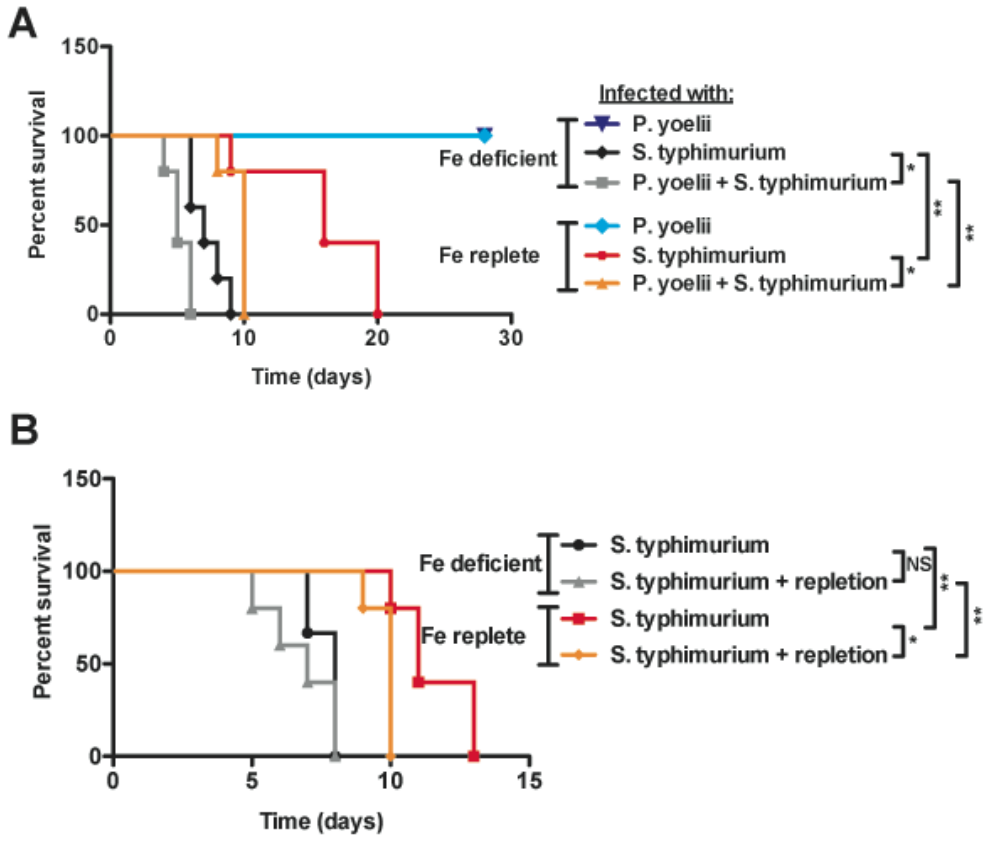
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Figure 3

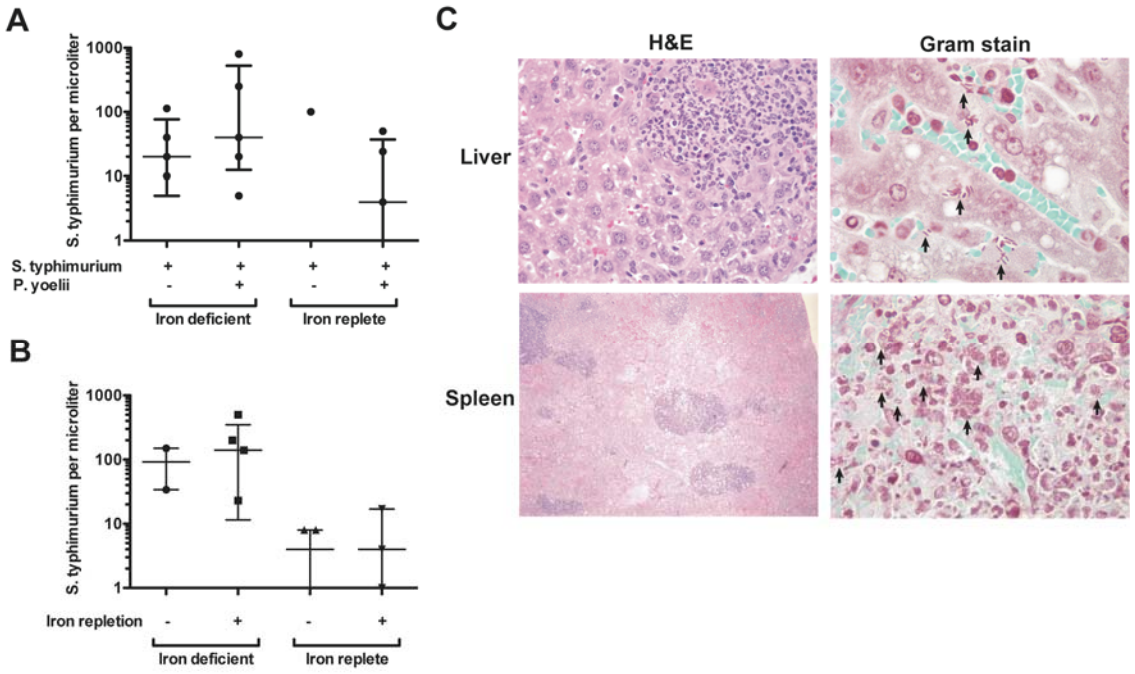
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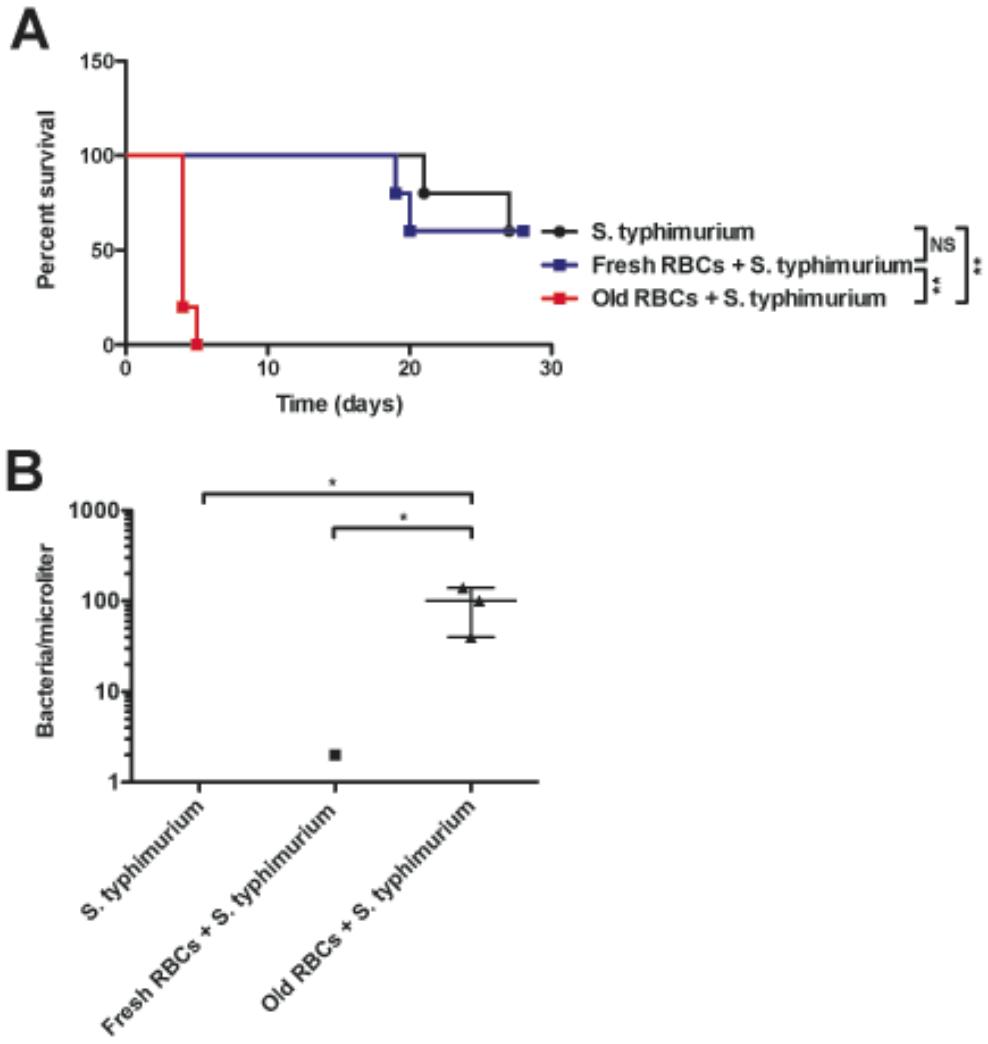
Figure 4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Time (days)
S. typhimurium
Fresh RBCs + S. typhimurium
Old RBCs + S. typhimurium
NS
**
**
B
Bacteria/microliter
1000
100
10
1
*
*
S. typhimurium
Fresh RBCs + S. typhimurium
Old RBCs + S. typhimurium