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# Evaluation of interleukin-3 in blood-stage immunity against murine malaria *Plasmodium yoelii*

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## Abstract

Malaria is a mosquito-borne infection caused by the parasitic protozoan *Plasmodium*. This disease infects over 200-300 million people and causes nearly 400,000 deaths every year. Our laboratory previously examined malarial infection caused by *Plasmodium berghei* NK65, a lethal rodent strain that induces symptoms similar to those observed in humans. The results of this previous study indicated that the hematopoietic growth factor and immunoregulatory cytokine interleukin-3 (IL-3) suppressed protective immunity against infection with *P. berghei* NK65. However, the extent to which IL-3 contributes to host defense against blood-stage malaria infection caused by other *Plasmodium* species remains to be determined. In the present study, we have examined the roles of IL-3 in host defense against blood-stage malaria by using IL-3-deficient or “knockout” (KO) mice infected with either nonlethal *P. yoelii* 17XNL or lethal *P. yoelii* YM parasites. Survival and parasitemia were measured to monitor the course of infection in wild-type (WT) and IL-3 KO mice. We also characterized parameters indicative of a protective immunity, such as the development of splenomegaly and parasitized red blood cell (pRBC) levels. Surprisingly, we found that IL-3 did not significantly alter the normal clinical course of infection caused by these two *Plasmodium* strains. These studies indicate that the ability of IL-3 to influence blood-stage malaria infection is dependent on the particular *Plasmodium* species used to infect animals. These studies also suggest that *Plasmodium* species may differ in their ability to stimulate IL-3 production and/or differ in their ability to induce a type of host immune response that is IL-3 dependent.

## Introduction

### Malaria introduction

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium*. In humans, there are four major species of *Plasmodium* that cause infection: *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae*. According to the World Health Organization (WHO), in 2015, the human-infectious *Plasmodium* species were responsible for nearly 300 million cases worldwide, with over 400,000 of these cases resulting in death (World Malaria Report, 2015). WHO thus ranks malaria infection as one of the biggest threats to global public health. This is in spite of decreased mortality rates, which have dropped by 47% since 2000. This progress has occurred due to the advent of antimalarial medicines and widespread multinational government investment in this disease. However, the lack of an effective vaccine as well as reports of widespread drug and insecticide resistance threaten the progress already achieved.

Throughout all species of malaria, the transmission and life cycle of the parasite are largely preserved (reviewed in Halder *et al.*, 2007). The parasites are initially transmitted as spindle-shaped sporozoites in the salivary fluids of the female *Anopheles* mosquito as the mosquito bites the host and takes a blood meal. Once inside the body, the highly motile parasites travel through the bloodstream, and into the liver within an hour of transmission. This begins what is known as the exoerythrocytic cycle, which takes place outside of the bloodstream and within hepatocytes.

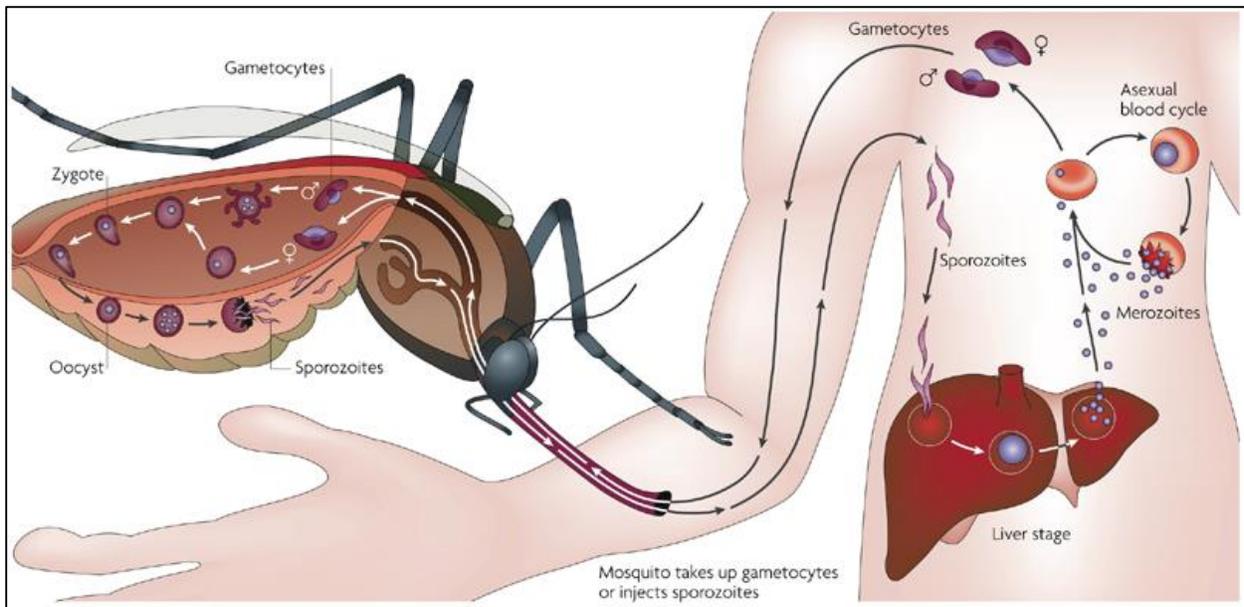
Following entry into the liver, the parasites divide asexually in parenchymal cells until the cell bursts and releases merozoites into the bloodstream. Each species of *Plasmodium* is unique in the time required to perform this division and each species

produces a varying number of progeny from each cell. The disease is further complicated in some species like *P. vivax* and *P. malariae*, where the parasites can remain dormant within the liver cells as hypnozoites. These hypnozoites are delayed in their development, which can lead to a relapse in the patient up to five years later. Once released from the liver parenchymal cells, the merozoites enter the circulation and infect red blood cells (RBCs), beginning what is known as the erythrocytic or blood-stage cycle of malaria infection (Figure 1).

Within RBCs, the parasites grows into a trophozoite, which often has a signature ring-like appearance sometimes called a “signet ring”. The parasite will continue to expand until it fills most of the cell. At this point the parasite undergoes asexual division into numerous merozoites. The merozoites are surrounded by a membrane and the entire structure is known collectively as an erythrocytic schizont. Within the RBC these parasites will feed on hemoglobin. The cycle is complete when the RBC ruptures and releases these merozoites, which proceed to invade other RBCs. Some merozoites may diverge from the cycle at this stage and develop into gametocytes that are then taken up by a mosquito so that the life cycle can continue in a new host. A generalized depiction of the *Plasmodium* life cycle is shown in Figure 1.

Both macrogametocytes and microgametocytes develop from the merozoites that invade RBCs. These sexual forms are ingested by the mosquito during a blood meal, and can only complete their development within the gut of the *Anopheles* mosquito. Additionally, this is the only instance of sexual reproduction for the malaria parasite. After entering the gut of the mosquito, both gametocytes shed their erythrocytic

**Figure 1.** *Plasmodium* life cycle (Su *et al.*, 2007).



membranes and the male gametocyte immediately begins to extend up to eight flagella-like gametes that can each fertilize a separate macrogametocyte. Each resulting zygote will penetrate the gut wall to lie under the basement membrane, where it will develop into an oocyst. Each oocyst is capable of developing into up to 1,000 sporozoites. These sporozoites mature over the next 10-14 days before escaping the oocyst and moving up into the salivary glands of the mosquito. When the mosquito takes another blood meal, the sporozoites will be injected into a new host.

Clinical aspects of the disease occur largely as a result of the blood-stage cycle and the rupture of RBCs. In addition to the expression of anemia, ruptured RBCs release pyrogenic compounds into the blood which result in the generation of a fever by the host. Chills and sweating often will subsequently follow. The specific disease symptoms and severity vary according to the *Plasmodium* strain but generally include headache, flu-like illness, splenomegaly, and muscle pain (Despommier *et al.*, 2005). Patients may also complain of nausea, vomiting, and diarrhea. There is some evidence that these host symptoms work to increase the attractiveness of the host to the mosquito vector, thus increasing the chance for further transmission (Nacher, 2005). For example, individuals experiencing a fever may have flushed skin and will bare more of their skin in order to cool down. These individuals may also have an increased respiration rate and will exhale more CO<sub>2</sub>, which is an attractant for mosquitos (Nacher, 2005).

Severe malaria infection caused by some species of *Plasmodium* such as *P. falciparum* is often characterized by the manifestation of cerebral malaria. This occurs as parasitized RBCs (pRBCs) travel through the capillaries of the brain and adhere to the endothelium, blocking the blood flow through that channel and promoting

inflammation at these areas through recruitment of immune cells to those sites. The appearance of histidine-rich protein “knobs” on the surface of pRBCs enables these cells to bind more tightly to the epithelium but are not necessarily required for adhesion (Wahlgren *et al.*, 1999). In many endemic areas, adult inhabitants develop a resistance to severe malaria symptoms, leaving children between six months and five years at the highest risk for developing these symptoms. Cerebral malaria in these cases is responsible for the majority of malaria-related deaths (Halder *et al.*, 2007).

Cytoadhesion may also occur within other organ systems, including the lungs, gastrointestinal (GI) tract, skin, and placenta of pregnant women. Lung sequestration may lead to respiratory distress, pulmonary edema, or bronchopneumonia in populations of all ages (Halder *et al.*, 2007). Placenta sequestration often leads to an increased rate of fetal mortality, fetal wastage, and low birthweight. This also occurs in mouse models of infection. For example, early infection of mice with *P. berghei* ANKA lead to an increased rate of abortion, while late infection led to symptoms similar to those found in human infection (Zuzarte-Luis *et al.*, 2014).

### **Mouse models of malaria**

Mouse models of malaria infection have made significant contributions to our understanding of the biology of both the parasite and host immunity. Studying human-infectious malaria species as they occur naturally in humans is often difficult, not only due to the inability to control all variables in these studies but also because many times these studies lack access to tissue, organ, and/or blood samples. Additionally, mechanistic studies of lethal malarial disease is often limited or unfeasible within human subjects. This makes additional approaches using *in vitro* or *in vivo* animal models both

appropriate and necessary. Mice are particularly suited to this role for several key reasons. The genome between humans and mice is largely conserved (Pennacchio, 2003). Mice provide an easily-accessible source of organ, blood, and tissue samples and can thrive in a controlled environment manipulated by the experimenter.

Additionally, mice are small and can be housed socially, making them an attractive economic option. Rapid gestation periods and large litter sizes in these species also provide a large pool of possible subjects.

Four species of *Plasmodium* are typically used to infect laboratory mice: *P. vinckei*, *P. yoelii*, *P. chabaudi*, and *P. berghei*. Each of these strains have different substrains that lead to a distinct immunopathology, developmental biology, and ability to cause a lethal outcome in mice (Zuzarte-Luis *et al.*, 2014). The outcomes of infection also vary widely according to both the parasite and mouse strain utilized, which has led to some investigators to question the applicability of mouse models to human immunity and disease (Langhorne *et al.*, 2011). However, this variety of outcomes in mice can also be seen as a reflection of the diversity found within human malarial disease and can therefore be seen as an arguable strength.

Due to the variability of symptoms found within the different combinations of both parasite and mouse strains, researchers often select their model based on their particular interest. For instance, *P. berghei* ANKA causes severe and fatal accumulation of inflammatory cells within the brain of C57BL/6 mice; these mice are most commonly used in studies concerning cerebral malaria. Another substrain *P. berghei* NK65 has provided key insights into the occurrence of malaria-associated liver and lung injury, blood-stage infection, and placental malaria (Halder *et al.*, 2007). The *P. vinckei vinckei*

strain is uniformly lethal in all mice and closely mimics the clinical symptoms and sequestration seen in many cases of human malaria (Li *et al.*, 2001). Strains like *P. yoelii* 17XNL and *P. chaubaudi chaubaudi* are most commonly used to study chronic malaria infection and immune mechanisms due to their nonlethal nature in most mice strains (Li *et al.*, 2001).

Among rodent *Plasmodium* parasites, *P. yoelii* family has proven crucial to providing an understanding of a wide variety of malarial symptoms and processes. *P. yoelii* YM is a highly lethal strain of malaria that has been used in mice to mimic the effect of *P. falciparum*. This strain is commonly used in laboratories to study possible vaccine targets and the appearance of hypoglycemia commonly associated with severe *P. falciparum* infection (Elased & Playfair, 1994; reviewed in Halder *et al.*, 2007). Another strain, *P. yoelii* 17XNL is often utilized for more extensive immunological studies (Khan & Vanderberg, 1991). The non-lethal nature of this strain allows the study of all stages of infection more closely as the mice develop and then overcome parasitemia. In particular, this strain has been repeatedly used to study sporozoite-induced infection during the exoerythrocytic stage (reviewed in Halder *et al.*, 2007; Khan & Vanderberg, 1991). This strain most closely mimics human infection, where an immune response is not mounted by the host until the hepatic schizonts burst and merozoites are released into the bloodstream.

*In vitro* studies of malarial infection are most commonly used to study molecular-level dynamics between certain host tissues and the malaria parasite. For example, the ability of several pantropical leaf and bark extracts to inhibit the growth of erythrocytic *P. berghei* was measured via *in vitro* studies (François *et al.*, 1997). These plants are

commonly used in endemic countries as herbal remedies for fever and malaria. Many of these plant species showed considerable antiplasmodial properties and are being investigated as a possible alternative to current antimalarial medicines, which are quickly becoming obsolete through parasite resistance.

Studying malarial infection *in vivo* is particularly valuable for gauging whole-organism responses and studying complex systems within the body. The mammalian immune response is one such system. *In vivo* studies in mouse models are generally performed through two means: through infection with mosquito-derived sporozoites or through intraperitoneal (i.p.) injection of pRBCs. Sporozoites first participate in the exoerythrocytic stage of infection and enable researchers to study this stage of infection, which is often a target of possible vaccines. Inoculation of mice with viable *P. berghei* NK65 sporozoites, for example, has been shown to produce some protective immunity against sporozoite infection from *P. yoelii* 17XNL, to which mice are nearly 2000 times more susceptible to (Vanderberg *et al.*, 1993). This study and others like it have been invaluable to understanding the exoerythrocytic cycle of malaria and the host immune response to mosquito-derived infection.

Mouse models have also been invaluable in furthering our understanding of blood-stage malaria infection. Blood-stage infection typically is induced experimentally through injection of pRBCs into the intraperitoneal cavity of the mouse. pRBCs are taken up into the blood directly, bypassing any liver involvement. This allows researchers to study the physical effects of the erythrocytic cycle directly, which produce all of the deleterious clinical manifestations associated with malaria infection. Although genetics clearly plays a role in susceptibility to murine malarial infection (Longley *et al.*, 2011), understanding

how the underlying mechanisms within an immune response come together can only be understood at the organism level.

### **Interleukin-3 and malaria**

The protein interleukin-3 (IL-3) is a part of a vast system of cytokines produced by the immune system in response to numerous types of infection. IL-3 was initially proposed to play an important role in regulating the proliferation and differentiation of hematopoietic cells (Donahue *et al.*, 1988). Since then, IL-3 has also been indicated to play a role in the migration and effector function of many of these hematopoietic cells (Broughton *et al.*, 2012). *In vitro* studies of this protein have reported that IL-3 is expressed primarily by activated CD4 T cells and mast cells, and to a lesser extent by basophils, eosinophils, NK T cells, and endothelial cells during infection (Schrader, 2003). IL-3 is amongst the first cytokines to be expressed following infection and has been shown to stimulate the differentiation of myeloid cells; these develop into granulocytes, dendritic cells, and macrophages (Broughton *et al.*, 2012). However, despite its apparent therapeutic potential in bone marrow transplants, this cytokine also causes the stimulation of basophils and mast cells (Lantz *et al.*, 1998), which under the right circumstance would be undesirable in those suffering from allergic reactions.

It is important to note that most of the attributes attributed to IL-3 have been based on *in vitro* studies and may not reflect what is occurring *in vivo*. For example, relatively few *in vivo* studies have been completed with IL-3-deficient (knockout [KO]) mice. An *in vivo* study using BALB/c IL-3 KO mice has shown that these mice did not have hematopoietic abnormalities under normal physiological conditions (Mach *et al.*, 1998). This study supports the idea that IL-3 functions are dispensable under baseline

conditions *in vivo*; this is likely because IL-3 is not produced constitutively under physiological conditions and is not essential to the regular steady-state maintenance of the body (Schrader, 2003). However, several studies have shown that IL-3 is expressed and plays a significant role during certain disease conditions. These cells make up a large part of the innate immune response to infection. Past experiments have shown that IL-3 KO mice have impaired contact hypersensitivity responses to haptens (Mach *et al.*, 1998) and mount attenuated mast cell and basophil responses to gastrointestinal nematode infection that result in compromised worm expulsion (Lantz *et al.*, 1998). IL-3 has also been shown to promote recovery from acute herpes simplex virus infection in mice (Chan *et al.*, 1990). Thus, depending on the disease condition, IL-3 can either promote or inhibit the development of an appropriate immune response.

Since the initial description of IL-3, only a limited number of reports have suggested a potential role of IL-3 in the pathophysiology of malaria. In 1998, increased serum levels of IL-3 in mice infected with *P. chaubaudi* were reported during the peak of infection (Helmbj *et al.*, 1998). The increase in levels of serum IL-3 promoted production of IL-4 and IL-6, which in turn supported the switch from a Th1 to a Th2 response; this Th2 response is vital for final parasite clearance from the blood. Thus, stimulation of immune cells by IL-3 is in this case beneficial to malarial parasite clearance. Alternatively, in *P. berghei*-infected CBA mice, administration of anti-IL-3 and anti-GM-CSF antibodies prolonged survival of these mice to nearly three weeks and caused a significant decrease in the occurrence of cerebral malaria (Grau *et al.*, 1988). Additionally, rises in serum TNF levels were prevented and macrophage accumulation in the spleen was reduced significantly. This study suggests that IL-3 and GM-CSF play

a detrimental role in the development of cerebral malaria in mice, which causes the majority of early mortalities during infection. More recently, a non-synonymous single nucleotide polymorphism (snSNP) of the human IL-3 gene on chromosome 5q31-33 was reported to be associated with risk of recurrent malaria attacks caused by *P. falciparum* (Meyer *et al.*, 2011). This study found that certain variants of the human IL-3 gene led to a more protective effect against malarial infection by *P. falciparum*. This supports the argument that IL-3 plays a critical role in the pathophysiology of human-infectious malaria and should thus be further studied within the murine model.

Our laboratory has recently reported on the ability of IL-3 to influence the course and outcome of disease in mice acutely infected with the lethal malaria strain *P. berghei* NK65 (Auclair *et al.*, 2014). IL-3 KO mice infected with this strain survived for significantly longer than wild type (WT) mice, despite both groups developing similar physical symptoms at around day seven. This difference in survival rate is correlated with the parasitemia observed during infection; IL-3 KO mice experienced significantly lower levels of parasitemia than did their WT counterparts (Auclair *et al.*, 2014). *P. berghei* NK65-infected IL-3 KO mice also displayed increased splenomegaly and had markedly reduced levels of serum interferon- $\gamma$  (IFN- $\gamma$ ) (Auclair *et al.*, 2014). These findings may be due to an enlarged splenic erythroid compartment in IL-3 KO mice, which is a major site of pRBC clearance and immune system activation. Additionally, the IL-3 KO mice displayed a more severe anemia at days 8 and 10 of infection although the reasons behind such a severe anemia in these mice is unclear (Auclair *et al.*, 2014). This anemia may represent an increase in the destruction of pRBCs and/or may be indicative of a greater rate of phagocytosis or antibody-mediated opsonization

by the host. Overall, this report indicates that IL-3 plays a critical role in suppressing protective immunity to *P. berghei* NK65. Further studies using this IL-3 KO model should be performed in order to more fully elucidate the role of IL-3 in malarial infection.

### **Project goals and specific aims**

Our laboratory has previously examined the role of IL-3 in mice acutely infected with *P. berghei* NK65. We found that IL-3 KO mice were more resistant to blood-stage malaria than were corresponding WT mice (Auclair *et al.*, 2014). Thus, it can be concluded that IL-3 functions in some way to decrease resistance to lethal infection with *P. berghei* NK65. However, the extent to which IL-3 contributes to host defense against blood-stage malaria infection caused by other lethal *Plasmodium* species remains to be determined. Furthermore, the role of IL-3 in chronic malaria infection has not been explored. My thesis project was devoted to answering these two major questions.

**Aim 1: Assess the extent to which IL-3 influences the course and outcome of disease in an experimental model of chronic malarial infection caused by *P. yoelii* 17XNL.**

To investigate the effects of IL-3 in chronic malaria infection caused by *P. yoelii* 17XNL, groups of WT and IL-3 KO mice were infected i.p. with  $10^5$ - $10^6$  pRBCs. For all experiments, groups of 5-7 mice of each genotype were used. Changes in levels of parasitemia were determined primarily through manual counts of parasitemia on blood smears stained with Giemsa, and were recorded as a percentage of pRBCs to total RBCs. Blood was taken and parasitemia recorded on every second to third day until the infection is cleared. The total body weight before and after infection was also recorded on a weekly basis along with measures of total white blood cells (WBCs). Mice typically

begin to lose weight during infection as internal reserves are depleted in order to fight the parasite and mice become too lethargic to continue to eat or drink. The degree of anemia was determined by hematocrit.

In separate experiments, additional groups of infected IL-3 KO and WT were sacrificed at various days post-infection prior to their clearance of parasites. These mice were used to assess the degree of splenomegaly and to obtain larger volumes of blood necessary to collect serum. As time allows, the serum collected from these mice will be used to determine if infected IL-3 KO and WT mice show differences in levels of IgA, IgG1, IgG2a, IgG2b, IgG3, and IgM antibodies. Antibody quantification will be performed using an antibody isotyping multiplex assay.

As time allows, the ability of these mice to resist a secondary infection will also be examined. For these studies, previously infected IL-3 KO and WT mice will be infected 60-90 days after the primary infection and the levels of anemia and parasitemia analyzed as described above. Antibody levels in these mice will also be examined three weeks following the secondary infection.

A group of uninfected mice were also sacrificed in a separate experiment. All data sets from infected mice were then compared to these values. This was done in order to provide controls or baseline values for all collected data.

**Aim 2: Assess the extent to which IL-3 influences the course and outcome of malarial disease in an experimental model of acute infection caused by *P. yoelii* YM.**

*P. yoelii* YM has been used formerly in mice to study possible protein targets for vaccine development (Narum *et al.*, 2000), and like *P. berghei* NK65, causes a lethal

blood-stage malaria infection in mice. We first performed mortality studies to determine if WT and IL-3 KO mice differ in their response to infection as we have previously seen in *P. berghei*-infected mice. All experiments took place with 5-7 mice of each genotype. Mice were infected i.p. with  $10^4$ - $10^5$  pRBCs and monitored daily until the mice succumbed to infection.

Additional experiments were also performed in which both infected IL-3 KO and WT mice were sacrificed at a time point just prior to when either genotype suffers significant mortality. These mice were used to characterize those parameters indicative of a protective host response to infection: the development of splenomegaly, parasitemia, RBC levels, and degree of weight loss.

A group of uninfected mice were sacrificed in a separate experiment. All data sets from infected mice were compared to the values generated from these mice. This was done in order to provide controls or baseline values for all collected data.

## Materials and Methods

### **Mice**

The production of BALB/c IL-3-deficient (knockout [KO]) mice and their phenotypic characteristics have been described in detail (Auclair *et al.*, 2014). IL-3 KO mice were bred at the Biology Department at James Madison University. Genotypes were verified by PCR of tail tissue. Age- and sex-matched BALB/cJ wild-type (WT) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Alternatively for some experiments, WT mice were bred at the Biology Department at James Madison University. All mice were between 8-13 weeks of age at the beginning of the experiments and were provided food and water *ad libitum*. Mice were housed in a positive, individually ventilated caging system (Allentown Inc., Allentown, NJ) under a 14-hr/10-hr light/dark cycle in the Department of Biology's animal facility. All mice were used and maintained in accordance with James Madison University's Institutional Animal Care guidelines.

### **Plasmodium parasites**

Cryopreserved parasitized red blood cells (pRBCs) were obtained from the American Type Culture Collection (ATCC). These parasites include *Plasmodium yoelii* 17XNL (MRA-593, MR4, ATCC® Manassas Virginia, contributed by Daniel Carucci), *Plasmodium yoelii yoelii* 17X (MRA-749), *Plasmodium yoelii yoelii* YM (MRA-755), and *Plasmodium vinkei vinkei* V67 (MRA-824, MR4, ATCC® Manassas Virginia, contributed by David Walliker). The parasites were propagated by intraperitoneal (IP) injection of

the parasite in uninfected 'incubator' mice. All strains were maintained by serial blood passage in mice for no more than 4 passages before being discarded.

### **Plasmodium infection and evaluation of disease**

Experimental mice were infected i.p. with  $10^3$ - $10^6$  pRBCs in PBS. Typically, blood used for experimental injections contained 15-35% pRBCs. Mice were monitored daily during the infection. Typical symptoms observed during the course of infection included ruffled fur, hunching posture, and limited mobility. For both the non-terminal and terminal evaluation of anemia using hematocrit, blood was collected in a heparinized tube from the retroorbital sinus while under gas anesthesia. Plasma or serum was collected and stored immediately after at  $-80^{\circ}\text{C}$ . Parasitemia was quantified via light microscopy of Giemsa-stained thin blood-smears. Approximately 300-500 RBCs were evaluated per slide. When monitoring parasitemia levels over the course of infection, multiple takings of tail blood were used instead of retroorbital blood. Spleens from uninfected control mice and from mice infected at various days post infection (p.i.) were weighed. Splenic index was calculated based on spleen weight (g)  $\times$  total body weight (g)  $\times$  1000. Mice used in mortality experiments were observed daily at roughly the same time each day.

### **Statistical analysis**

All data is presented as the mean of the data  $\pm$  the standard error (SEM). Survival curves were compared using a Mantel-Cox (log-rank) test. An unpaired t-test was used to compare one variable between two groups. A significance level of  $P < 0.05$  was used

for all comparisons. All analyses were completed using GraphPad Prism or Microsoft Excel software programs.

## Results

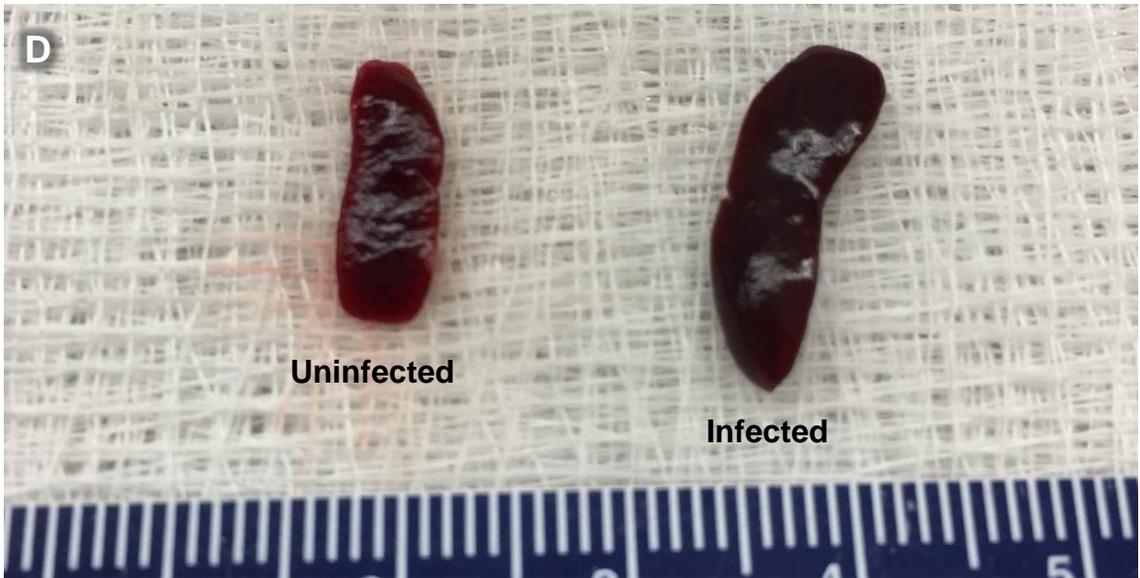
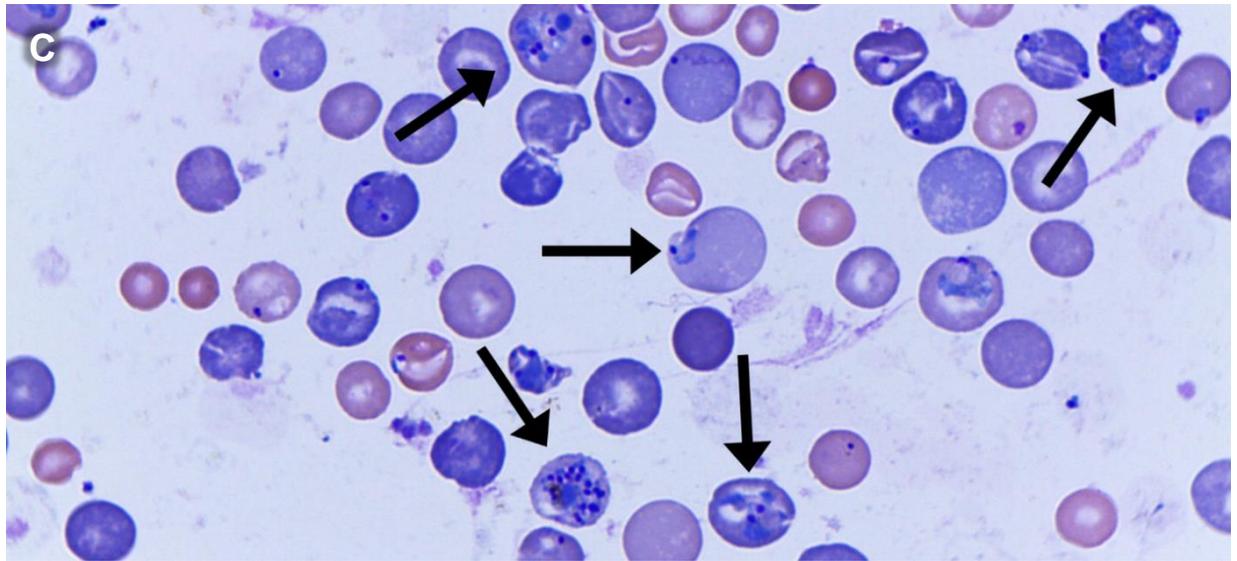
IL-3 is a cytokine produced primarily by activated T cells and mast cells and plays a role in the proliferation, differentiation, migration, and effector function of several immune cells (Donohue *et al.*, 1988). A past study completed by our laboratory reported on the ability of IL-3 to influence the course and outcome of disease in mice infected with the lethal malaria strain *P. berghei* NK65 (Auclair *et al.*, 2014). IL-3 KO mice infected with this strain possess on average a larger splenic index, decreased parasitemia, and significantly prolonged survival as compared to similarly infected WT mice. This indicates that IL-3 plays a critical role in suppressing protective immunity to *P. berghei* NK65. However, further research is still needed using the IL-3 KO model to more clearly resolve the role of IL-3 in *Plasmodium* infection.

A successfully implemented experimental malarial infection causes infected mice to exhibit a scruffy, unkempt appearance whereas healthy mice are generally fastidious groomers. Infected mice also become lethargic and exhibit a hunched posture (Figure 2A and B). Infection is indicated by the invasion of *Plasmodium* merozoites into the host's RBCs, as depicted in Figure 2C. In a typical blood-stage malarial infection, mice experience several systemic symptoms caused by the rupture of RBCs and entry of pyrogens into the bloodstream. These symptoms manifest physically in the mice in several ways. Infected mice also experience an enlarged spleen or splenomegaly as shown in Figure 2D.

### **Chronic (Non-lethal) Malarial Infection**

**Parasitemia in *P. yoelii* 17XNL-infected male IL-3 KO and WT mice.** In order to understand the role of IL-3 in chronic malarial infection, we first needed to understand

**Figure 2.** Representative images of uninfected and infected mice. Representative images of (A) an uninfected and (B) *P. yoelii*-infected BALB/c mouse at day 8 p.i., (C) peripheral blood from a *P. yoelii*-infected BALB/c mouse at day 19 post infection (arrows indicate pRBCs), and (D), spleens from an uninfected and *P. yoelii*-infected BALB/c mouse at day 8 post-infection.

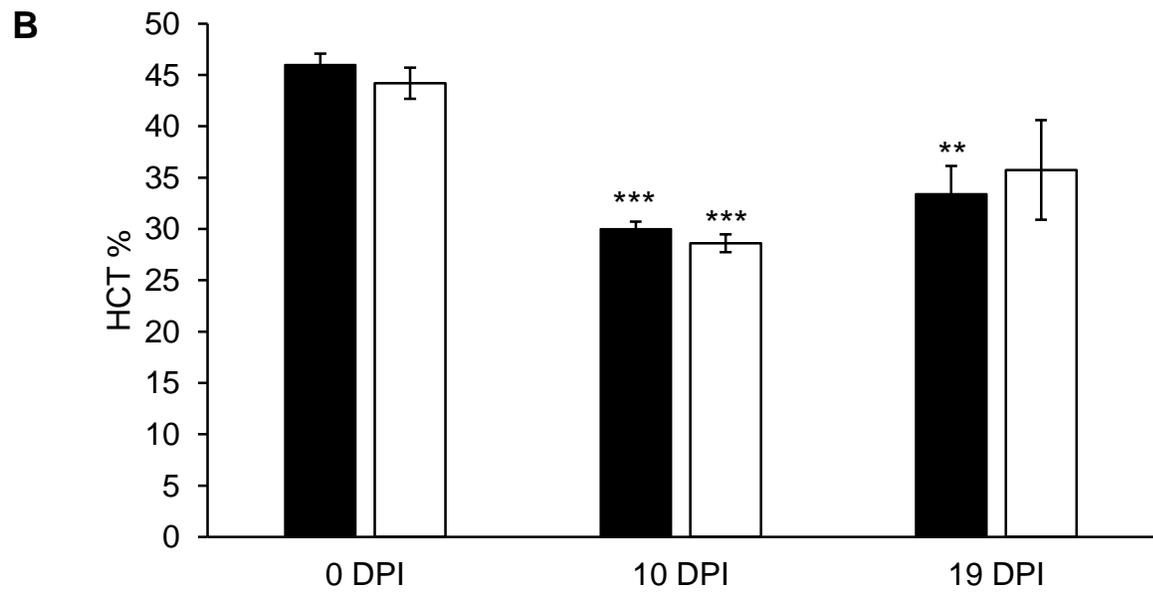
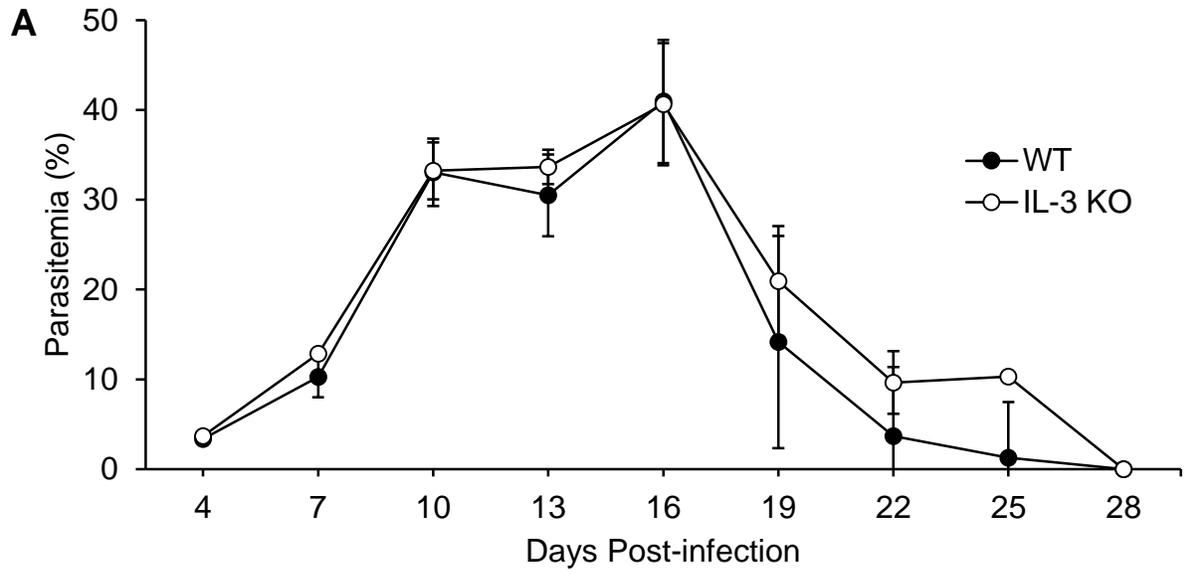


the length of time the parasite is able to persist within the blood-stage cycle before being cleared by the host. Groups of male WT and IL-3 KO mice ( $n = 5$  of each genotype) were infected i.p. with  $10^6$  pRBCs of *P. yoelii* 17XNL. Mice were then monitored daily for physical signs of distress and the percent parasitemia was recorded every 3 days. Blood for calculating the percent parasitemia was taken via tail snips. Twice during the infection on days 10 and 19 p.i., hematocrits were obtained using blood collected from the retro-orbital sinus in order to determine the extent of anemia.

Infection with non-lethal *P. yoelii* strains has been shown to preferentially invade reticulocytes (Fahey *et al.*, 1984), and this was observed in both WT and IL-3 KO mice (Figure 2C). Both genotypes began to exhibit a parasitemia around day 4 p.i., and parasitemia levels continued to climb until day 16 p.i., where both groups of mice exhibited an average parasitemia of ~41%. After day 16 p.i., both genotypes exhibited a steadily declining parasitemia until day 28 p.i., when both groups cleared the infection and no parasites were seen in the blood (Figure 3A). Both WT and IL-3 KO mice began to exhibit a rough hair coat at day 6 p.i. All mice appeared prostrate and lethargic from day ~7 p.i. through day ~22 p.i. (data not shown). Hematocrits taken from these mice at days 10 and 19 p.i. showed that infection did induce anemia, however, there was no significant difference between infected WT and IL-3 KO mice at either point in time (Figure 3B).

**Parasitemia, anemia, and splenomegaly in *P. yoelii* 17XNL-infected IL-3 KO and WT mice as measured on day 8 p.i.** In order to evaluate the degree to which IL-3 influences splenomegaly, we infected male WT and IL-3 KO BALB/c mice ( $n = 6$  for each genotype) with  $10^5$  pRBCs of *P. yoelii* 17XNL and sacrificed them on day 8 p.i.

**Figure 3.** Parasitemia and anemia in mice infected with  $10^6$  *P. yoelii* 17XNL pRBCs. (A) Time course of parasitemia in male WT and IL-3 KO BALB/c mice infected with  $10^6$  *P. yoelii* 17XNL pRBCs. No significant difference in survival between the genotypes were observed ( $P > 0.05$ ). (B) Hematocrit values of uninfected and *P. yoelii*-infected WT and IL-3 KO mice at days 0, 10, and 19 p.i. (labelled as days post infection or DPI in figure). All data are presented as means  $\pm$  SEM from 5 mice per group analyzed individually.  $P < 0.001$  (\*\*\*) and  $P < 0.01$  (\*\*) versus corresponding values for uninfected mice.



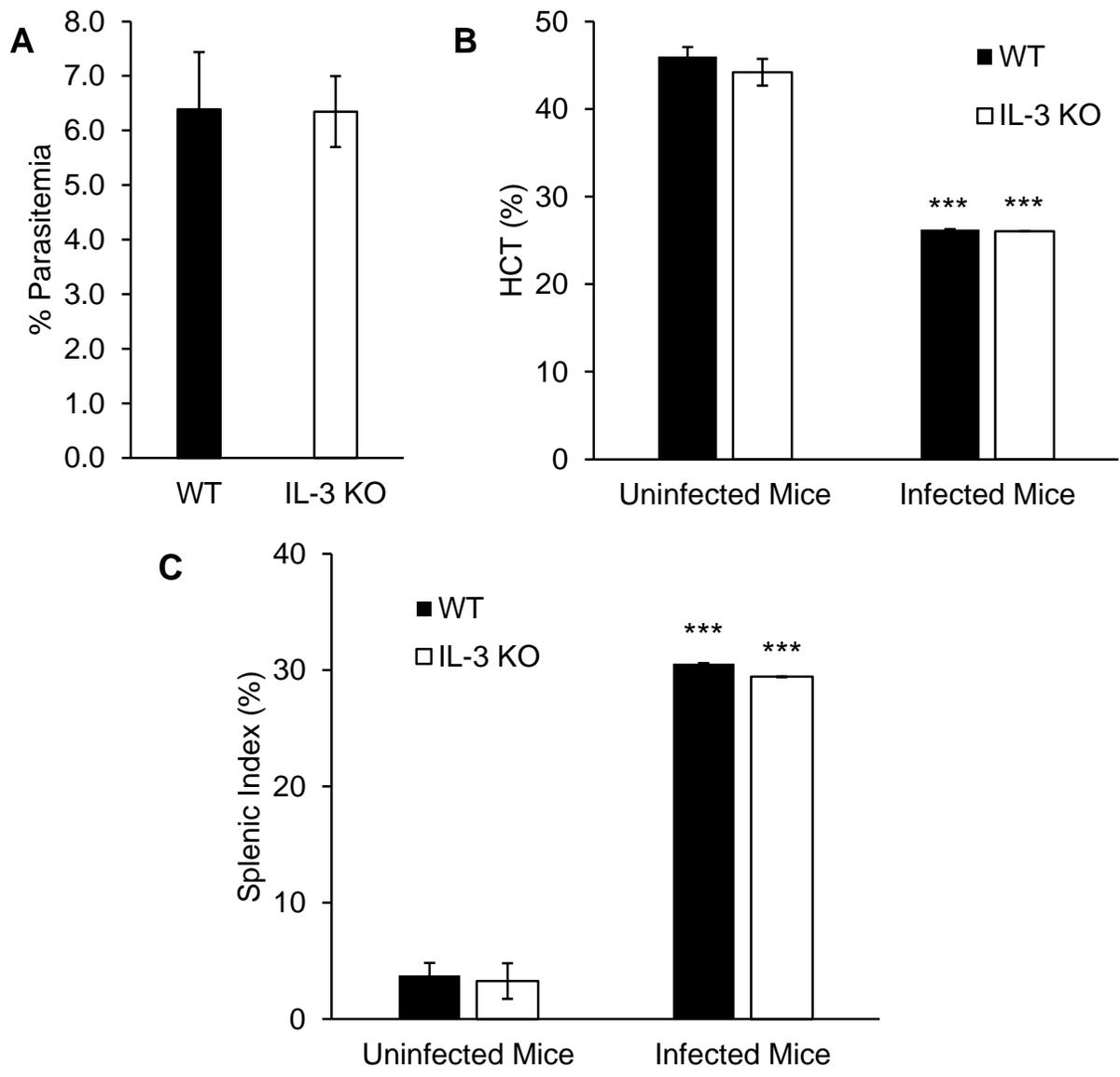
Day 8 of infection is ideal for such a sacrifice because mice have begun to experience the outward symptoms of infection but have only just started to produce antibodies against the parasite. Thus, the mice are primarily mounting an innate immune response against infection. It is noteworthy that around this day, mice infected with lethal strains of malaria begin to succumb to infection. Both chronically infected WT and IL-3 KO mice exhibited a similar parasitemia of 6.3% at day 8 p.i. (data not shown). As expected, both genotypes were significantly more anemic than their uninfected counterparts by day 8 p.i. (Figure 4B) and both genotypes possessed a significantly enlarged spleen (Figure 4C).

### **Acute (Lethal) Malarial Infection**

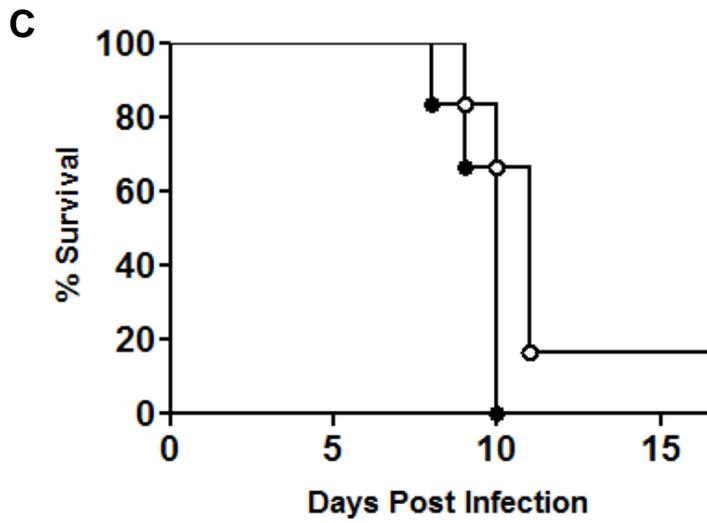
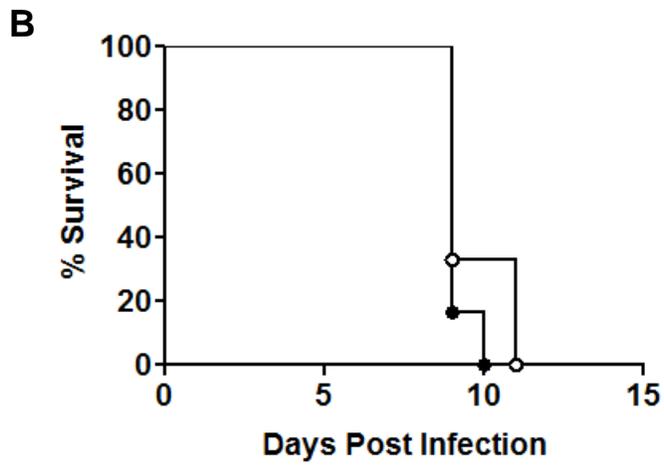
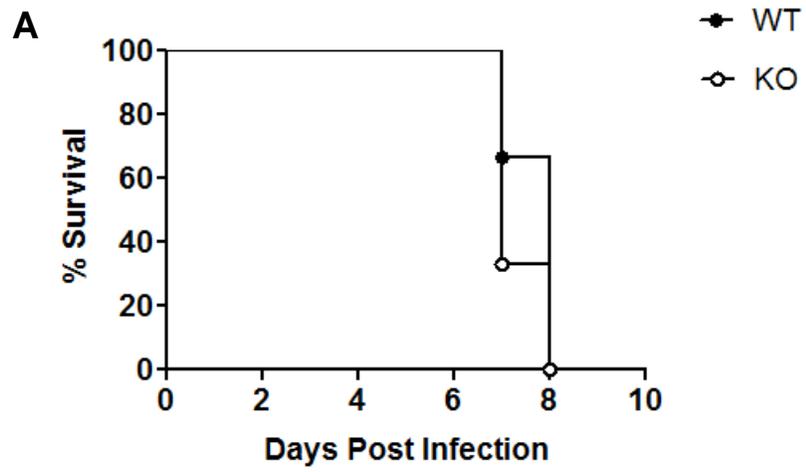
**Mortality rates of *P. yoelii* YM-infected male IL-3 KO and WT mice.** Mice infected with *P. yoelii* YM typically do not clear the infection and usually die between days 6-15 p.i. In order to determine if IL-3 plays a role in prolonging survival of these mice, male WT and IL-3 KO BALB/c mice ( $n = 6$  of each genotype) were infected intraperitoneally with *P. yoelii* YM pRBCs. Mice were then monitored daily for physical signs of illness or death.

Three separate experiments were performed to examine the survival of WT and IL-3 KO mice infected with varying numbers of pRBCs. Mice infected with  $10^5$  *P. yoelii* YM pRBCs survived 7 to 8 days p.i., (Figure 5A). Around day 6 p.i. both genotypes experienced some lethargy and rough hair coats but no other outward symptoms of advanced malarial infection were observed. Mice infected with  $10^4$  *P. yoelii* YM pRBCs survived 9 to 11 days p.i. (Figure 5B). Between days 7 and 8 p.i., mice declined quickly and were observed to have advanced malarial symptoms, including lethargy, prostrate

**Figure 4.** Parasitemia, anemia, and splenomegaly in mice infected with  $10^5$  *P. yoelii* 17XNL pRBCs. Percent parasitemia, hematocrit (HCT) values, and splenic index in male WT and IL-3 KO BALB/c mice at baseline and/or following infection at day 8 p.i. (A) Parasitemia in *P. yoelii* 17XNL-infected WT and IL-3 KO mice. (B) Hematocrit values in uninfected and *P. yoelii* 17XNL-infected WT and IL-3 KO mice. (C) Splenic index in uninfected and *P. yoelii* 17XNL-infected WT and IL-3 KO mice. The splenic was determined as the ratio of spleen weight to total body weight. All data are presented as means  $\pm$  SEM from 6 mice per group analyzed individually. \*\*\*,  $P < 0.001$  versus corresponding values in uninfected mice.



**Figure 5.** Survival rate of male WT and IL-3 KO BALB/c mice infected with *P. yoelii* YM ( $n = 6$  mice/group). Groups of mice were infected with either (A)  $10^5$  pRBCs, (B)  $10^4$  pRBCs, or (C)  $10^3$  pRBCs of *P. yoelii* YM. No statistical significance was observed between the genotypes when infected with  $10^5$  and  $10^4$  pRBCs. IL-3 KO mice infected with  $10^3$  pRBCs survived for significantly longer than their WT counterparts ( $P = 0.038$ ).

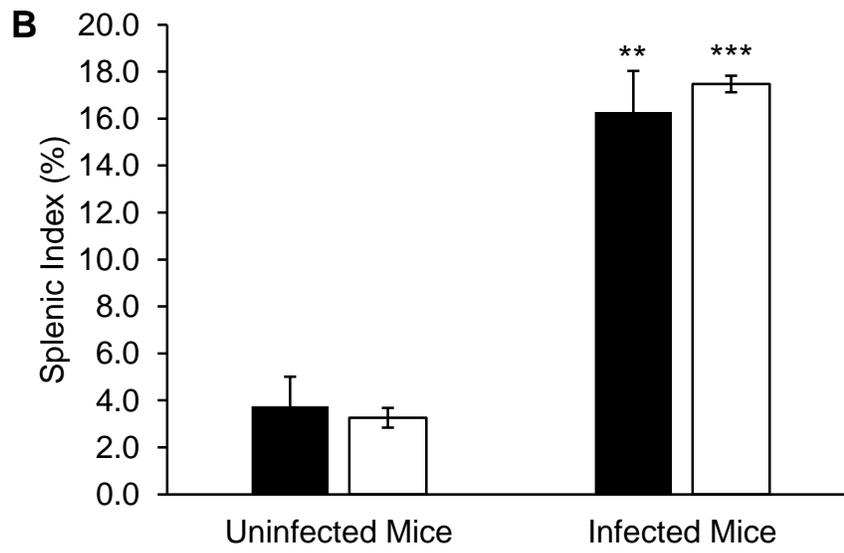
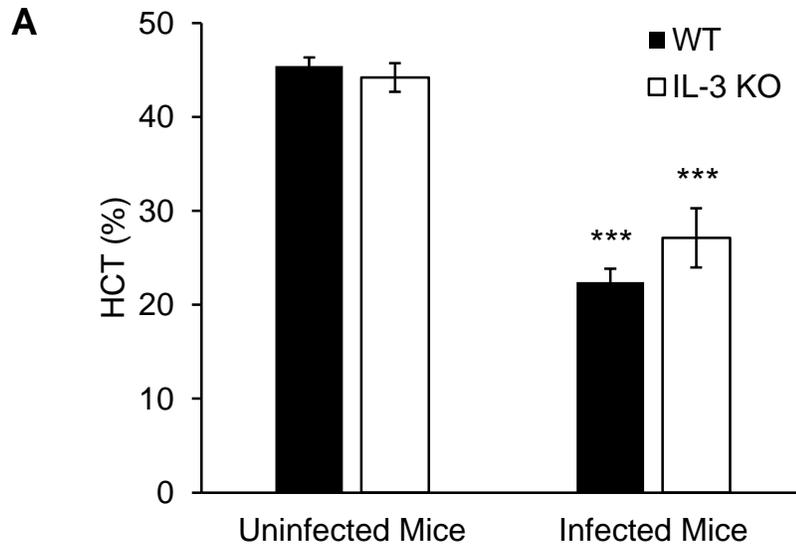


position, and rough hair coat. No statistical difference was observed in survival between the genotypes. Most mice infected with  $10^3$  *P. yoelii* YM pRBCs survived 8 to 11 days p.i., (Figure 5C). Interestingly, IL-3 KO mice survived for significantly longer (~1 day longer,  $P = 0.0388$ ) than did their WT counterparts, with one IL-3 KO mouse overcoming infection completely.

**Anemia and splenomegaly in *P. yoelii* YM-infected IL-3 KO and WT mice as measured on day 8 p.i.** Mice infected with malaria typically exhibit anemia as a major symptom. In addition, mice characteristically exhibit splenomegaly. The increase in spleen size is due to a number of reasons. First, because the spleen is a major secondary lymphoid organ, there is increased proliferation of cells like lymphocytes in response to infection. Second, the spleen is a major site where infected and damaged RBCs accumulate and are removed by phagocytic cells such as macrophages. Finally, the spleen becomes a major site of erythropoiesis whenever there is a significant demand for the production of new RBCs. Previously, IL-3 KO mice infected with *P. berghei* NK65 experienced a significantly higher anemia and splenic index than did infected WT mice (Auclair *et al.*, 2014). However, a role for IL-3 in infections caused by other lethal strains of *Plasmodium* parasites had not yet been assessed.

We infected male WT and IL-3 KO mice ( $n = 5$  per genotype) with  $10^4$  pRBCs of the lethal strain *P. yoelii* YM and examined the degree of anemia and extent of splenomegaly at day 8 p.i. Infection caused significant anemia and splenomegaly in both genotypes (Figure 6A). However, there was no significant difference between infected WT and IL-3 KO mice.

**Figure 6.** Anemia and splenomegaly in mice infected with  $10^4$  *P. yoelii* YM pRBCs. Hematocrit (HCT) values and splenic index in male WT and IL-3 KO BALB/c mice at baseline or at day 8 p.i. (A) Hematocrit values in uninfected and *P. yoelii* YM-infected WT and IL-3 KO mice. (B) Splenic index in uninfected and *P. yoelii* YM-infected WT and IL-3 KO mice. The splenic was determined as the ratio of spleen weight to total body weight. All data are presented as means  $\pm$  SEM from 5 mice per group analyzed individually.  $P < 0.001$  (\*\*\*) and  $P < 0.01$  (\*\*) versus corresponding values in uninfected mice.



## Discussion

IL-3 is a cytokine produced by the body in response to certain infections and has been proposed to play a role in regulating the production, differentiation, and migration of various types of hematopoietic cells (Donahue *et al.*, 1988). IL-3 is produced primarily by activated mast cells and CD4 T cells and has been shown to play a critical role in the function of basophils and mast cells during nematode infection (Lantz *et al.*, 1998). Since the initial discovery of the cytokine, very few studies have described the *in vivo* activities of IL-3. Those which have been most informative are those which utilized IL-3-deficient (IL-3 KO) mice. In contrast to results obtained by *in vitro* studies, mice that lack the IL-3 gene do not possess hematopoietic abnormalities under normal physiological conditions, likely because IL-3 is not produced constitutively under these conditions. However, several studies have shown IL-3 plays a critical role in immune response during certain disease conditions. Our laboratory recently published one such article that supports a critical role of IL-3 in malarial infection caused by *P. berghei* NK65 (Auclair *et al.*, 2014). IL-3 KO mice infected with this lethal strain of malaria survived for significantly longer than WT mice, experienced lower levels of parasitemia, and secreted reduced levels of serum IFN- $\gamma$  early in infection. Additionally, IL-3 KO mice developed a splenomegaly that was significantly greater than their WT counterparts. This report indicates that IL-3 plays a major role in the immune response to *P. berghei* NK65 infection. However, further studies are needed in order to determine if IL-3 also influences the outcome of infection in other *Plasmodium* strains.

In order to determine if IL-3 influences mortality in *Plasmodium* strains other than *P. berghei* NK65, we first infected male WT and IL-3 KO mice with  $10^5$  pRBCs of the lethal

strain *P. yoelii* YM. This was the same dosage of pRBCs used by Auclair *et al.* (2014). Our initial experiments found that there was no significant difference in survival between the two genotypes (Figure 5). We therefore reasoned that  $10^5$  pRBCs was perhaps too high an infectious dose and that the effects of IL-3 might be more noticeable at a lower dose. Therefore, we performed additional experiments in which we infected the mice with fewer parasites. We found that although the survival of these mice was prolonged in comparison to those mice infected with  $10^5$  pRBCs, a dosage of  $10^4$  pRBCs did not result in there being significant differences in mortality between the genotypes (Figure 5B). However, when mice were injected with  $10^3$  pRBCs, IL-3 KO mice survived for significantly longer than their WT counterparts (Figure 5C). Further trials of this experiment should be performed to ascertain the reliability of these results.

Next, a kinetic study evaluating parasitemia using the non-lethal *P. yoelii* 17XNL parasite was performed in order to determine if IL-3 influenced the percentage of infected RBCs during the course of a chronic blood-stage infection. Monitoring parasitemia is a good indicator of susceptibility to infection and provides insight into how efficiently the animal can clear the pRBCs from the peripheral circulation. Previously, Auclair *et al.* (2014) found that IL-3 KO mice infected with *P. berghei* NK65 had significantly lower parasitemias at day 8 and 10 p.i. when compared to infected male WT mice. In contrast with this study, we found no significant difference in parasitemia between the two genotypes at any time point following chronic infection (Figure 3).

In addition to parasitemia, we measured the degree of anemia present at day 10 and 19 p.i. in WT and IL-3 KO mice chronically infected with *P. yoelii* 17XNL (Figure 3). Anemia occurs as a major symptom during *Plasmodium* infection likely because the

merozoites utilize RBCs as a source of nutrition and proliferation, which eventually results in their rupture. Auclair *et al.* (2014) previously reported that IL-3 KO mice infected with *P. berghei* NK65 were more anemic than their WT counterparts. Furthermore, Auclair reported that this difference in anemia was likely due to an increased ability of IL-3 KO mice to destroy pRBCs and/or as a result of increased phagocytosis or antibody-mediated opsonization of pRBCs. As expected, we observed a significant increase in anemia in both genotypes that were infected with *P. yoelii* 17XNL (Figure 3), likely due to RBC rupture by the circulating parasites. Anemia fluctuated with the degree of parasitemia observed. However, we did not observe any significant difference in anemia between WT and IL-3 KO mice at any time point. Likewise, in a separate experiment, we did not observe any difference in anemia between WT and IL-3 KO mice infected with the lethal strain *P. yoelii* YM at day 8 p.i.

A common manifestation of malaria infection in both humans and animals is the appearance of splenomegaly, or an enlarged spleen. We therefore examined the degree of splenomegaly in WT and IL-3 KO mice infected with non-lethal *P. yoelii* 17XNL or lethal *P. yoelii* YM by determining the splenic index of these mice at day 8 p.i. Both genotypes developed a significantly larger spleen over the course of infection when compared to uninfected mice (Figures 4 and 6). However, there was no significant difference in splenic index between infected WT and IL-3 KO mice. Both genotypes infected with the non-lethal *P. yoelii* 17XNL experienced a splenic index of around 30, while mice infected with the lethal *P. yoelii* YM experienced a splenic index of around 18. Such a large difference in splenic index between *P. yoelii* strains may allude to different mechanisms at work during infection even within the same species.

Overall, these results indicate that IL-3 may not play a significant role in host defense against malarial infection with either lethal or non-lethal *P. yoelii* parasites in BALB/c mice. This is in contrast to what has been recently reported in mice infected with *P. berghei* NK65 (Auclair *et al.*, 2014). Auclair *et al.* (2014) reported on IL-3-dependent differences in survival, spleen size, anemia, and parasitemia in male mice infected with *P. berghei* NK65. Upon examination of these attributes in mice infected with either *P. yoelii* 17XNL or YM, no genotypic differences were observed except when infected with  $10^3$  pRBCs of *P. yoelii* YM, an extremely low dose. This suggests that there are key differences in the host response against different *Plasmodium* species. Although the exact reason(s) for these differences is unknown at the time, there are a number of possibilities which might explain these results.

Most obviously, the study present here and that by Auclair *et al.* (2014) differ in the species of parasite used. It is not surprising that the host response, including the production and actions of IL-3, may differ in response to infection caused by different parasites. Significant differences in the immune response to infection based on the major strain of *Plasmodium* used have been previously reported. For example, clear differences in host response have been observed in regard to the host production of various cytokines, parasite-specific antibodies, and other immune components such as Toll-like receptors and dendritic cells (Angulo & Fresno, 2002; Keswani, 2015; Schofield & Grau, 2005; Zuzarte-Luis *et al.*, 2014). In addition to the differences in the host response to the parasites, there are known differences in the biology of the parasites themselves. For example, *P. berghei* and *P. yoelii* differ in their life cycle in regard to their preference for invading immature RBCs called reticulocytes, the duration of the

asexual blood-stage cycle, and the time it takes for the parasite to generate gametocytes (Carter & Diggs, 1977; Killick-Kendrick, 1978; Landau & Boulard, 1978).

The lack of effect from IL-3 on the clinical outcome of *P. yoelii* infection as described by these results may also reflect the inability of *P. yoelii* infection to induce the production of significant levels of biologically active IL-3. It is important to note that IL-3 is not produced constitutively under baseline physiological conditions in healthy mice. The most potent inducer of IL-3 production in mice is infection with gastrointestinal nematodes (Lantz *et al.*, 2008). However, limited evidence exists in the literature regarding the ability of *protozoan* parasites to induce IL-3 induction. One study published in 1998 reported on an elevation in serum IL-3 levels in mice infected with *P. chabaudi* (Helmsby *et al.*, 1998). The only other study providing evidence that *Plasmodium* infection induces IL-3 was by Auclair *et al.* in 2014. In this study, IL-3 production in *P. berghei* NK65-infected mice was detected as early as day 4 p.i. However, the levels of IL-3 detected in these mice was low in comparison to what is normally observed in nematode-infected mice. Based on these observations, infection of mice with *P. yoelii* may induce little to no biologically active IL-3. If true, the results presented in this report would not be totally unexpected.

Any one of these factors may play the deciding role in encouraging or limiting the influence of IL-3 in infection with *Plasmodium*. Additionally, the data reflected within this report does not confirm the absolute absence of a role for IL-3 in *P. yoelii* infection. For instance, plasma levels of interferon gamma (IFN- $\gamma$ ) and chemokine CXCL9 have been shown to be critical in malarial infection with other species of *Plasmodium* (Li *et al.*, 2001) and were previously shown by our laboratory to be influenced by IL-3 (Auclair *et*

*al.*, 2013). Due to time constraints we were unable to quantify levels of any serum cytokines or antibodies.

In conclusion, many questions remain regarding the ability of IL-3 to influence disease outcome in mice infected with *Plasmodium* strains other than *P. berghei* NK65. The role of IL-3 in both lethal and non-lethal *P. yoelii* remains unclear but at this time does not appear to have a major effect on the outcome of either lethal or chronic infection as monitored by survival, percent parasitemia, anemia, or splenomegaly. Future experiments that address other indicators of disease outcome like serum levels of cytokines like IFN- $\gamma$  or CXCL9 should be performed. These further studies, in addition to the ones provided within this report and published by Auclair *et al.* (2014), will help to more clearly elucidate the role that IL-3 plays in infection with both rodent and possibly human malaria.

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