Spring 2014

Role of interleukin-3 in blood-stage malaria infection caused by *Plasmodium berghei* ANKA

Abdalla Sheikh
James Madison University

Role of interleukin-3 in blood-stage malaria infection caused by

Plasmodium berghei ANKA

_____

A Project Presented to

the Faculty of the Undergraduate

College of Science and Mathematics

James Madison University

_____

in Partial Fulfillment of the Requirements

for the Degree of Bachelor of Science

_____

by Abdalla Sheikh

May 2014

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science

FACULTY COMMITTEE:

Project Advisor: Chris Lantz, Ph.D.,
Professor, Biology

Reader: Pradeep Vasudevan, Ph.D.,
Assistant Professor, Biology

Reader: Steven Cresawn, Ph.D.,
Associate Professor, Biology

HONORS PROGRAM APPROVAL:

Barry Falk, Ph.D.,
Director, Honors Program

Dedication

To my parents with love – without whose encouragement and support I could not have finished my work on time.

Acknowledgments

I would like to express many thanks to my research advisor, Dr. Chris Lantz, for his support and guidance throughout my undergraduate research. Without his influence and expertise this thesis would not have been completed and I would not have grown to love research with a passion. I would also like to thank my committee members, Drs. Steven Cresawn and Pradeep Vasudevan for their valuable advice and providing constructive criticism towards the completion of my work. In addition, I would like to express my deepest appreciation to graduate student Sarah Auclair for bearing with me whenever I needed help and to Dr. Roth for his experience and humor and to my lab colleagues and dearest friends for their assistance in completing my experiments and boosting my morale. Finally, I would like to thank the Department of Biology at James Madison University for providing me with the space and environment for research.

Table of Contents

List of Figures 6

Abstract 7

Introduction 9

Malaria 9

Overview of malaria 9

Plasmodium life cycle 10

Clinical disease 11

Mouse models of malaria 15

Interleukin-3 and malaria 16

Project goals 18

Materials and method 20

Mice 20

Plasmodium berghei ANKA parasites 20

Plasmodium berghei ANKA infection and evaluation of disease 20

Statistical analysis 21

Results 23

Mortality rates of *P. berghei* ANKA-infected IL-3 -/- and WT mice 23

Parasitemia in *P. berghei* ANKA-infected IL-3 -/- and WT mice 26

Anemia in *P. berghei* ANKA-infected IL-3 -/- and WT mice 30

Development of splenomegaly in *P. berghei* ANKA-infected IL-3 -/- and WT mice 33

Discussion 36

References 42

List of Figures

Figure 1 *Plasmodium* life cycle……....12

Figure 2 Survival rate of *P. berghei* ANKA-infected male (A) and female (B) WT and IL-3 -/- (KO) mice (*n* = 6-9 mice/group)……..…24

Figure 3 Parasitemia values in *P. berghei* ANKA-infected male and female WT and IL-3 -/- (KO) mice…….....27

Figure 4 Hematocrit values in uninfected and *P. berghei* ANKA-infected male and female WT and IL-3 -/- (KO) mice…….……..……31

Figure 5 Splenic index in uninfected and *P. berghei* ANKA-infected male and female WT and IL-3 -/- (KO) mice……..…..…34

Abstract

Malaria is a blood-borne disease caused by protozoans of the genus *Plasmodium*. Currently, 300-500 million cases are reported each year and 1.3 million deaths occur world-wide, with the majority taking place in developing nations. Four major species of *Plasmodium* cause disease in humans, the most wide-spread of them being *Plasmodium falciparum.* Typical symptoms of malaria include fever, lethargy, splenomegaly, and anemia, and in severe cases, neurological symptoms may arise. Interleukin-3 (IL-3), a cytokine released primarily by activated T-cells and mast cells, is known for its hematopoietic growth functions. It has also been shown to play a protective role in nematode infections and in promoting delayed-type contact hypersensitivity responses. In this study, we investigated the extent to which IL-3 plays a role in the host response to *Plasmodium* infections. We induced a blood-stage malaria infection in wild-type (WT) and IL-3-deficient (IL-3 -/-) mice using *P. berghei* ANKA, a lethal strain of *Plasmodium* that infects rodents. We show that infected female IL-3 -/- mice, but not male mice, presented with lower numbers of circulating parasitized red blood cells, were more anemic, and had increased splenomegaly compared to corresponding WT mice. However, there was no difference in the survival rate between infected male or female mice of either genotype. Together these data indicate that IL-3 might act to suppress protective immune responses to *P. berghei* ANKA in female mice, but these actions were not enough to prolong survival. Our findings that gender influences the disease indicators likely suggest hormonal and immunological differences in the sexes. These findings are in contrast to similar studies by our laboratory using *P. berghei* NK65-infected IL-3 -/- and WT mice in which it was shown that IL-3 plays a

critical role in suppressing protective immunity in both male and female mice, and prolongs survival in male mice. We believe that the studies presented here, together with those reported for *P. berghei* NK65, will help to eventually elucidate the relative contribution of IL-3 in the resulting morbidity and mortality associated with both rodent and human malaria.

Introduction

Malaria

Overview of malaria

Malaria is a parasitic infection caused by a protozoan of the genus *Plasmodium* and is transmitted among humans by female mosquitoes of the genus *Anopheles*. The most common *Plasmodium* species that infect humans are *P. falciparum, P. vivax, P. ovale and P. malariae.* Each year, 300-500 million cases of malaria are reported and 1.3 million deaths occur worldwide, mainly among children under the age of 5; it takes the life of 2000 children daily in Africa alone (National Institute of Allergy and Infectious Diseases, 2011). Although malaria is not currently a problem in developed countries such as the United States, almost half of the world's population lives in areas where due to economic situations malaria is endemic. It occurs mainly in sub-Saharan Africa, Southeast Asia, India and South America. (National Institute of Allergy and Infectious Diseases, 2011).

Malaria is a serious and sometimes fatal infection. Individuals infected with malaria present with a variety of symptoms but generally include high fever, shaking, chills, headaches, and anemia, and flu-like illness. All the clinical symptoms of malaria are caused by asexual blood stage parasites. When the parasites develop in the RBCs, numerous toxic substances accumulate and are released when the infected cells are lysed. The release of these toxic substances stimulates a variety of immune and non-immune cells which in turn contribute to pathology. With some *Plasmodium spp.*, parasitized RBCs adhere to the vascular endothelium of blood vessels and do not freely

circulate. When this occurs in vessels of the brain, a serious and often fatal condition known as cerebral malaria (CM) can develop. Although malaria is a curable disease if diagnosed and treated properly, control and eradication of the disease has presented with various obstacles, mainly due to the lack of an effective vaccine and the development of drug resistant strains. A better understanding of the biology of *Plasmodium* is therefore critical to identifying meaningful targets for vaccines, therapeutics and diagnostics.

Plasmodium **life cycle**

Among the *Plasmodium spp.* four are known to infect humans: *P. falciparum, P. vivax, P. ovale,* and *P. malariae.* While *P. falciparum*, found worldwide, is the most virulent among these species and is known to cause severe fatal malaria, *P. vivax* is the most prevalent *Plasmodium* species, causing high morbidity and high socioeconomic impact. Other *Plasmodium spp.* have been reported to infect rodents, including *P. chabaudi, P. vinckei, P. yoelii and P. berghei.*

Plasmodium spp. exhibit a complex life cycle that involves female *Anopheles* mosquitoes and a vertebrate host. This life cycle is similar in both humans and rodents. The *Plasmodium* life cycle is generally divided to two parts within the vertebrate host: the exo-erythrocytic and erythrocytic stages. When an infected female mosquito takes a blood meal from a vertebrate host, saliva containing vasodilation compounds and parasite sporozoites are injected into the bloodstream of the host. Sporozoites travel through the bloodstream and enter the parenchymal cells of the liver, initiating the exo-erythrocytic stage, where they divide asexually forming thousands of merozoites. The

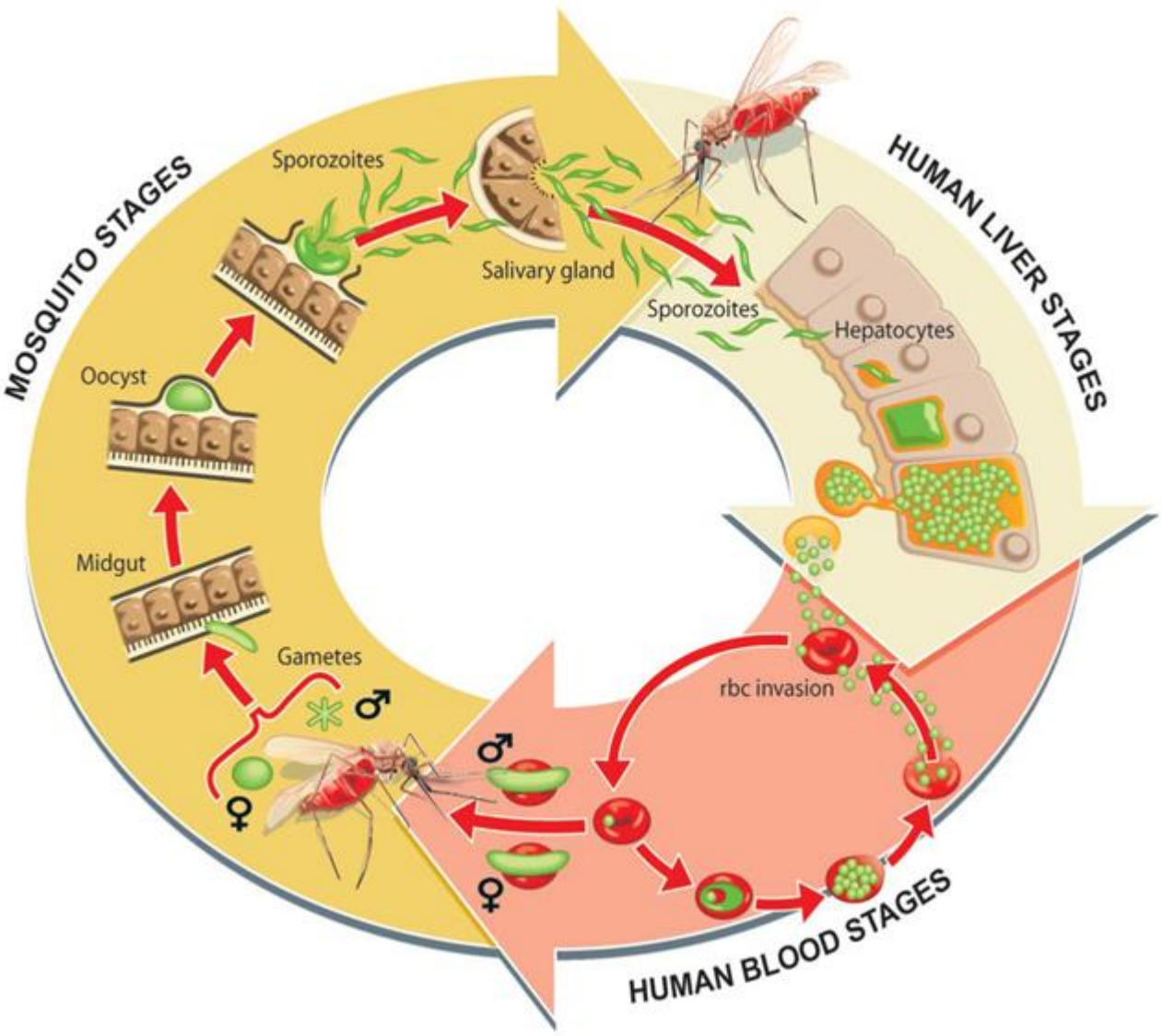
number of merozoites produced varies between the different species. When the merozoites fill the entire volume of a cell they burst out into the bloodstream to start the erythrocytic stage by invading red blood cells (RBCs) (Sylvie *et al*., 2008).

Within RBCs, merozoites develop into a ring stage (early trophozoites) and undergo asexual division to form a schizont. Eventually the RBCs burst and release numerous newly formed merozoites into the bloodstream to infect more RBCs. The erythrocytic cycle is repeated every 48 hours for most species. Merozoites depend on RBCs for nutrition as they utilize hemoglobin after breaking the complex and releasing a pigmented product, hemozoin, which is primarily responsible for most symptoms of disease, especially fever (Goldberg, *et al*., 1990). Some merozoites advance into the sexual stage and form into macro gametocytes (female) or microgametocytes (male). These stages can develop further only in the gut of a female mosquito. Once the gametocytes are ingested by a mosquito during a second blood meal they fuse and form zygotes which become oocytes. The oocytes mature into sporozoites which migrate to the salivary glands of the mosquito. When the mosquito infects another host, the life cycle continues. The generalized life cycle of *Plasmodium* is shown in Figure 1.

Clinical disease

Clinical symptoms of malaria result mainly from the erythrocytic stage of *Plasmodium* infection and commonly include enlargement of the spleen (splenomegaly), anemia, headache, myalgia and a periodic outbreak of fever and chills (Haldar *et al*., 2007). Many of the studies in malaria identify a severe malaria infection to be one that

FIG 1. Plasmodium life cycle (Derbyshire *et al.,* 2011).


MOSQUITO STAGES
Sporozoites
Salivary gland
Oocyst
Midgut
Gametes
♂
♀
HUMAN LIVER STAGES
Sporozoites
Hepatocytes
rbc invasion
HUMAN BLOOD STAGES

includes a cerebral pathogenesis, severe anemia and splenomegaly. It is thought that the fever and chills are mainly caused by the release of hemozoin, a byproduct of hemoglobin breakdown, and other toxic factors which induce macrophages and other immune cells to produce inflammatory cytokines and other inflammatory mediators leading to fever and tremors (chills).

Since malaria is a blood borne pathogen, the spleen plays a major role as a secondary lymphoid tissue. Within the spleen, immune cells encounter antigens and proliferate into cytokine-producing effector cells. This proliferation process is believed to be the reason the spleen enlarges (Haldar *et al*., 2007). There is much debate on how CM occurs in a *Plasmodium* infection. It is thought that it is mainly caused due to the sequestration of parasitized RBCs and immune cells (mainly neutrophils) in the microvasculature of the central nervous system (Porcherie *et al.,* 2011). Along with blockage, other factors are thought to play a role in CM pathogenesis. For instance, TNF-α, IFN-γ, IL-1 and IL-6 are inflammatory cytokines pivotal in the early response to malarial infection and have also been implicated in CM (Kwiatkowski *et al*., 1990). Severe infection, characterized by hyperparasitemia, hypoglycemia and severe anemia, were also observed with patients who had higher levels of TNF-α (Schaffer *et al*., 1991). In some studies, suppression of the inflammatory cytokines IFN-γ, TNF-α and IL-12 inhibited the development of CM in infected murine models (Yanez *et al*., 1996), although the mechanism by which these cytokines result in these outcomes is unclear.

Another great cause of morbidity and mortality in malaria is anemia. The process by which anemia occurs is still unclear. It is thought that the two likely mechanisms are due to the clearance of parasitized and possibly non-parasitized RBCs and/or

suppression of erythropoiesis (Haldar *et al*., 2007). Waitumbi *et al.,* (2000) suggested that as parasitized RBCs lyse as part of the *Plasmodium* life cycle, antigens are released that bind to non-parasitized RBCs, which consequently become eliminated from the bloodstream by phagocytosis mediated by immunoglobulin G or by complement mediated opsonization. The symptoms of malaria, specially fever and chills, occur in a periodical manner due to the nature of the *Plasmodium* life cycle, where large number of merozoites break out of RBCs in a repetitive cycle and the time elapsed depends on the strain of *Plasmodium*.

Mouse models of malaria

Mice have provided investigators with a useful model suitable for the study of the interactions between the host and *Plasmodium* parasites. A number of *Plasmodium spp*. that infect mice and other rodents have been extensively studied. The major species used in mouse models of infection include *P. chabaudi, P. vinckei, P. yoelii*, and two subspecies of *P. berghei*, NK65 and ANKA. There are numerous small differences between the major species in regard to their life cycle, morphology and host interactions. For example, *P. chabaudi* is generally considered to be a non-lethal parasite whereas *P. berghei* causes significant mortality by 2 weeks post-infection (p.i.). Therefore, the decision to work with a particular parasite will depend on the goals of the research in question.

Mouse experiments with *Plasmodium* can be conducted by using mosquitoes to infect the mice. This method of infection can be utilized to study the exo-erythrocytic stage of the *Plasmodium* infection. To study the blood stages, mice are normally

injected with parasitized RBCs. Parasitized RBCs can be introduced into the blood stream by injecting them intraperitoneally (IP) into the abdomen, or intravenously. *P. berghei* is a commonly used strain because the resulting pathology is similar to that seen in humans: severe anemia, splenomegaly and respiratory distress. *P. berghei* NK65*,* a subspecies, is used commonly because it mimics the effects of *P. falciparum* in humans which can serve as an essential tool for studying malaria. Mice injected I.P. with parasitized RBCs develop a significant parasitemia that is generally lethal by 12 days p.i.

Infection with a different subspecies, *P. berghei* ANKA, presents with similar symptoms, but it varies from that of *P. berghei* NK65 in that it also causes CM. CM is the most consequential manifestation of severe malaria that often involves impairment of consciousness, seizures, coma, and other neurological abnormalities. Although the etiology of CM is not clear, it is thought to be caused by blockage of the cerebral capillaries with parasitized RBCs, which adhere to the endothelium. In mice, experimental CM induced by *P. berghei* ANKA also leads to blockage of cerebral capillaries. Furthermore, it has been shown that neutrophils, not mast cells or basophils, play a central role in inducing inflammatory responses in CM (Porcherie *et al*., 2011). Importantly, involvement of neutrophils was not seen in mice infected with *P. berghei* NK65, a strain that does not induce CM.

Interleukin-3 and malaria

Interleukin-3 (IL-3) is a 28 kDa glycoprotein monomer functioning as a cytokine, released primarily by activated T-cells and mast cells working primarily as a

hematopoietic growth factor. It has been documented to cause the *in vitro* differentiation of murine stem cells into granulocytes, erythrocytes and mast cells (Ihle, 1992). IL-3 has also been documented to play a role in the differentiation of monocytes into macrophages (Young *et al*., 1990) and *in vitro* studies have shown that IL-3 induces TNF- α production by antigen-activated monocytes (Cannistra *et al*., 1988). In addition, IL-3 has also been used in *in vivo* studies by administering it to mice, primates and humans which resulted in increased hematopoiesis (Ihle, 1992). Moreover, IL-3 has been documented to promote mast cell growth and release of pro-inflammatory mediators in allergic reactions (Hu *et al.,* 2007).

Notwithstanding the functions of IL-3, studies show that BALB/c IL-3-deficient (IL-3 -/-) mice do not have abnormalities in hematopoiesis, presenting with normal basophil and mast cell numbers under normal physiological conditions (Mach *et al*., 1998). This is likely because IL-3 is not produced constitutively. However, IL-3 -/- mice have shown impaired delayed-type contact hypersensitivity responses to hapten antigens (Mach *et al*., 1998) and delayed expulsion of gastrointestinal nematodes (Lantz *et al*., 1998).

Since the initial description of IL-3, there has been no direct evidence in a published literature of a role for IL-3 in the pathophysiology of malaria. Grau *et al*., (1988) have shown that the use of anti-IL-3 and anti-GM-CSF antibodies on *P. berghei* ANKA-infected CBA mice prevents the development of CM and extends their survival, possibly by preventing increase in TNF-α. Furthermore, IL-3 has been shown to play a role in increasing adhesion of parasitized RBCs to endothelial cells leading to severe

malaria (Carlson *et al.,* 1990). Meyer *et al*., (2011) have also shown that IL-3 polymorphisms are associated with chances of recurrent malaria infections in humans.

Recently, in our laboratory, we studied the role of IL-3 in host immunity during a *Plasmodium* infection using IL-3 -/- and wild-type (WT) BALB/c mice. We have found that *P. berghei* NK65-infected IL-3 -/- mice presented with larger spleens (perhaps due to significant immune cell expansion) and lower hematocrit (more anemic) at day 8 p.i. compared to similarly infected WT mice. Interestingly, we have demonstrated in separate experiments that IL-3 -/- male mice presented with significantly increased survival and lower peak parasitemia compared to similarly infected WT mice. We also found that infected IL-3 -/- male mice were able to produce more erythrocyte progenitors (CFU-E and BFU-E) compared to infected WT male mice. Furthermore, WT mice had higher levels of the inflammatory cytokine IFN-γ at day 4 p.i. in their plasma. All these results indicate the importance of IL-3 during a *Plasmodium* infection and necessitate further studies.

Project goals

After having discovered that IL-3 plays a significant role in a mouse model of *P. berghei* NK65 infection, we think a similar study using a different strain of *Plasmodium* is necessary. This is justified by the differences in symptomatology implicated by the various strains on the host. *P. berghei* ANKA, a mouse model of *Plasmodium*, is known to cause CM unlike *P. berghei* NK65. CM occurs as a result of cellular blockage (see above) which is a significantly different form of pathogenesis compared to that which

occurs with *P. berghei* NK65. This necessitates the completion of a similar study using *P. berghei* ANKA to provide an understanding of the role of IL-3 in CM.

We intend to study the blood stage form of *P. berghei* ANKA infection by injecting 10^5 parasitized RBCs IP into 8-12 WT and IL-3 -/- male and female mice. We will then examine the spleen sizes and the hematocrits. In addition, we intend to do a survival study accompanied by a kinetic study to examine parasitemia levels at different time points (preferably at days 2, 4, 6, 8 and 10). As time allows, we also plan to perform an analysis of cytokine levels in serum using a multiplex assay.

The specific aims of this project are as follows:

Aim 1 – Assess the extent to which IL-3 influences the course and outcome of disease in P. berghei ANKA-infected BALB/c mice. We will employ both male and female BALB/c IL-3 -/- and WT mice infected with *P. berghei* ANKA to test the hypothesis that endogenous IL-3 influences blood parasitemia and survival.

Aim 2 – Assess the extent to which IL-3 influences the host immune response of P. berghei ANKA-infected BALB/c mice. We will characterize parameters that are indicative of a host protective response to infection in BALB/c IL-3 -/- and WT mice infected with *P. berghei* ANKA: the development of splenomegaly, RBC levels, and cytokine production.

Materials and method

Mice

IL-3-deficient (IL-3 -/-) BALB/c mice were generated as described by (Mach *et al*., 1998) and housed in the animal holding facility of the Department of Biology at James Madison University. The wild-type (WT) BALB/c mice used were ordered from The Jackson Laboratory (Bar Harbor, ME).The mice were maintained and used in accordance with James Madison University's Institutional Animal Care guidelines. Both male and female mice were used and ranged in age between 8-12 weeks at the start of the experiments.

Plasmodium berghei ANKA parasites

Cryopreserved *P. berghei* ANKA parasites were obtained from BEI Resources (Manassas, VA; stock number MRA311) in the form of parasitized RBCs. Before using the parasites experimentally, they were passaged through WT BALB/c mice. Blood from these mice was routinely collected and used to infect experimental animals. Frozen stocks of *P. berghei* ANKA were stored in liquid nitrogen.

P. berghei ANKA infection and evaluation of disease

P. berghei ANKA parasites were obtained from cryopreserved vials stored in liquid nitrogen. After a single passage through a WT BALB/c mouse, the parasites were ready to be used on experimental mice. Specifically, the parasites were thawed and injected intraperitoneally (I.P.) into a single mouse. 7 days post-infection (p.i.) blood was

collected from the retro-orbital sinus of the mouse and prepared for the experimental infection. Approximately 10^5 parasitized RBCs were injected I.P. into each mouse. In each experiment, IL-3 -/- and WT male or female BALB/c mice (n = 6 per group) were used. 8 days p.i. the mice were weighed and blood was collected from their retro-orbital sinus. Thin smears of the peripheral blood were prepared and stained with Giemsa to examine the percentage of parasitized RBCs (% parasitemia) using light microscopy. Blood was also collected into heparinized hematocrit capillary tubes to evaluate anemia. Additionally, the remaining blood was centrifuged for 8 minutes at 1000 x g to collect serum to be stored at -80°C for future cytokine level analysis. The spleens were also extracted and weighed immediately after euthanizing the mice. The splenic indices were calculated using the formula (spleen weight (mg)/ body weight (g)) x 1000.

For each survival experiment, male and female IL-3 -/- and WT mice (n = 7-9 per group) were injected with 10^5 parasitized RBCs I.P. The health and behavior of each mouse was monitored and recorded at approximately 24 hour intervals. Blood was also drawn from male mice through tail snips every two days beginning from days 0 to 10 p.i. to prepare thin blood smears for kinetic parasitemia evaluation.

Statistical analysis

Data are presented as the means ± standard deviations from the means (SD). To assess the statistical significance between mean values influenced by two variables, a two-way or repeated analysis of variance (ANOVA) followed by Bonferroni posttest was performed; an unpaired *t* test was used to compare one variable between two groups. Survival data were analyzed using the log-rank (Mantel-Cox) test. A significance level of

0.05 was used for all comparisons. All statistical analyses were performed using GraphPad Prism, version 5.02 (GraphPad Software, La Jolla, CA).

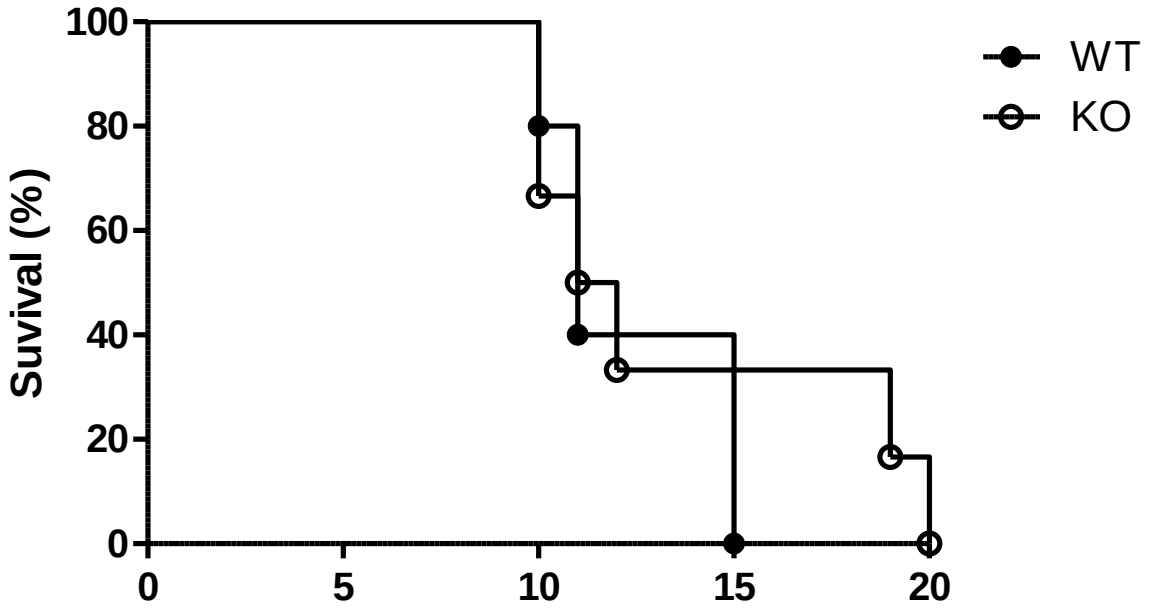
Results

Mortality rates of *P. berghei* ANKA-infected IL-3 -/- and WT mice. Mice infected with *P. berghei* ANKA usually do not clear their infection and tend to die 8-12 days post-infection (p.i.). In order to determine if IL-3 plays a role in the survival of *P. berghei* ANKA-infected mice, male and female IL-3 deficient (IL-3 -/-) and wild-type (WT) mice (n = 6-9 of each genotype) were infected intraperitoneally (I.P.) with 10^5 parasitized RBCs. Mice were then monitored daily for signs of illness and death. The objective of this experiment was to conduct a survival comparison for the two genotypes.

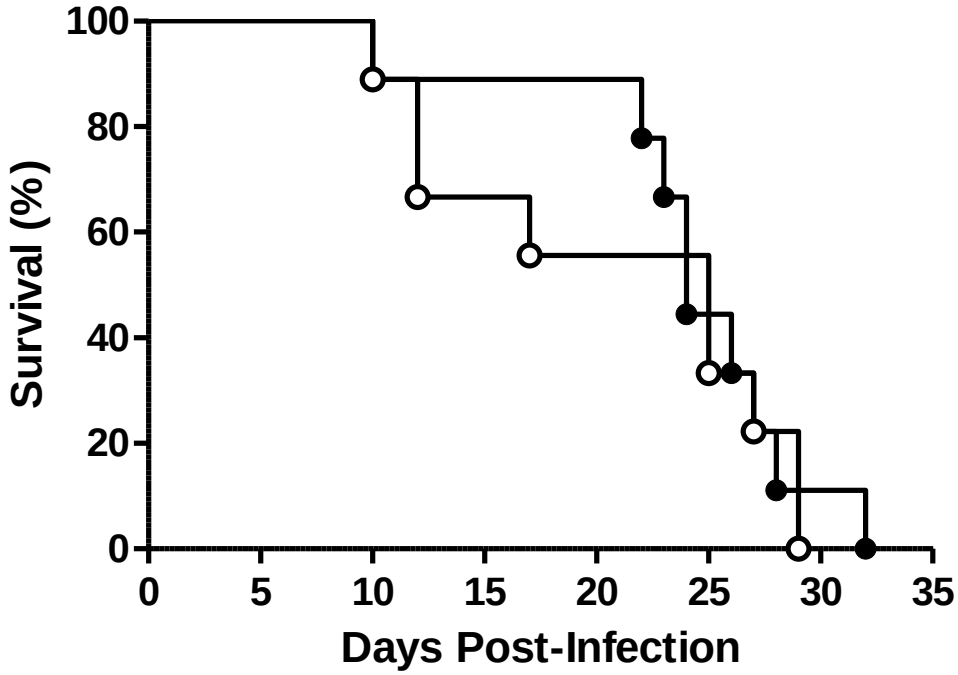
Neither sex presented with a statistically significant difference in mortality between the IL-3 -/- and WT mice as can be seen in Figs. 2A and 2B. Male mice of both genotypes started dying on day 10, and by day 15 all the WT mice were dead while two IL-3 -/- mice survived until day 20 (Fig 2A). Similar to male mice, female mice of both genotypes started dying on day 10 p.i. but lived as long as day 30-35. All the mice had rough hair coats by day 7 p.i. but still had normal outward physical activity. By day 9 p.i. all the mice exhibited advanced malaria symptoms of lethargy and were prostrate and inactive by day ~12 with some periodically active days.

FIG 2. Survival rate of *P. berghei* ANKA-infected male (A) and female (B) WT and IL-3 -/- (KO) mice (*n* = 6-9 mice/group). There was no statistically significant difference in survival between infected WT and IL-3 -/- mice ($P > 0.05$).

A


100
80
60
40
20
0
Suvival (%)
0
5
10
15
20
WT
KO

B


100
80
60
40
20
0
Survival (%)
0
5
10
15
20
25
30
35
Days Post-Infection

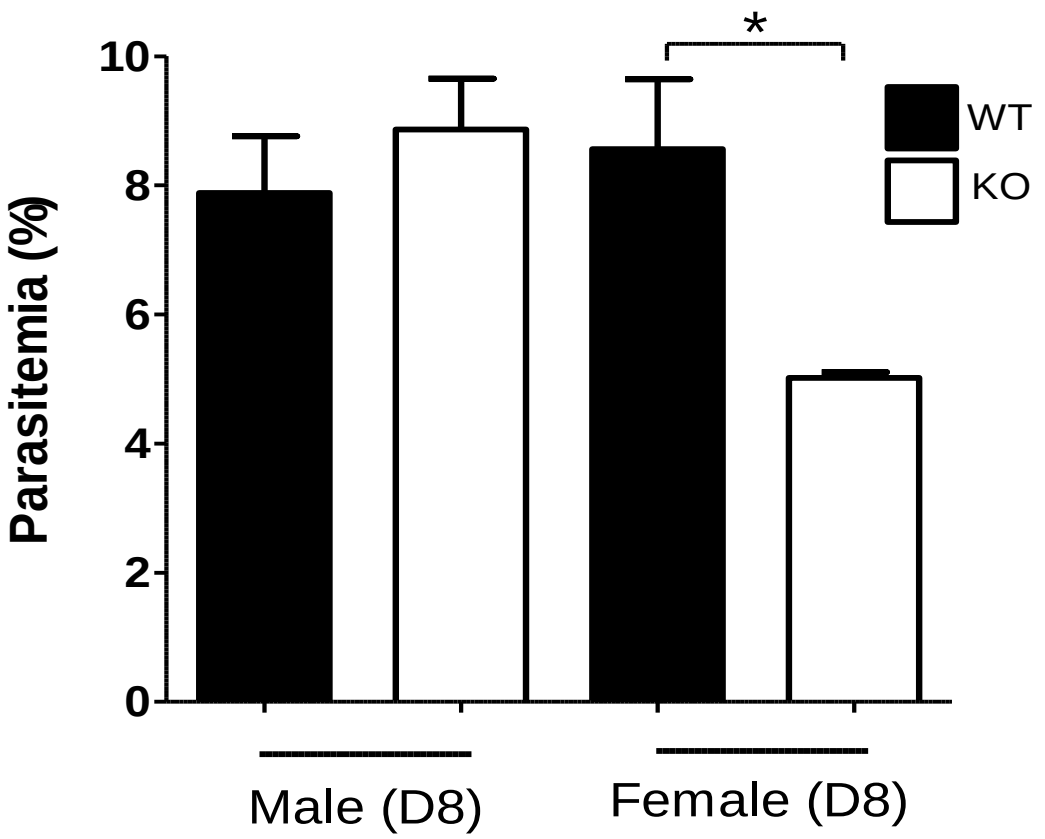
Parasitemia in *P. berghei* ANKA-infected IL-3 -/- and WT mice. A successfully induced experimental malaria infection is indicated by the invasion of RBCs by *Plasmodium* merozoites. Plasmodium parasites benefit from this parasitic relationship by using hemoglobin as a nutrition component and by undergoing asexual division in blood cells. Since parasitic burden is a good indication of the severity of infection, measurement of parasitemia levels was evaluated in *P. berghei* ANKA-infected WT and IL-3 -/- mice. To evaluate the degree to which IL-3 influences parasitemia levels, we infected male and female IL-3 -/- and WT mice (n = 6 of each genotype) with 10^5 parasitized RBCs IP and sacrificed them on day 8 p.i. Blood was then collected just prior to sacrifice through retro-orbital bleeding. Day 8 of infection is ideal for collecting blood and tissue samples since the mice are experiencing advanced outward symptoms of malaria but have not yet succumbed to infection. Parasitemia counts were determined by examining peripheral blood smears stained with Giemsa.

Fig. 3A illustrates the parasitemia levels of male and female infected mice at day 8 p.i. No statistically significant difference was observed between male IL-3 -/- and WT mice, with both genotypes having approximately 8-9% parasitemia. However, female WT mice were more susceptible to parasite invasion (~9%) than their IL-3 -/- counterparts (~5%) on day 8 p.i. as evidenced by the statistically higher parasitemia.

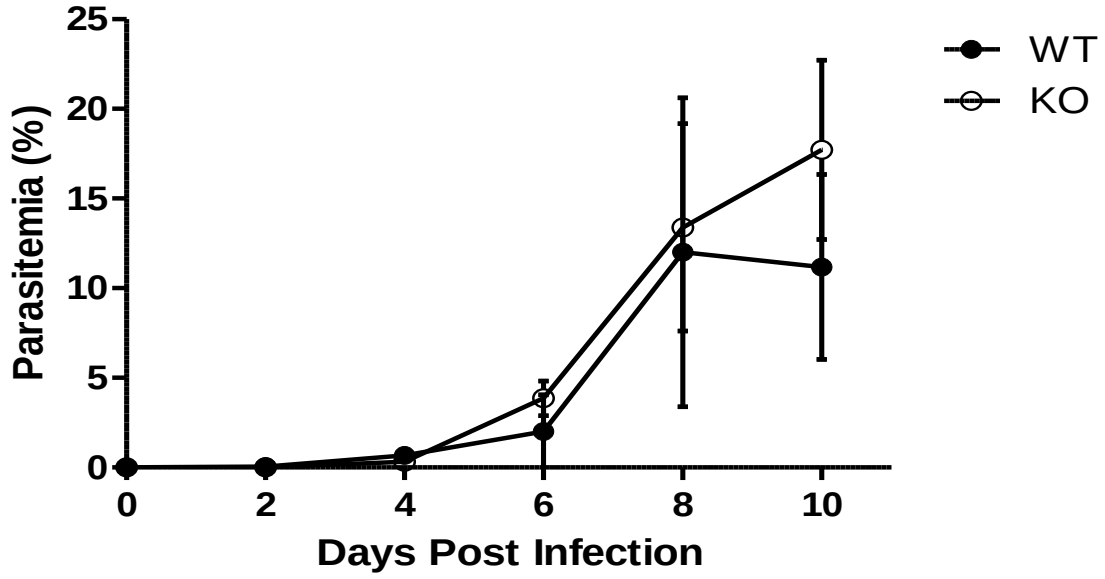
In order to determine if IL-3 influenced parasitemia levels at other times points during infection, a kinetic experiment was performed using male mice. To make this analysis, blood was drawn from tail snips of male mice shown in Fig. 2A at days 0, 2, 4, 6, 8 and 10 p.i. As shown in Fig. 3B male WT mice reached a peak parasitemia of ~12 % on day 8 p.i. followed by a reduced parasitemia on day 10 p.i. IL-3 -/- mice had a

FIG 3. (A) Parasitemia values in *P. berghei* ANKA-infected male and female WT and IL-3 -/- (KO) mice. Parasitemia of infected mice was determined on day 8 (male and female). All data are presented as mean ± SD from 6 mice per group analyzed individually. *, $P < 0.05$ for mice as indicated by the square brackets versus corresponding values for female mice of the other genotype. (B) Time course of parasitemia in *P. berghei* ANKA-infected male WT and IL-3 -/- (KO). All data are presented as mean ± SD from 6-9 mice per group analyzed individually.

A


*
10
8
6
4
2
0
Parasitemia (%)
WT
KO
Male (D8)
Female (D8)

B

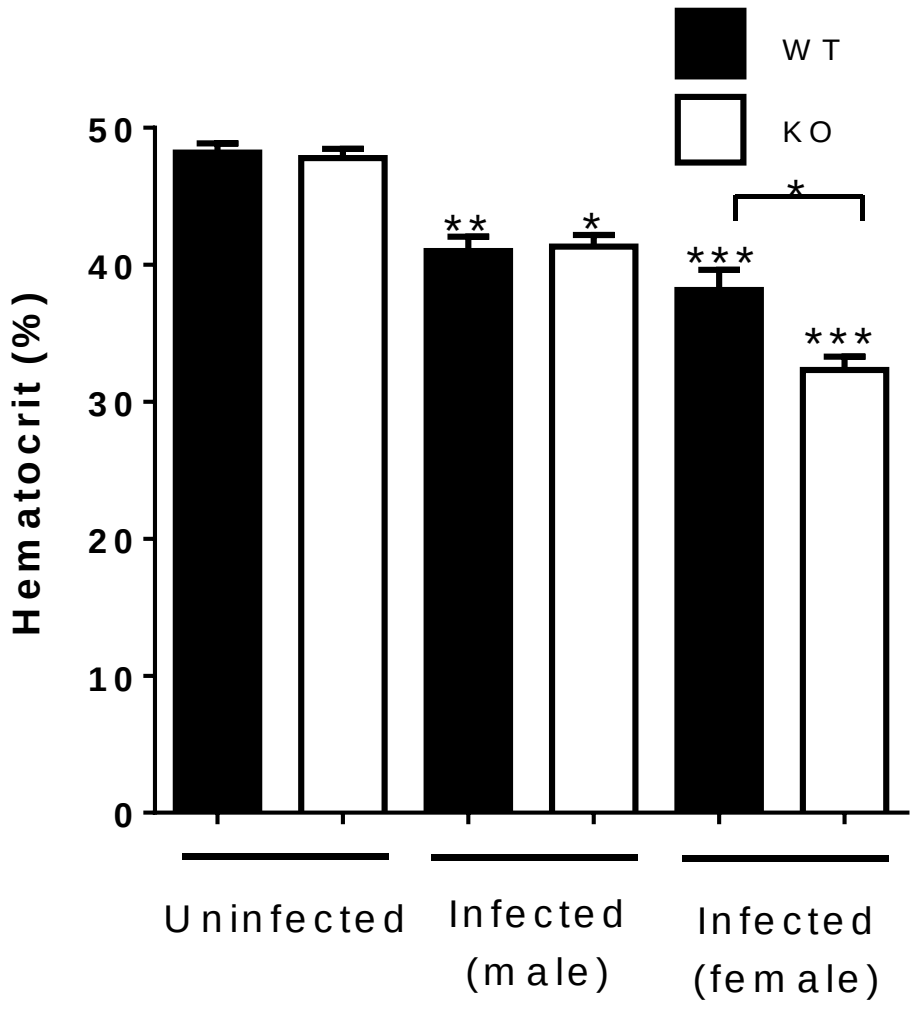

25
20
15
10
5
0
Parasitemia (%)
0
2
4
6
8
10
Days Post Infection
WT
KO

peak parasitemia of ~18 % on day 10 p.i. However, the two groups of mice did not present with a significant difference in parasitemia throughout the course of the infection.

Anemia in *P. berghei* ANKA-infected IL-3 -/- and WT mice. Typically in *Plasmodium* infections, anemia occurs as a major symptom, largely due to the fact that merozoites utilize RBCs for nutrition and proliferation, eventually resulting in their rupture. The clearance of invaded and damaged RBCs by the spleen or suppression of erythropoiesis also contributes to anemia. In order to determine if IL-3 influenced the degree of anemia resulting from infection, we measured hematocrit levels in *P. berghei* ANKA-infected male and female WT and IL-3 -/- mice at day 8 p.i. Mice used for obtaining these results are the same mice as those shown in Fig. 3A.

As illustrated by Fig. 4, uninfected WT and IL-3 -/- mice presented with similar hematocrit levels as previously reported (Mach *et al.,* 1998). All infected mice had significantly lower hematocrit values compared with baseline values. However, male IL-3 -/- and WT mice did not have a statistically significant difference in hematocrit levels. In fact, both groups had levels between 40-41%. In contrast, female IL-3 -/- and WT mice infected similarly had statistically significant difference in anemia, with IL-3 -/- mice being more anemic (~33%) than WT mice (~38%).

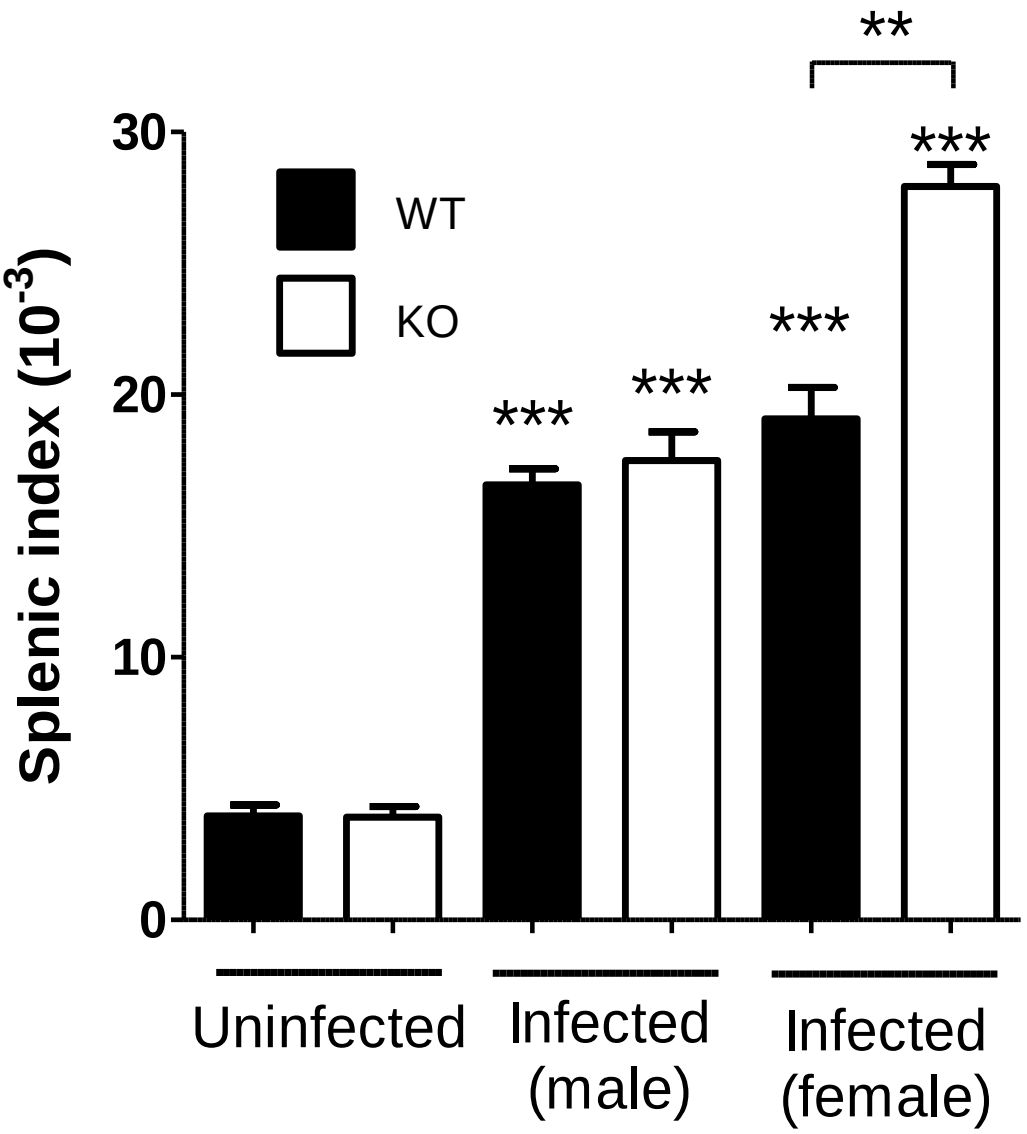
FIG 4. Hematocrit values in uninfected and *P. berghei* ANKA-infected male and female WT and IL-3 -/- (KO) mice. Hematocrits of infected mice were determined on day 8 p.i. Data from uninfected male and female mice of each genotype were not significantly different and were pooled (Day 0). All data are presented as means ± SD from 6 mice per group analyzed individually. $P < 0.001$ (***), $P < 0.01$ (**), and $P < 0.05$ (*) versus corresponding values for uninfected mice or (as indicated by the square brackets) versus corresponding values for mice of the other genotype.


WT
KO
Hematocrit (%)
50
40
30
20
10
0
**
*
***
*
***
Uninfected
Infected (male)
Infected (female)

Development of splenomegaly in *P. berghei* ANKA-infected IL-3 -/- and WT mice. As a major secondary lymphoid organ, the spleen plays an important role in controlling blood-borne infections. During a *Plasmodium* infection, mice typically present with enlarged spleens caused by immune cell proliferation in response to antigens encountered in the splenic white pulp and/or as a result of RBC destruction occurring in the splenic red pulp. Although IL-3 had no influence over the mortality rate of *P. berghei* ANKA-infected mice, its role in influencing spleen pathology was yet to be determined. We investigated whether IL-3 plays a role in *P. berghei* ANKA infection by examining spleen sizes of infected IL-3 -/- and WT mice. We infected male and female mice of both genotypes (n = 6 pergroup) with 10^5 parasitized RBCs I.P. and sacrificed them on day 8 p.i. to determine if there were differences in spleen size as determined by calculating the splenic index. Mice used for obtaining these results are the same mice as those shown in Figures 3A and 4.

We found that infected mice of both genotypes had significantly higher splenic indices than corresponding uninfected mice (Fig. 5). Male IL-3 -/- and WT mice did not have a significant difference in spleen size with both groups having a splenic index of ~16. Interestingly, there was a significant difference between infected female mice. We observed that female IL-3 -/- had a higher splenic index (~27) than similarly infected WT mice (~18).

FIG 5. Splenic index in uninfected and *P. berghei*-infected male and female WT and IL-3 -/- (KO) mice. Spleen weights of infected mice were determined on day 8 p.i. The splenic index was determined as the ratio of spleen weight to body weight. Data from uninfected male and female mice of each genotype were not significantly different and were pooled. All data are presented as means ± SD from 6 mice per group analyzed individually. $P < 0.001$ (***) and $P < 0.01$ (**) versus corresponding values for uninfected mice or (as indicated by the square brackets) versus corresponding values for mice of the other genotype.


**
***
***
***
***
WT
KO
Splenic index (10-3)
30
20
10
0
Uninfected
Infected (male)
Infected (female)

Discussion

IL-3 is a cytokine produced primarily by activated T-cells and mast cells and has been shown to be critical for nematode-induced mast cell and basophil proliferation, and in promoting delayed-type hypersensitivity reactions (Mach *et al*., 1998). Since the discovery of IL-3 in 1981 (Ihle *et al*., 1981), there have only been a limited number of reports suggesting a role for IL-3 in the pathophysiology of protozoan infections, including malaria. Recently, Auclair *et al.* (2013) have reported that IL-3-deficieint (IL-3 -/-) mice have increased resistance to blood-stage malaria caused by *P. berghei NK65* (Auclair *et al.,* 2013). However, only one strain of *Plasmodium* was examined in this report. *P. berghei* ANKA is a lethal strain of *Plasmodium* that induces symptoms similar to *P. berghei* NK65. However, unlike *P. berghei* NK65, *P. berghei* ANKA also causes cerebral malaria (CM) in mice. Thus, further work is necessary to determine if IL-3 influences the outcome of infection in other *Plasmodium* strains besides *P. berghei* NK65.

In order to understand the significance of IL-3 during a *P. berghei* ANKA infection, we first infected IL-3 -/- and wild-type (WT) mice of both sexes and determined if there were any differences in their survival. We found that there was no significant difference in survival between IL-3 mice and their corresponding WT counterparts (Fig. 2). This is in contrast to results found using *P. berghei* NK65 in which it was observed that male, but not female mice, IL-3 -/- survived significantly longer that male WT mice (Auclair *et al.,* 2013). Although there was no significant difference in mortality between female IL-3 -/- mice and WT mice, we did observe that female mice of both genotypes survived significantly longer than corresponding male mice. A similar finding was

observed in *P. berghei* NK65-infected mice. Rodent malaria mortality rates are generally higher in males than in females and may relate to immunological and sex steroid differences in the sexes (Klein, 2004).

In addition to survival, measurement of blood parasitemia levels are used to monitor the course and severity of infection. The percentage of parasitized RBCs present in an infected animal allows one to determine the susceptibility to infection and the ability of the animal to clear parasitized RBCs. We first examined parasitemia levels in male *P. berghei* ANKA-infected IL-3 -/- and WT mice at days 0, 2, 4, 6, 8, and 10 post-infection p.i. There was no statistically significant difference in parasitemia levels between male IL-3 -/- and WT mice at any time point examined (Fig. 3b). These results correlate with our observation that there is no difference in survival between these infected mice. This is in contrast to results found with *P. berghei* NK65-infected mice, where male IL-3 -/- mice had significantly lower parasitemia at day 8 and 10 p.i. compared to infected male WT mice.

To begin studies examining the influence of IL-3 on the degree of parasitemia present in female mice, we infected both female and male mice with *P. berghei* ANKA and examined parasitemia at day 8 p.i., a time point just prior to when mice begin to suffer significant mortality. As expected, we did not detect a significant difference in parasitemia between male infected IL-3 -/- and WT mice at day 8 p.i. (Fig. 3a). However, we observed that female IL-3 -/- mice had a significantly lower parasitemia at this time point than did female WT mice. Despite there being fewer parasitized RBCs in IL-3 -/- mice, this does not lead to increased survival of the mice as noted above. It is interesting to note that no similar difference was observed in female IL-3 -/- and WT

mice infected with *P. berghei* NK65 (Auclair *et al.,* 2013). A kinetic study of parasitemia in *P. berghei* ANKA-infected IL-3 -/- and WT mice is planned to further characterize our findings in female mice.

The severity of anemia often correlates with the severity of *Plasmodium* infection in mice. Malarial anemia appears to be multifactorial. It involves the destruction of circulating parasitized RBCs, premature destruction of non-parasitized RBCs, as well as suppression of erythroid development in the bone marrow and mouse spleen (Haldar *et al*., 2007). Furthermore, there is a strong correlation between severity of disease and an observed increase in splenic erythropoiesis. We have previously reported that both male and female *P. berghei* NK65-infected IL-3 -/- mice were more anemic as determined by hematocrit values than similarly infected WT mice (Auclair *et al.,* 2013). Furthermore, the decreased anemia was not due to insufficient erythropoiesis occurring in the bone marrow or spleen since it was found that IL-3 -/- mice actually had higher numbers of colony-forming unit erythrocytes (CFU-Es) and burst-forming unit erythrocytes (BFU-Es) present in these organs (Auclair *et al.,* 2013). Therefore, it was hypothesized that IL-3 -/- mice may be more anemic due to increased destruction of parasitized RBCs and/or non-parasitized RBCs by mechanisms such as antibody-mediated opsonization and phagocytosis. We therefore wanted to determine the effects of IL-3 on anemia in *P. berghei* ANKA-infected mice.

We examined hematocrit levels in uninfected and in IL-3 -/- and WT mice infected 8 days with *P. berghei* ANKA. As previously reported, there were no differences in hematocrit levels between uninfected IL-3 -/- and WT mice (Fig. 4). As expected, infection caused a significant decrease in hematocrit levels in all mice compared to

uninfected controls. However, no difference in anemia was observed between infected IL-3 -/- and WT male mice. In contrast, female IL-3 -/- mice were significantly more anemic than corresponding WT mice at day 8 p.i. These results, as well as our findings that infected female IL-3 -/- mice have decreased parasitemia, are difficult to interpret at this time. These results may suggest that IL-3 exerts its effects in *P. berghei* ANKA-infected female, but not male mice, and that the absence of IL-3 results in more efficient removal of parasitized RBCs from the peripheral circulation. Future experiments need to be performed to examine this possibility.

Spleen enlargement or splenomegaly is a common manifestation of malaria in both humans and rodents. Reason for its increase in size is likely due to multiple factors. The spleen is an important site of emergency erythropoiesis, as well as a site of parasitized RBC clearance and immune system activation in response to blood-stage malaria (Lamikanra *et al*., 2007). During infection, parasitized RBCs are normally removed by increasing numbers of macrophages and dendritic cells primarily located in the splenic red pulp. At the same time, there is expansion of parasite-specific lymphocytes occuring in the white pulp of the spleen. These processes together are thought to contribute to the development of splenomegaly (Ing *et al.,* 2006; Mizobuchi *et al.,* 2014).

Because the degree of splenomegaly often impacts the host's ability to mount a successful response to parasites, splenomegaly was examined in male and female IL-3 -/- and WT mice infected with *P. berghei* ANKA by determining the splenic index on day 8 p.i. As shown in Fig. 5, there was no difference in the splenic index of uninfected IL-3 -/- and WT mice. In response to infection, all strain of mice experienced significant

splenomegaly, but the splenic index was significantly higher in female IL-3 -/- mice as compared to infected female WT mice. No difference was observed between male IL-3 -/- and WT mice. Increased splenomegaly in female IL-3 -/- mice does correlate with our findings that female IL-3 -/- mice had lower numbers of parasitized RBCs (parasitemia) and increased anemia. Although it is difficult to speculate at this time, the increased splenomegaly seen in IL-3 -/- mice might contribute to increased clearance of parasitized RBCs, leading to a decrease in the number of circulating RBCs as measured by hematocrit. However, it is important to note that despite these findings, infected female mice did not survive longer than corresponding WT mice.

Overall, these results suggest that IL-3 plays a minor role in susceptibility of female, but not male mice, to blood-stage malaria caused by *P. berghei* ANKA. The fact that IL-3 plays a critical role in suppressing protective immunity to *P. berghei* NK65 but not *P. berghei* ANKA suggests there are key differences in host IL-3-dependent response to these pathogens. Both are lethal murine malarial strains and it is well-known that *P. berghei* ANKA infection leads to the development of experimental CM whereas *P. berghei* NK65 does not. CM caused by *P. berghei* ANKA is believed to be a major cause of mortality and involves increased cytoadherence of parasitized RBCs in the host brain microvasculature, leading to inflammation dominated neutrophils (Porcherie *et al*., 2011). The exact cause of death in mice infected with *P. berghei* NK65 is not known but may be a combination of several factors, including severe anemia and excessive cytokine production. Interestingly, *P. berghei* NK65 does have a higher preference for invading reticulocytes and induces greater liver damage than does *P. berghei* ANKA infection. Whether or not these differences account for the varied

influence of IL-3 on the host response to these two strains of *P. berghei* remains to be determined.

Many questions remain regarding the underlying basis of how IL-3 influences disease outcome during both *P. berghei* ANKA and *P. berghei* NK65 infection. Due to time limitations, many of the time-consuming studies described with *P. berghei* ANKA will need to be repeated since they reflect only one experiment. It will also be important to initiate studies to determine the cellular source IL-3 during infection, the kinetics of IL-3 production, and the target cell(s) which mediate IL-3 actions. We have already initiated studies using multiplex cytokine assays to determine if there are differences in serum levels of cytokines between IL-3 -/- and WT mice infected *P. berghei* ANKA. These are important questions that must be answered in order to determine the mechanism by which IL-3 is functioning, either directly and/or indirectly, in the context *P. berghei* infection. We believe that the studies presented here, together with those reported for *P. berghei* NK65, will help to eventually elucidate the relative contribution of IL-3 in the resulting morbidity and mortality associated with both rodent and human malaria.

References

Auclair, S. R., Roth, K. E., Saunders, B. L., Ogborn, K. M., Sheikh, A. A., Naples, J., Young, A. M. P., Boisen, D. K., Tavangar, A. T., Welch, J. E., Lantz, C. S. (2014). Interleukin-3-deficient mice have increased resistance to blood-stage malaria. *Infection and Immunity* 82: 1308-1314.

Cannistra, S., Vellenga, E., Groshek, P., Rambaldi, A., Griffin, J. (1988). Human granulocyte-monocyte colony-stimulating factor and interleukin 3 stimulate monocyte cytotoxicity through a tumor necrosis factor- dependent mechanism. *Blood* 71(3): 672-676.

Carlson, J., Helmby, H., Hill, A., Brewster, D., Greenwood, B., Wahlgren, M. (1990). Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *Lancet* 336: 1457-1460.

Derbyshire, E. R., Mota, M. M., Clardy, J. (2011). The next opportunity in anti-malaria drug discovery: the liver stage. *Public Library of Science Pathogens* 7: e1002178.

Goldberg, D. E., Slater, A. F., Cerami, A. & Henderson, G. B. (1990). Hemoglobin degradation in the malaria parasite Plasmodium falciparum: an ordered process in a unique organelle. *Proceedings of the National Academy of Sciences* 87: 2931-2935.

Grau G.E., Kindler V., Piguet P.F., Lambert P.H., Vassalli P (1988). Prevention of experimental cerebral malaria by anticytokine antibodies. Interleukin 3 and granulocyte macrophage colony-stimulating factor are intermediates in increased tumor necrosis

factor production and macrophage accumulation. *The Journal of Experimental Medicine* 168: 1499-1504.

Haldar, K., Murphy, S. C., Milner, D. A., & Taylor, T. E. (2007). Malaria: Mechanisms of erythrocytic infection and pathological correlates of severe disease. *Annual Review of Pathology: Mechanisms of Disease 2*(1): 217-249.

Hu, Z., Zhao, W., Shimamura, T. (2007). Regulation of mast cell development by inflammatory factors. *Current Medicinal Chemistry* 14: 3044-3050.

Ing, R., Segura, M., Thawani, N., Tam, M., Stevenson, M. M. (2006). Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation, and antigen presentation. *The Journal of Immunology* 176: 441-450.

Ihle, J. N., Pepersack, L., Rebar, L. (1981). Regulation of T cell differentiation: In vitro induction of 20 alpha-hydroxysteroid dehydrogenase in splenic lymphocytes from athymic mice by a unique lymphokine. *The Journal of Immunology* 126(6): 2184-2189.

Ihle, J. N. (1992). Interleukin-3 and hematopoiesis. *Chemical Immunology* 51: 65-106

Klein, S. L. (2004). Hormonal and immunological mechanisms mediating sex differences in parasite infection. *Parasite Immunology* 26(6-7): 247-264.

Kwiatkowski, D., Hill, A., Sambou, I., Twumasi, P., Castracane, J., Manogue, K., Cerami, A., Brewster, D., Greenwood, B. (1990). TNF concentrations in fatal cerebral, non-fatal cerebral, and uncomplicated plasmodium falciparum malaria. *Lancet* 336: 1201-1204.

Lamikanra, A. A., Brown, D., Potocnik, A., Casals-Pascual, C., Langhorne, J., Roberts, D.J. (2007). Malarial anemia: of mice and men. *Blood* 110: 18-28.

Lantz, C.S, Boesiger, J., Song, C.H, Mach, N., Kobayashi, T., Mulligan, R.C., Nawa, Y., Dranoff, G., Galli, S.J. (1998). Role for interleukin- 3 in mast cells and basophil development and immunity to parasites. *Nature* 392: 90-93.

Mach, N., Lantz, C. S., Galli, S. J., Reznikoff, G., Mihm, M., Small, C., Granstein, R., Beissert, S., Sadelain, M., Mulligan, R.C., Dranoff, G. (1998). Involvement of interleukin-3 in delayed-type hypersensitivity. *Blood* 91(3): 778-783.

Meyer, C.G., Calixto Fernandes, M.H., Intemann, C.D., Kreuels, B., Kobbe, R., Kreuzberg, C., Ayim, M., Ruether, A., Loag, W., Ehmen, C., Adjei, S., Adjei, O., Horstmann, R.D., May, J. (2011). IL3 variant on chromosomal region 5q31-33 and protection from recurrent malaria attacks. *Human Molecular Genetics* 20: 1173-11.

Mizobuchi, H., Yamakoshi, S., Omachi, S., Osada, Y., Sanjoba, C., Goto, Y., Matsumoto, Y. (2014). The accumulation of macrophages expressing myeloid-related protein 8 (MRP8) and MRP14 in the spleen of BALB/cA mice during infection with plasmodium berghei. *Experimental Parasitology* 138(0): 1-8.

National Institute of Allergy and Infectious Diseases. (n.d). *Research agenda for malaria*. Retrieved 19 October 2011 from http://www.niaid.nih.gov/topics/Malaria/Documents/researchagenda.pdf.

Porcherie, A., Mathieu, C., Peronet, R., Schneider, E., Claver, J., Commere, P., Kiefer-Biasizzo, H., Karasuyama, H., Milon, G., Dy, M., Kinet, J., Louis, J., Blank, U., Mecheri,

S. (2011). Critical role of the neutrophil-associated high-affinity receptor for IgE in the pathogenesis of experimental cerebral malaria. *Journal of Experimental Medicine* 208: 2225-2236.

Schaffer, N., Grau, G. E., Hedberg, K., Davachi, F., Lyamba, B., Hightower, A. W., Breman, J. G., Nguyen-Dinh, P. (1991). Tumor necrosis factor and severe malaria. *Journal of Infectious Diseases* 163(1): 96-101.

Silvie, O., Mota, M. M., Matuschewski, K., & Prudêncio, M. (2008). Interactions of the malaria parasite and its mammalian host. *Current Opinion in Microbiology* 11(4): 352-359.

Waitumbi, J. N., Opollo, M. O., Muga, R. O., Misore, A. O., & Stoute, J. A. (2000). Red cell surface changes and erythrophagocytosis in children with severe plasmodium falciparum anemia. *Blood* 95(4): 1481-1486.

Yanez, D. M., Manning, D. D., Cooley, A. J., Weidanz, W. P., & van der Heyde, H. C. (1996). Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *The Journal of Immunology* 157(4): 1620-1624.

Young, D. A., Lowe, L. D., & Clark, S. C. (1990). Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture. Analysis of macrophage antibody-dependent cellular cytotoxicity. *The Journal of Immunology* 145(2): 607-615.