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Interleukin-3-deficient mice have increased resistance to blood-stage malaria

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Dedication

This thesis is dedicated to my family in thanks for all of their love and support throughout my education and my life.

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Abstract

The contribution of interleukin-3 (IL-3), a hematopoietic growth factor and immunoregulatory cytokine, to resistance to blood-stage malaria was investigated by infecting IL-3-deficient (knockout [KO]) mice with *Plasmodium berghei* NK65. The results show that endogenous IL-3 significantly alters the susceptibility of BALB/c mice to *P. berghei* NK65 infection as reflected by measures of survival, anemia, parasitemia, and splenomegaly. Male IL-3 KO mice, but not female mice, were more resistant to infection than wild-type (WT) mice, as evidenced by lower peak parasitemia and prolonged survival. Both male and female IL-3 KO mice had increased splenomegaly and were more anemic than corresponding WT mice. Anemia was compensated for by an increase in bone marrow and splenic erythropoiesis in male IL-3 KO mice, as evidenced by higher levels of erythroid progenitors. *P. berghei* NK65 infection was also shown to induce IL-3-dependent changes in plasma cytokine levels: plasma levels of gamma interferon (IFN-γ) and chemokine (C-X-C motif) ligand 9 (CXCL9 or monokine induced by IFN-γ [MIG]) were found to be significantly reduced in male IL-3 KO mice during early stages of infection. In contrast, granulocyte colony-stimulating factor (G-CSF) levels were significantly higher, and the percentage of peripheral blood neutrophils lower, in *P. berghei* NK65-infected male IL-3 KO mice than in WT counterparts. *P. berghei* NK65 infection induced degranulation of splenic mast cells in both male IL-3 KO and WT mice; however, there was no difference in mast cell numbers between genotypes. Overall, our results indicate that IL-3 plays a critical role in suppressing protective immunity to *P. berghei* NK65 infection and that it is involved in inhibiting the development of splenomegaly, anemia, and erythropoiesis. IL-3 also influences IFN-γ,

CXCL9, and G-CSF production in response to infection. The abnormal responses seen in infected IL-3 KO mice may be due to the lack of IL-3 during development, to the lack of IL-3 in the infected mature mice, or to both.

Introduction

Malaria:

Malaria is a tropical infectious disease caused by protozoan parasites of the genus *Plasmodium* and spread by *Anopheles* mosquitoes. The World Health Organization estimates that malaria infected 207 million people and killed 627,000 in 2012 (WHO, 2013), ranking this disease among the greatest threats to global public health.

Four species of *Plasmodium* have infected humans throughout history: *P. falciparum, P. vivax, P. ovale,* and *P. malariae*; *P. falciparum* is the most virulent species and accounts for most malaria-related mortality (Neghina *et al.,* 2010). In recent years the primate malaria, *P. knowlesi,* has also emerged as a zoonotic human pathogen (Müller and Schlagenhauf, 2014). Malaria – classically characterized by periodic chills, rigors, and high fevers followed by profuse sweating – has been described in the Chinese literature as far back as 2700 B.C., and has had a major impact on human history ever since (Neghina *et al.,* 2010).

Although it was originally observed that malaria was associated with swampy areas and frequently disappeared when the swamps were filled, this was commonly attributed, not to the mosquitoes who bred in the swamps, but to "bad air," giving rise to the Italian name for the disease - *mal aria* (Neghina *et al.,* 2010). The true cause of malaria was not discovered until 1880, when the French military surgeon Alphonse Laveran described parasites found in the blood of malaria patients. Shortly thereafter, British doctor Ronald Ross made the Nobel Prize-winning discovery that malaria was transmitted by mosquitoes (Neghina *et al*., 2010).

The first effective treatment for malaria was quinine, which was isolated from the bark of the Peruvian Chinchona tree by French chemists Joseph Pelletier and Jean Bienaime Caventou in 1820 (Butler *et al.,* 2010). During WWII, American scientists developed a synthetic antimalarial drug called chloroquine, which quickly became the standard of treatment for malaria (Butler *et al.*, 2010). By the 1970s, however, resistance to chloroquine and its derivatives was becoming widespread (Butler *et al.,* 2010). The current treatment of choice for malaria is artemisinin, which is isolated from *qing hao*, an herb long used to treat intermittent fever in traditional Chinese medicine (Butler *et al.*, 2010). The World Health Organization recommends combining artemisinin with other drug therapies in order to prevent the development of resistance. However, the first strains of artemisinin-resistant strains of *Plasmodium* have already been identified at the Thailand-Cambodia border in a series of studies conducted between 2001 and 2009 (WHO 2011).

The intensive Roll Back Malaria Partnership launched in 1998 has reduced malaria cases by 50% in 43 countries over the past decade; unfortunately, the development of artemisinin-resistant strains puts these gains at risk, renewing interest in the development of a malaria vaccine (WHO, 2011). In spite of the fact that studies conducted in the 1970s indicated that humans could be immunized against malaria by the bites of large numbers of irradiated, infected mosquitoes (Clyde *et al.,* 1975), attempts to create a practical malaria vaccine have been largely unsuccessful. The most promising vaccine candidate to date is the sporozoites subunit vaccine RTS,S, which has shown 30 – 50% efficacy in clinical trials; however, protection is relatively short-lived (Arama *et*

al., 2014). The development of an effective vaccine depends on obtaining a detailed understanding of the immune response to malaria infection.

Malaria Life Cycle:

Malaria infection begins when an infected female *Anopheles* mosquito takes a blood meal from a human and injects salivary fluids containing motile, spindle-shaped sporozoites into the wound (Despommier *et al.,* 2006). The sporozoites migrate to the liver, where they invade liver cells and undergo asexual division until the host cells burst, releasing thousands of merozoites into the bloodstream (Despommier *et al.,* 2006). The merozoites invade red blood cells (RBCs), feeding on hemoglobin and growing into the larger, ring-shaped trophozoites. The parasite then undergoes asexual division to become a schizont: a large, multinucleate cell containing more merozoites. The RBC eventually ruptures, releasing the merozoites into the bloodstream to infect more RBCs and continue the cycle (Despommier *et al.,* 2006). Some of the merozoites differentiate into gametocytes, the parasite's sexual form. Gametocytes can only complete their life cycle within the gut of an *Anopheles* mosquito. Once ingested as part of the mosquito's blood meal, the "male" microgametocytes fertilize the "female" macrogametocytes (Despommier *et al.,* 2006). The resulting zygotes develop into oocysts, each containing over a thousand sporozoites. Once the sporozoites emerge from the oocyst, they migrate to the mosquito's salivary glands, ready to infect the mosquito's next blood meal (Despommier *et al.,* 2006) (Figure 1).

Clinical Aspects and Pathology of Malaria:

Several species of *Plasmodium* infect humans, but *P. falciparum* causes the most severe disease and nearly all mortality (Despommier *et al.,* 2006). The pathogenic

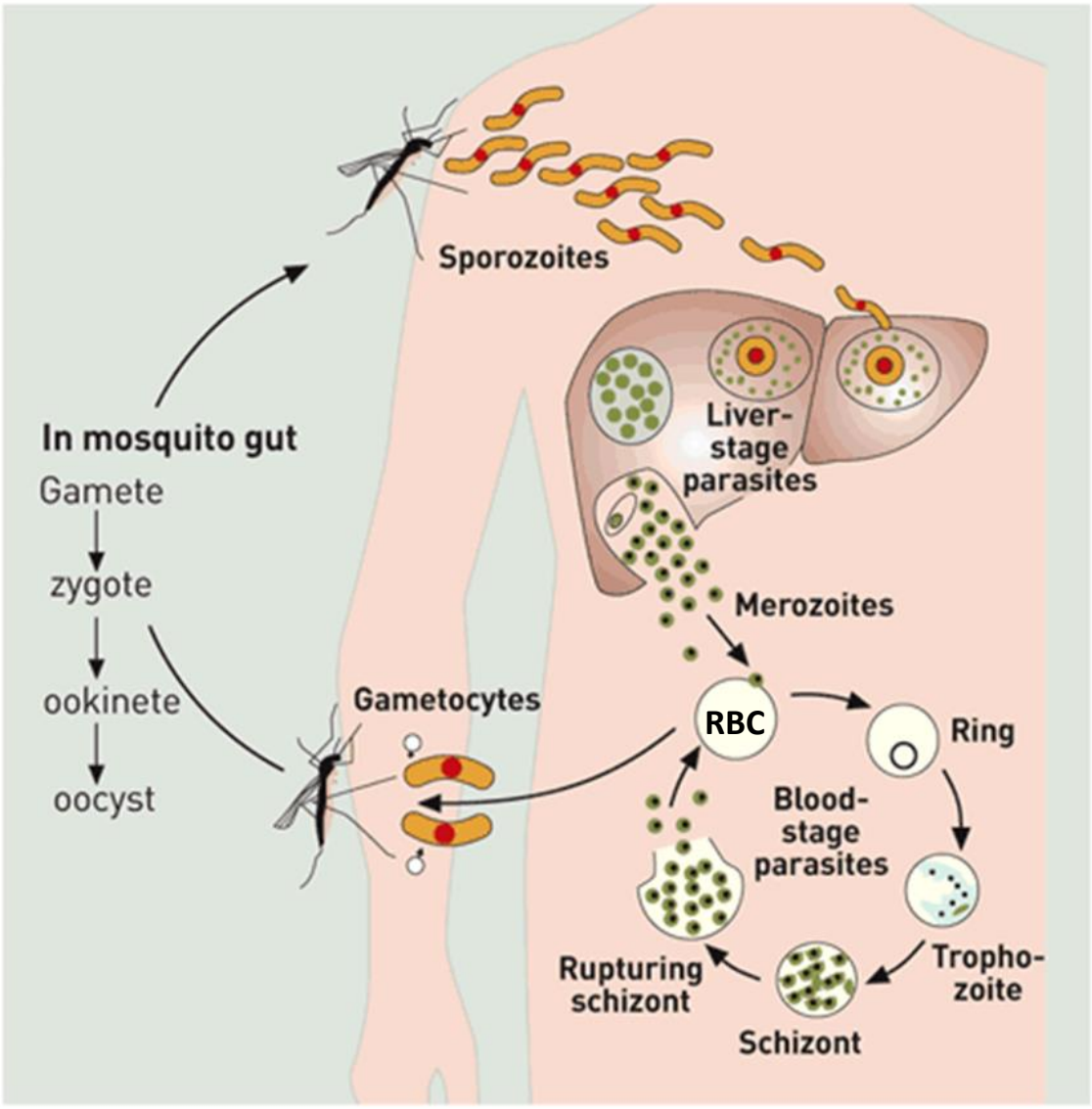

Sporozoites
Liver-
stage
parasites
In mosquito gut
Gamete
zygote
ookinete
oocyst
Merozoites
Gametocytes
RBC
Ring
Blood-
stage
parasites
Rupturing
schizont
Tropho-
zoite
Schizont

Figure 1. Malaria life cycle in both the human host and the mosquito vector (adapted from Thayer, 2005.)

consequences of malaria infection are caused by the erythrocytic cycle (also called the "blood-stage"), during which circulating merozoites repeatedly infect and lyse RBCs. The lysed cells release pyrogens – inflammatory substances which cause the periodic chills, fever, and sweating which characterize the acute phase of malaria infection (Despommier *et al.,* 2006). About 10% of *P. falciparum* infections develop serious complications, including severe anemia, respiratory distress, and cerebral malaria. The over-all mortality rate of *P. falciparum* is 1%, but severe malaria involving one of these complications has a mortality rate of 10 – 50% (Wiser, 2011).

The pathology of anemia involves a number of factors: the severity of anemia correlates with the degree of parasitemia and is caused by the destruction of RBCs, bone marrow suppression, and increased osmotic fragility of uninfected RBCs (Lamikanra *et al.,* 2007). Respiratory distress syndrome has only relatively recently been recognized as an important malaria complication, and is actually a better predictor of mortality than severe anemia or cerebral malaria (Wiser, 2011). As RBCs are destroyed, the uptake of oxygen and release of carbon dioxide becomes impaired. Excess carbon dioxide raises the acidity of the blood, a condition known as metabolic acidosis. The disturbed pH balance causes fluid to build up in the lungs, leading to difficulty breathing and eventual respiratory failure (Taylor *et al.,* 2012).

Cerebral malaria, long considered the most severe complication of malaria infection, is caused by a complex interplay of parasite virulence, host susceptibility, and immunopathology that is still not completely understood. Cerebral malaria is characterized by mechanical occlusion and inflammation of the cerebral microvasculature

and breakdown of the blood-brain barrier (Shikani *et al.*, 2012). Briefly, parasitized RBCs (pRBCs) adhere to the inner walls of capillaries in the brain, causing local over-production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) (Wiser, 2011). The pRBCs also adhere to one another, forming large clumps called *rosettes* which can physically block capillaries (Wiser, 2011). Although both of these phenomena have been associated with cerebral malaria, the relative contribution of each, and the exact mechanisms by which they lead to clinical disease, have not yet been fully explicated (Wiser, 2011). The symptoms of cerebral malaria include severe headache and fever progressing to impaired consciousness and other neurological symptoms such as convulsions, vomiting, and respiratory distress. If not treated, cerebral malaria can quickly progress to coma and death (Wiser, 2011).

Malaria Immunology and Immunopathology:

The initial response to malaria infection is a cellular response characterized by the release of pro-inflammatory cytokines such as IFN-γ and TNF-α, which activate macrophages to more efficiently phagocytize parasites and pRBCs (Angulo and Fresno, 2002; Langhorne *et al.*, 2008). Although IFN-γ and TNF-α have both been shown to be essential to parasite clearance, over-production of these cytokines can cause pathology, including cerebral malaria (Angulo and Fresno, 2002; Langhorne *et al.*, 2008). At 7 - 10 days postinfection (p.i.), the anti-inflammatory Th2 cytokine IL-4 begins to down-regulate cellular immunity, including the production of IFN-γ, and stimulate humoral immunity and antibody production (Helmby *et al.*, 1998). As *Plasmodium* parasites are both intra- and extra-cellular at different stages of their life cycle, both cellular and

humoral immunity are believed to be essential to parasite clearance (Angulo and Fresno, 2002; Langhorne *et al.,* 2008).

Studies indicate that the most effective anti-malaria antibodies are the opsonizing antibodies IgG1 and IgG3, which enhance the phagocytosis of parasites and infected RBCs (Perlmann and Troye-Blomberg, 2000; Langhorne *et al.*. 2008). In contrast, IgE antibodies have been shown to exacerbate pathology by forming immune complexes which cross-link receptors on monocytes and endothelial cells, stimulating them to release TNF-α (Perlmann and Troye-Blomberg, 2000). Overproduction of TNF-α in the blood vessels of the brain can lead to inflammation and cerebral malaria (Perlmann and Troye-Blomberg, 2000).

Unlike many diseases, recovery from malaria infection does not result in classical "sterile" immunity, in which previously encountered pathogens are quickly eliminated before causing symptoms. This is due in part to the fact that *Plasmodium* parasites change their surface antigens to avoid recognition by the adaptive immune system (Wiser, 2011). However, people living in endemic areas do develop a degree of resistance to the clinical manifestations of malaria infection, a phenomenon known as premunition (Angulo and Fresno, 2002; Langhorne *et al.,* 2008). This is why the majority of patients who die of malaria are young children – they have not yet experienced enough exposures to develop premunition (Langhorne *et al.,* 2008).

Mouse Models of Malaria:

Animal studies, primarily in rodents, have played a key role in elucidating the mechanisms by which *Plasmodium* infection causes disease as well as the host immune response and immunopathology. Four species of *Plasmodium* naturally infect African

rodents: *P. vinckei, P. berghei, P. chabaudi,* and *P. yoelii* (Stephens *et al.*, 2012); the last three of these are commonly used to infect mice in the laboratory (Zuzarte-Luis *et al*., 2014). Mice may be infected via mosquito bite in order to study both the exoerythrocytic and the erythrocytic stages of *Plasmodium* infection; alternately, mice may be infected via intravenous (i.v.) or intraperitoneal (i.p.) injection in order to study the erythrocytic cycle alone (Zuzarte-Luis *et al*., 2014).

The course and outcome of infection differ depending on both the *Plasmodium* species and the strain of mouse used. For example, *P. berghei* ANKA causes cerebral malaria in C57BL/6 but not BALB/c mice, although it is fatal in both species (Zuzarte-Luis *et al*., 2014). *P. chabaudi* is non-lethal in most mouse strains, while *P. yoelii* may be lethal or non-lethal depending on the parasite strain (Stephens *et al.,* 2012; Zuzarte-Luis *et al*., 2014).

P. berghei NK65 does not cause cerebral malaria but is fatal in BALB/c mice, typically within 2 weeks postinfection (Lacerda-Queiroz *et al.,* 2011). Mice infected with *P.berghei* NK65 develop many of the same symptoms as humans, including weight loss, severe anemia, splenomegaly, and immune-mediated liver injury (which is caused by blood-stage malaria infection as distinct from the destruction of hepatocytes during the exoerythrocytic stage) (Zuzarte-Luis et al., 2014). Although *P. berghei* NK65 infection does not cause the neurological symptoms of classical experimental cerebral malaria (ataxia, paralysis, convulsions, and coma followed by death), *P. berghei* NK65-infected BALB/c mice do show increased cerebral vascular permeability and levels of leukocyte rolling and adhesion comparable to that seen in *P. berghei* ANKA infection (Lacerda-

Queiroz et al., 2011). Infected mice typically develop a rough hair coat and lethargy, eventually progressing to complete prostration shortly before death. (Figure 2).

Interleukin-3:

Interleukin-3 (IL-3) is a 28-kDa glycoprotein of 133 amino acids in humans and 140 amino acids in mice (Schrader, 1998). It was first discovered as a factor produced by activated T cells and the murine myelocytic leukemia cell line WEHI-3B, which induced the expression of the T cell associated, progesterone-reducing enzyme 20α-hydroxysteroid dehydrogenase in nude mouse splenocytes *in vitro* (Ihle, 1992; Weinstein, 1977). It was shown that this factor induced the *in vitro* proliferation of cells of the myeloid lineage in cell culture: mast cells, neutrophils, macrophages, eosinophils, megakaryocytes, and erythroid progenitors (Schrader, 1998). IL-3 has a 4-helix-bundle structure which it shares with a number of colony stimulating factors, growth factors, and cytokines (Schrader, 1998). In particular, IL-3 shares a number of structural and functional similarities with interleukin-5 (IL-5) and granulocyte macrophage colony stimulating factor (GM-CSF), with which it forms a cytokine family (Broughton *et al.*, 2012). IL-3 exerts its biological effects through a receptor that is composed of a ligand-specific α (IL3RA) subunit and a signal transducing β subunit (IL3RB) common to IL- 3/IL-5/GM-CSF. Unlike in humans, the mouse IL-3 receptor has two distinct β subunits, one that functions only in IL-3-mediated cell signaling (βIL3) and a second that is shared with IL-5 and GM-CSF (IL3RB) (Itoh *et al.*, 1990).

IL-3 is expressed mainly by activated T lymphocytes and mast cells, however, eosinophils, keratinocytes, NK cells and endothelial cells have been reported to secrete this cytokine under some circumstances (Broughton *et al*., 2012). IL-3 is not produced


A


B

Figure 2. Image of a healthy (A) and a *P. berghei* NK65-infected (B) BALB/c mouse.

constitutively and is dispensable for steady-state hematopoiesis. *In vivo* administration of IL-3 can enhance hematopoiesis in mice and experimental primates (Marone *et al.,* 2000). In addition to supporting the survival and proliferation of myeloid progenitor cells, IL-3 also enhances the functions of a variety of cell types. In *in vitro* studies, IL-3 enhances macrophage antigen presentation (Frendl and Beller, 1990), cytotoxicity, and adherence (Mach *et al.,* 1998), promotes mast cell growth, differentiation, and mediator release (Hu *et al.,* 2007, Gurish and Boyce, 2002), and promotes basophil development and enhances the release of histamine, IL-4, and IL-6 (Voehringer, 2012, Karasuyama *et al.,* 2011, Lantz *et al.,* 2008). Mast cell precursors are particularly sensitive to the growth promoting properties of IL-3. For example, when mouse hematopoietic cells (e.g. bone marrow cells) are maintained *in vitro* with IL-3 as the only exogenous cytokine, an essentially homogeneous population of mast cells develop within approximately 3-4 weeks. IL-3 also enhances inflammation by up-regulating the expression of adhesion molecules on endothelial cells (Khew-Goodall *et al.,* 2006) and promoting the adhesion of basophils and eosinophils to the endothelium in preparation for tissue infiltration (Lim *et al.,* 2006). Finally, IL-3 has been shown to promote the secretion of the important inflammatory cytokine TNF-α by antigen-activated monocytes (Cannistra *et al.,* 1988), and modulates the development of regulatory T (Treg) cells (Srivastava *et al.,* 2011). As several of the functions of IL-3 enhance the inflammatory immune response, IL-3 may influence the outcome of malaria infection by enhancing the inflammatory immune response. This could be beneficial or detrimental depending on the strength and timing of the inflammatory response (Angulo and Fresno, 2002; Langhorne *et al.,* 2008).

In order to obtain a better understanding of the role of endogenous IL-3 *in vivo,* IL-3-deficient ("knockout" or KO) mice were generated by homologous recombination in embryonic stem cells in (Mach *et al.,* 1998). As expected, IL-3 KO mice showed no deficiency in steady-state hematopoiesis and had normal numbers of basophils and tissue mast cells (Mach *et al.,* 1998; Lantz *et al.,* 1998). These mice did, however, have an attenuated delayed-type contact hypersensitivity response to epicutaneously applied haptens (Mach *et al.,* 1998). In addition, IL-3 KO mice infected with the intestinal nematode *Strongyloides venezuelensis* have an attenuated immune response that results in delayed worm expulsion and prolonged shedding of parasite eggs in IL-3 KO mice (Lantz *et al.,* 1998). *S. venezuelensis* infection in mice is normally resolved with a T cell-dependent immune response that includes extensive mast cell hyperplasia in the intestinal mucosa as well as increased numbers of basophils in the bone marrow (Lantz *et al.,* 1998). *S. venezuelensis*-infected IL-3 KO mice, however, showed no increase in bone marrow basophils over baseline and substantially decreased mast cell hyperplasia (Lantz *et al.,* 1998). Similar experiments using IL-3 KO mice infected with the intestinal nematode *Nippostrongylus brasiliensis* showed that IL-3 was necessary for increased basophil numbers in blood and bone marrow as well as enhancing the production of IL-4 by IgE-stimulated basophils (Lantz *et al.,* 2008). Over-all, these results support the hypothesis that IL-3 acts as a link between the hematopoietic and immune systems, and is particularly important as a promoter of the development, survival, and functions of mast cells and basophils (Marone *et al.,* 2000).

Interleukin-3 and Malaria:

Since the initial description of IL-3 in the early 1980s, a potential role for IL-3 in the pathophysiology of malaria has rarely been reported. An increase in IL-3 serum levels was detected in *P. chabaudi*-infected mice preceding and around the time of peak parasitemia (Helmby *et al.,* 1998). This increase was accompanied by an increase in non-B non-T cells (later recognized as basophils) secreting IL-4 and IL-6 in response to stimulation with IL-3, suggesting that IL-3 could play a role in the switch from Th1 to Th2 immunity (Helmby *et al.,* 1998, Poorafshar *et al.,* 2000). Likewise, IL-3-dependent mast cells and their products have also been shown to influence the outcome of malaria infection. Increases in splenic mast cells have been reported in response to malaria infection, and mast cell-derived TNF-α appears to exert a protective effect on immunity to malaria (Furuta *et al.,* 2006). Infection-activated mast cells also release Flt3 ligand, which is necessary for the expansion of dendritic cells in response to malaria infections (Guermonprez *et al.,* 2013). Grau *et al*. have shown that treatment of *P. berghei* NK65-infected CBA mice with anti-IL-3 and anti-GM-CSF antibodies prevents development of cerebral malaria and prolongs survival, possibly by preventing increases in TNF-α (Grau *et al.,* 1988). It was also demonstrated that IL-3 may increase adhesion of parasitized erythrocytes to endothelial cells and promotes pRBC rosetting, a phenomenon associated with the pathophysiology of severe malaria (Carlson *et al.,* 1990). More recently, a genetic survey of Ghanian children found that certain single nucleotide polymorphisms (SNPs) in the IL-3 gene exert a protective effect against recurrent malaria attacks (Meyer *et al.,* 2011). However, it is not yet known whether these SNPs result in gain, loss, or

change of IL-3 function, so it cannot be ascertained whether IL-3 exerts a protective or detrimental effect on malaria infection (Meyer *et al.,* 2011).

General Research Strategy:

A limited number of studies have reported that IL-3 may be involved in the susceptibility of mice to infection with *Plasmodium.* However, the precise role of IL-3 in malaria infection has yet to be addressed. For this project, BALB/c IL-3 KO mice and their wild type (WT) counterparts were used to test two major but related hypotheses: 1) that endogenous IL-3 significantly alters the susceptibility of BALB/c mice to *Plasmodium berghei* NK65 infection as reflected by measures of survival, anemia, parasitemia, and splenomegaly, and 2) that *Plasmodium berghei* NK65 infection induces IL-3-dependent changes in the levels of plasma cytokines and tissue mast cells.

Aim 1. Assess the extent to which IL-3 influences the course and outcome of disease in *Plasmodium berghei* NK65-infected BALB/c mice. BALB/c IL-3 KO mice and WT mice infected with *P. berghei* NK65 were used to test the hypothesis that endogenous IL-3 influences the course and outcome of the infection as monitored by survival, anemia, parasitemia, splenomegaly, and mature and immature RBC levels. The kinetics of IL-3 production during the course of the infection were evaluated by assaying IL-3 secretion from antigen-stimulated splenocyte cultures using cells obtained from *P. berghei* NK65-infected WT mice.

Aim 2. Assess the extent to which IL-3 influences cytokine levels and tissue mast cells in *Plasmodium berghei* NK65-infected BALB/c mice. BALB/c IL-3 KO mice and WT mice infected with *P. berghei* NK65 were used to test the hypothesis that endogenous IL-3 alters the levels of plasma cytokines as measured by a 32-analyte

multiplex cytokine immunoassay, and that the absence of IL-3 during infection results in reduced numbers of detectable splenic mast cells.

Materials and Methods

Mice. The production of BALB/c IL-3-deficient mice and many of the phenotypic characteristics of these mice have been described in detail (Mach *et al.,* 1998). Male and female BALB/c mice (purchased from The Jackson Laboratory, Bar Harbor, ME) were used as wild-type (WT) controls. For these studies, mice were 8 to 12 weeks of age at the beginning of the experiments. In individual experiments, mice of the same approximate age (within 1 week) and size were used. Mice were housed in a positive, individually ventilated caging system (Allentown Inc., Allentown, NJ) in the Department of Biology's animal facility under a 14-h/10-h light/dark cycle and were maintained and used in accordance with James Madison University's Institutional Animal Care guidelines.

***Plasmodium berghei* NK65 infection and evaluation of disease.** Cryopreserved *P. berghei* NK65A (ATCC® 50175™) was passaged once through WT BALB/c mice before being used to infect experimental animals. Mice were infected with blood stages of *P. berghei* NK65 by injecting 10^5 pRBC i.p. Blood collected from the retro-orbital sinus into heparinized tubes was used to determine hematocrit, and the plasma was stored frozen at -80°C. Parasitemia was monitored by examination of Giemsa-stained blood films using light microscopy. Spleens from uninfected and infected mice were harvested at the indicated days postinfection with *P. berghei* NK65. The splenic index for each individual mouse was calculated based on the spleen weight (mg)/body weight (g) x 1000. Infected mice used for mortality studies were monitored daily beginning at day 1 p.i.

Quantification of blood neutrophils and splenic mast cells. The proportion of neutrophils in blood was determined by counting 200 total leukocytes present in May-Grünwald Giemsa-stained blood films. Spleen sections were fixed in 10% buffered formalin, and 5 µm paraffin sectioning and staining was performed by Histo-Scientific Research Laboratories (Mt. Jackson, VA). Pinacyanol erythrosinate stain was used to identify splenic mast cells, and the numbers were quantified according to area (mm^2) using computer-generated image analysis (Nikon NIS Elements software, version 3.22.11).

Hematoxylin and eosin staining. Additional slides were stained with hematoxylin and eosin (H&E) for general histological evaluation. Spleen sections were fixed in 10% buffered formalin, and 5 µm paraffin sectioning and staining was performed by Histo-Scientific Research Laboratories (Mt. Jackson, VA).

Erythroid progenitor assays. Single-cell suspensions were prepared from bone marrow and spleen in Iscove's modified Dulbecco's medium (Lonza, Walkersville, MD) containing 5% fetal bovine serum (FBS), and erythrocytes were lysed with RBC lysis solution (Miltenyi Biotec Inc., Auburn, CA). Cell suspensions were pooled according to genotype, and colony assays were performed by plating cells in MethoCult (StemCell Technologies, Vancouver, BC, Canada) containing appropriate cytokines according to the manufacturer's instructions. For CFU-erythroids (CFU-Es), cells were plated in M3334 MethoCult already containing erythropoietin; for mature burst-forming-unit-erythroids (BFU-Es), cells were plated in M3436 MethoCult, which contains a proprietary combination of cytokines, including erythropoietin. Bone marrow cells were plated at 3 x 10^5/35-mm dish and spleen cells were plated at 2 x 10^5/35-mm dish, and the dishes were

incubated in triplicate at 37°C and 5% CO^2. CFU-E and BFU-E colonies were enumerated using an inverted microscope on days 2 and 10 of culture, respectively.

Preparation of *P. berghei* NK65 antigens. *Plasmodium berghei* NK65 crude antigen, used to stimulate splenocytes *in vitro*, was prepared as described previously (Amante, 2001). Briefly, *P. berghei* NK65 pRBCs collected from 10 WT mice at day 8 p.i. were centrifuged twice in PBS, and cells were lysed twice by adding distilled water and centrifuging at 290 x g, 4°C, for 10 min. The suspension was then centrifuged twice in PBS at 16,000 x g, 4°C, and the pellet was subjected to three cycles of freezing at -80°C and thawing at 37°C in 10 ml PBS. The lysate was further disrupted by passing through a 1-ml syringe with a 26-gauge needle seven times. RBCs collected from uninfected WT mice were subjected to the same procedure in order to obtain control antigen. All lysates were quantified using the Micro-BCA (bicinchoninic acid) protein assay kit (Thermo Scientific, Rockford, IL) and frozen at -80°C until use.

IL-3 production by cultured splenocytes. Spleens were removed aseptically at the indicated times, and single-cell suspensions were prepared by homogenizing through a 70 µm cell strainer. Following hematolysis with RBC lysis solution, the cell suspensions were washed and suspended in RPMI 1640 medium (Lonza BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS along with 100 U penicillin and 100 µg of streptomycin per ml. The cells were cultured at 5 x 10^6 cells/well in 1 ml of complete RPMI 1640 medium in 24-well plates for 48 h at 37°C and 5% CO^2. Cells were cultured either in the presence of *P. berghei* NK65 crude antigen (100 µg/ml), in medium alone, or with control antigen prepared from uninfected RBCs. Concanavalin A (2 µg/ml)

was used as a positive control. Supernatants were frozen at -80°C until assayed for IL-3 by enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA).

Quantification of cytokines. Plasma cytokine/chemokine levels were assayed in duplicate using a 32-analyte multiplex cytokine immunoassay based on xMAP technology (no. MCYTMAG-70K-PX32; Millipore Milliplex, Billerica, MA) on a MAGPIX multiplexing instrument (Luminex Technologies, Austin, TX). Mouse cytokines/chemokines assayed included eotaxin, G-CSF, GM-CSF, gamma interferon (IFN-γ), IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, keratinocyte-derived chemokine (KC), leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), monocyte chemoattractant protein 1 (MCP-1), macrophage colony stimulating factor (M-CSF), monokine induced by IFN-γ (MIG or CXCL9), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, MIP-2, RANTES, TNF-α, and vascular endothelial growth factor (VEGF).

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 post-test 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

Male, but not female, *P. berghei* NK65-infected IL-3 KO mice have prolonged survival and reduced parasitemia compared to WT counterparts.

P. berghei NK65 is uniformly fatal in BALB/c mice, often within 10 days p.i. In order to test the effect of IL-3 deficiency on mortality, 7 WT and KO mice of each sex were infected with 10^5 *P. berghei* NK65-infected RBCs via i.p. injection. While all 7 male WT mice died 8 – 13 days p.i., male IL-3 KO mice survived significantly longer, with 4 dying 14 – 19 days p.i. and the remaining 3 surviving 3 weeks or longer before succumbing to death (Figure 3A). In contrast, there was no significant difference between female WT and KO mortality, with the majority of mice in both genotypes surviving past 3 weeks (Figure 3B). Correlating with their prolonged survival, male IL-3 KO mice also have lower % parasitemia compared to WT mice, with the difference becoming significant by days 8 - 10 p.i., shortly before the death of the WT mice (Figure 3C.) In contrast, no significant difference in parasitemia was detected in female WT and KO mice at day 10 p.i. (WT: 15.9 ± 4.2; KO: 17.6 ± 2.9; n = 7/group; p = 0.40).

Both male and female *P. berghei* NK65-infected IL-3 KO mice have increased splenomegaly compared to WT counterparts, but male mice had no increase in splenic mast cells.

The spleen plays a major role in the host immune response to malaria infection, and spleen enlargement (splenomegaly) is a major symptom of malaria infection in both humans and rodents. The effect of IL-3 deficiency on the development of splenomegaly in *P. berghei* NK65-infected mice was determined by measuring the splenic indices of both male and female IL-3 KO and WT mice at day 8 p.i. (males) and day 10 p.i.

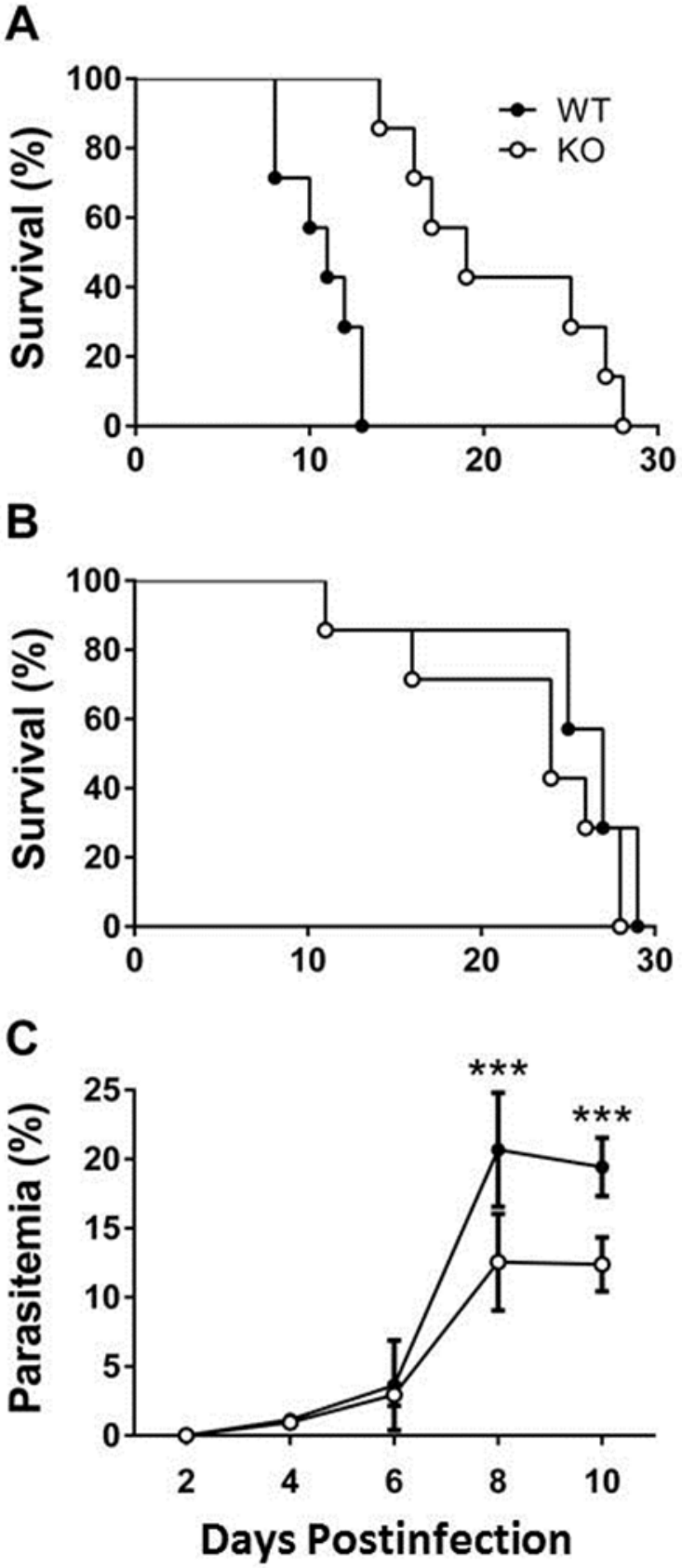

A
Survival (%)
100
80
60
40
20
0
0
10
20
30
WT
KO
B
Survival (%)
100
80
60
40
20
0
0
10
20
30
C
Parasitemia (%)
25
20
15
10
5
0
2
4
6
8
10
***
***
Days Postinfection

Figure 3. Survival rate of *P. berghei* NK65-infected male (A) and female (B) WT and IL-3 KO mice (n = 7 mice/group). A statistically significant difference in survival between infected male WT and IL-3 KO mice was observed ($P = 0.0002$). (C) Time course of parasitemia in *P. berghei* NK65-infected male WT and IL-3 KO. ***, $P < 0.001$ versus corresponding values for mice of the other genotype. Data are presented as means ± SD and are representative of two (C) or three (A and B) independent experiments**.**

(females). As shown in Figure 4, there is no significant difference between the splenic indices of uninfected male or female mice of either genotype. Infected males and females of both genotypes developed significant splenomegaly compared to uninfected mice, with infected IL-3 KO mice having significantly higher splenic indices than infected WT mice (Figure 4).

Mast cells have been shown to influence the immunopathology of malaria infection in rodents (Furuta *et al.*, 2006; Guermonprez *et al*., 2013). Since IL-3 supports the proliferation of mast cells, mast cells were counted in pinacyanol erythrosinate stained spleen sections taken from uninfected and *P*. *berghei* NK65-infected male WT and KO mice (Figure 5). WT and KO mice had similar numbers of mast cells/mm^2 of tissue both before and after infection; however, both WT and KO had a significant decrease in splenic mast cell counts after infection (Table 1.)

Hematoxylin-eosin (H&E) staining was also performed on spleen sections in order to compare the relative areas of red and white pulp between uninfected and *P. berghei* NK65-infected male WT and KO mice (Figure 6). Spleens of uninfected WT and KO mice were similar, with well-defined areas of white pulp surrounded by a visible marginal zone separating them from the red pulp. After infection, however, the spleens of both WT and KO mice showed a dramatic loss of definition between the red and white pulp and shrinkage of the marginal zone (Figure 6).

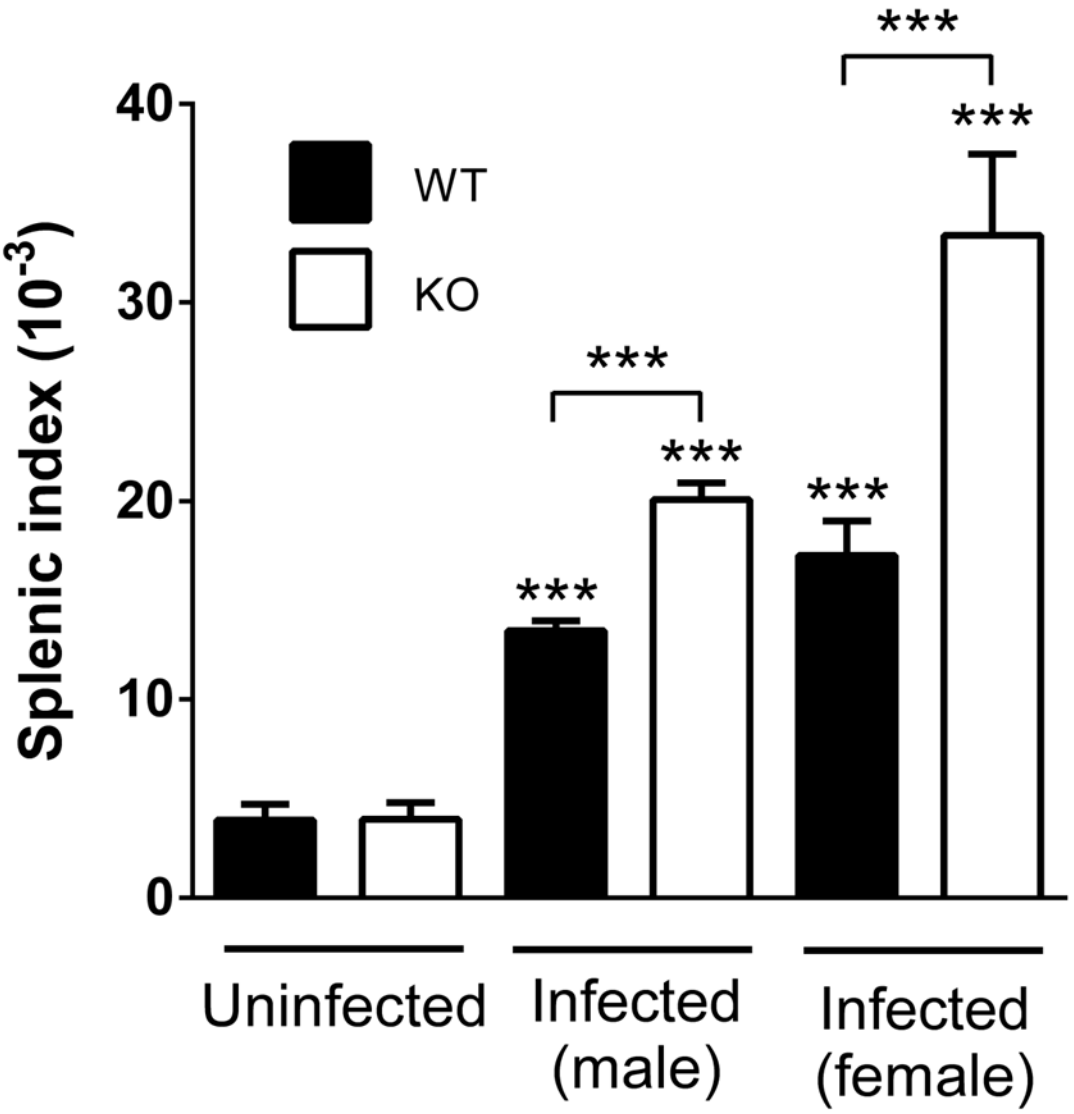

Splenic index (10^{-3})
40
30
20
10
0
WT
KO
***
***
***
***
***
Uninfected
Infected (male)
Infected (female)

Figure 4. Splenic index in uninfected and *P. berghei* NK65-infected male and female WT and IL-3 KO mice. Spleen weights of infected mice were determined on day 8 (male) and day 10 (female) 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 4 to 6 mice per group analyzed individually. ***, $P < 0.001$ versus corresponding values for uninfected mice or (as indicated by the square brackets) versus corresponding values for mice of the other genotype. The data are representative of three independent experiments.

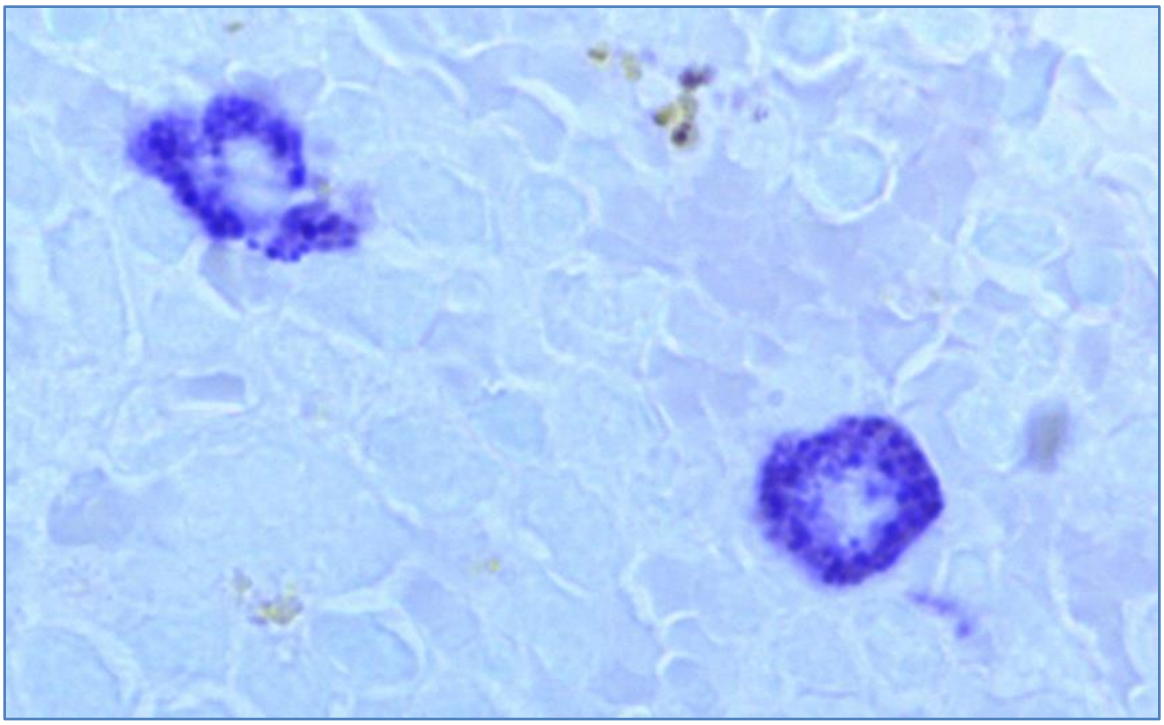


Figure 5. Granulated mast cells shown in a 5µm spleen section. Spleen sections were paraffin-embedded, formalin-fixed, stained with pinacyanol erythrosinate, and viewed at 1000X magnification.

Table 1. Mast cells/mm^2 counted in 5 µm spleen sections taken from uninfected and *P. berghei* NK65-infected (day 8 p.i.) male WT and KO mice. Spleen sections were paraffin-embedded, formalin-fixed, and stained with pinacyanol erythrosinate.

	Mast cells/mm^2	
	WT	KO
Uninfected	14.1 ± 2.7	16.0 ± 4.1
Infected	2.6 ± 2.0[a]	1.9 ±0.6[a]

[a]Statistically significant difference compared to uninfected mice of the same genotype ($P < 0.01$).

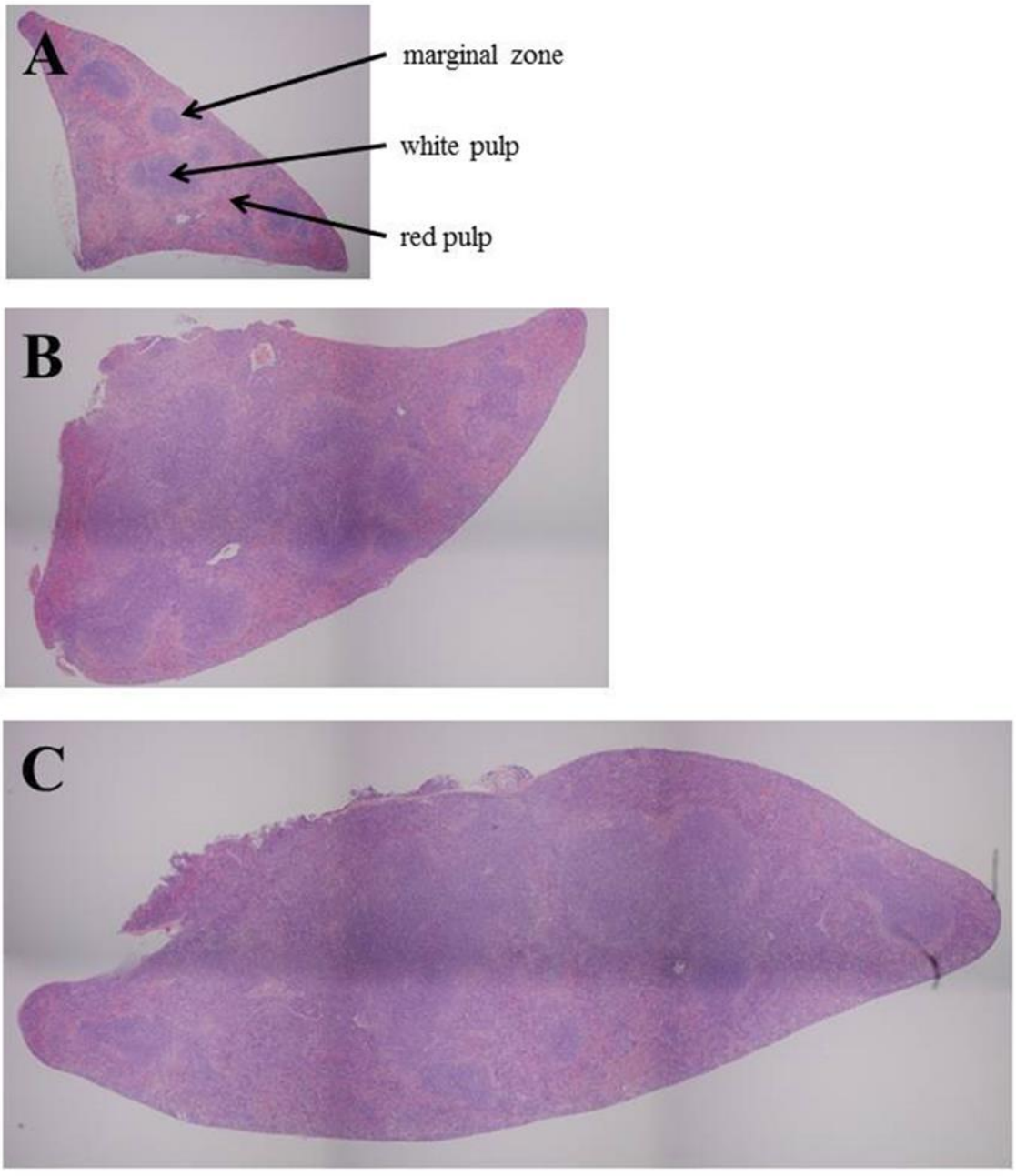

A
marginal zone
white pulp
red pulp
B
C

Figure 6. H&E-stained spleen sections from (A) uninfected, (B) *P. berghei* NK65-infected male WT, and (C) *P. berghei*-NK65 infected male KO mice. Sections viewed at 100X magnification.

P. berghei NK65-infected IL-3 KO mice have increased anemia and erythropoiesis compared to WT counterparts

A major cause of both morbidity and mortality in *Plasmodium*-infected humans and rodents is anemia due to the destruction of infected and uninfected RBCs as well as infection-induced suppression of erythropoiesis. In order to determine the effect of IL-3 on *P. berghei* NK65-induced anemia, hematocrits were measured in male and female mice of both genotypes at day 8 p.i. (males and females) and at day 10 p.i. (females). Male and female mice of both genotypes had decreased hematocrits compared to uninfected counterparts, but *P. berghei* NK65-infected IL-3 KO mice had significantly lower hematocrits than similarly infected WT mice of the same gender (Figure 7). Some studies have suggested that IL-3 is involved in erythropoiesis (Change and Stevenson, 2004). Thus, the potential ability of IL-3 to act as an erythropoietic growth factor makes suppression of erythropoiesis an especially pertinent factor in the development of anemia in response to malaria infection. In order to determine the effect of IL-3 deficiency on erythropoiesis, we determined the number of erythrocyte precursors in the bone marrow and spleens of male WT and IL-3 KO mice at 8 days p.i. Prior to infection, there was no significant difference in the number of blast-forming units erythroid (BFU-Es) or colony-forming units erythroid (CFU-Es) in either spleen or bone marrow (Figure 8, Table 2). At day 8 p.i., BFU-Es were significantly elevated in KO, but not WT, spleens and bone marrow. CFU-Es were significantly elevated in the spleen and bone marrow of both WT and KO mice, but KO mice had significantly higher levels of CFU-Es in both bone marrow and spleen compared to WT mice (Table 2).

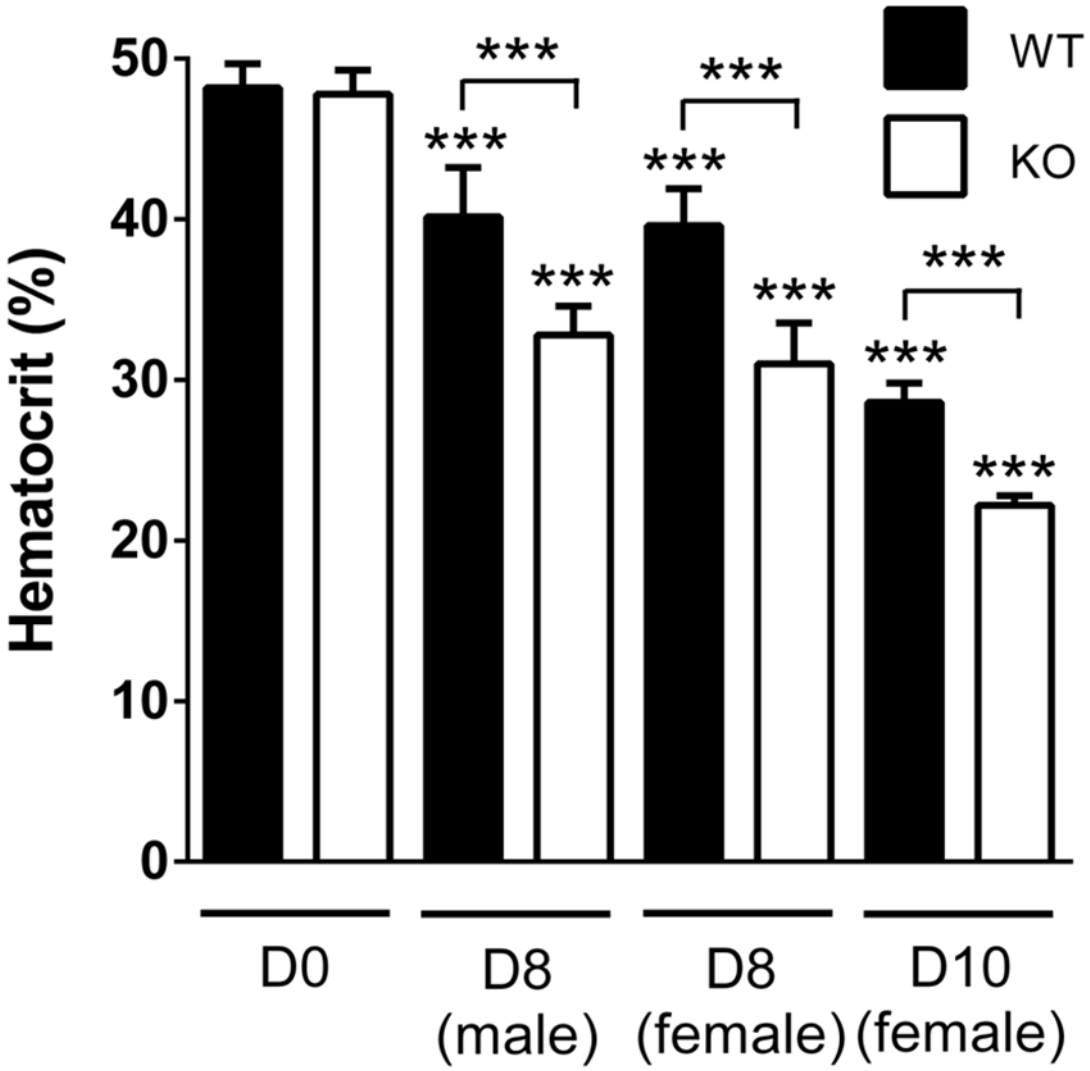
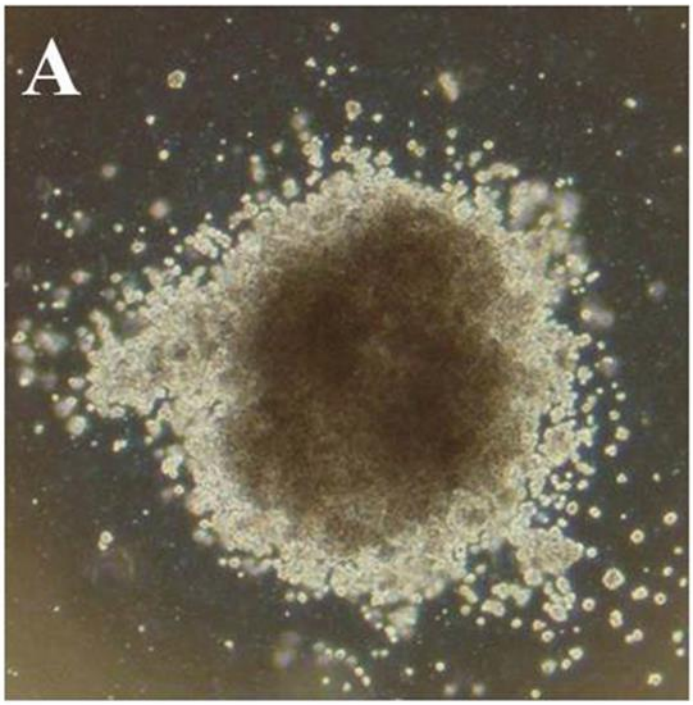

Hematocrit (%)
50
40
30
20
10
0
WT
KO
***
***
***
***
***
***
***
***
D0
D8
(male)
D8
(female)
D10
(female)

Figure 7. Hematocrit values in uninfected and *P. berghei* NK65-infected male and female WT and IL-3 KO mice. Hematocrits of infected mice were determined on day 8 (male and female) and day 10 (female) p.i. Data from uninfected male and female mice of each genotype were not significantly different and were pooled (D0). All data are presented as means ± SD from 4 to 6 mice per group analyzed individually. ***, $P < 0.001$ versus corresponding values for uninfected mice or (as indicated by the square brackets) versus corresponding values for mice of the other genotype. The data for male (D8) and female (D10) mice are representative of three and two independent experiments, respectively.


A

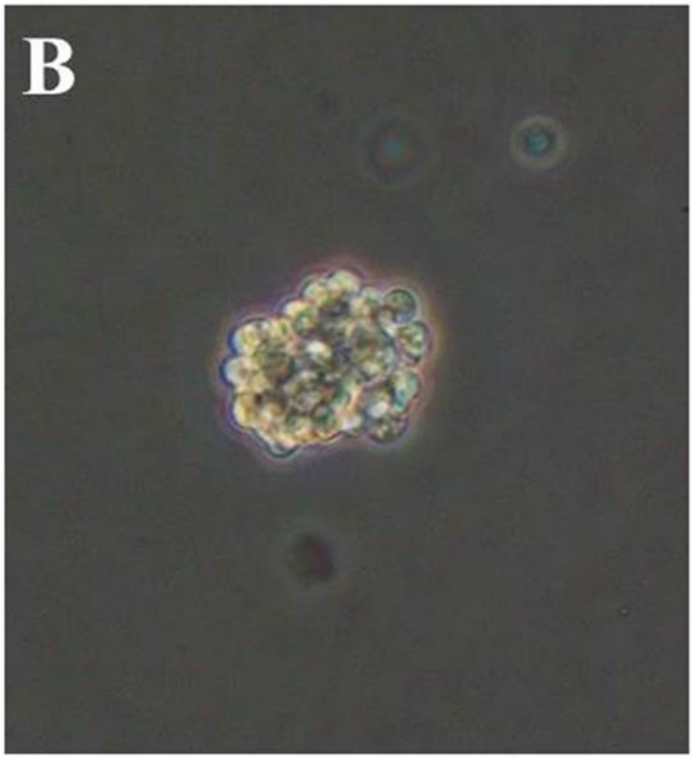

B

Figure 8. Representative images of (A) a blast forming unit-erythroid colony at 100x magnification, and (B) a colony forming unit-erythroid colony at 400x magnification.

Table 2. Erythropoiesis in male WT and IL-3 KO mice during *P. berghei* NK65 infection.

Mice	No. of progenitor cells[a]			
	Bone marrow		Spleen	
	BFU-E	CFU-E	BFU-E	CFU-E
Uninfected				
WT	26.7 ± 1.5	27.0 ± 4.4	9.0 ± 2.0	7.7 ± 2.9
KO	23.3 ± 8.1	20.7 ± 4.0	7.0 ± 2.6	4.3 ± 3.1
Infected				
WT	21.7 ± 5.5	54.0 ± 8.7[b]	4.0 ± 1.0	236.0 ± 20.0[c]
KO	48.0 ± 5.6[be]	150.7 ± 7.6[ce]	12.3 ± 4.2[d]	478.0 ± 41.4[ce]

[a]Means ± SD from triplicate cultures for pooled cells obtained from male WT and IL-3 KO uninfected mice (n = 4/group) and WT and IL-3 KO mice (n = 6/group) infected for 8 days with *P. berghei* NK65. Statistical differences were measured using two-way ANOVA with Bonferroni posttest. The data are representative of two independent experiments.

[b]Statistically significant difference compared to uninfected mice of the same genotype ($P < 0.01$).

[c]Statistically significant difference compared to uninfected mice of the same genotype ($P < 0.001$).

[d]Statistically significant difference compared to similarly infected WT mice ($P < 0.05$).

[e]Statistically significant difference compared to similarly infected WT mice ($P < 0.001$).

P. berghei NK65-infected IL-3 KO mice have higher levels of G-CSF, and lower levels of IFN-γ and CLCX9, than WT counterparts

A number of cytokines are known to affect the course and outcome of *Plasmodium* infection, with the host immune response depending upon the optimal balance and timing of both inflammatory and anti-inflammatory cytokines. In order to determine the effect of IL-3 deficiency on cytokine production in *Plasmodium*-infected mice, a multiplex immunoassay was used to determine the levels of 32 lymphokines, interferons, colony-stimulating factors, and chemokines present in the plasma of *P. berghei* NK65-infected male WT and IL-3 KO mice at days 0, 4, and 8 p.i. Of the 32 analytes tested, 15 were increased in infected mice compared to uninfected (GM-CSF, IL-1α, IL-1β, IL-6, IL-9, IL-10, IL-12 [p70], IL-13, IP-10, KC, MCP-1, M-CSF, MIP-1α, MIP-1β, and TNF-α; data not shown), but only three showed significant differences in expression between male WT and IL-3 KO mice: IFN-γ, CXCL9, and G-CSF.

Uninfected male WT and IL-3 KO mice produce nearly undetectable levels of IFN-γ. At day 4 p.i., both male WT and KO mice had increased levels of IFN-γ, but WT mice had significantly higher levels than IL-3 KO mice. By day 8 p.i., KO levels increased to be approximately equal to those of WT mice (Figure 9A). The IFN-γ-induced monokine CXCL9 followed a similar pattern, with male WT mice having higher levels than KO mice at day 4 p.i. but no significant difference at day 8 p.i. (Figure 9B).

Prior to *Plasmodium* infection, here is no statistically significant difference between male WT and IL-3 KO levels of G-CSF; by day 8 p.i. both WT and IL-3 KO mice have significantly increased levels compared to uninfected, with IL-3 KO mice having significantly higher levels than WT mice (Figure 9C). Since G-CSF supports the proliferation and function of neutrophils, May-Grünwald-stained thin blood smears taken

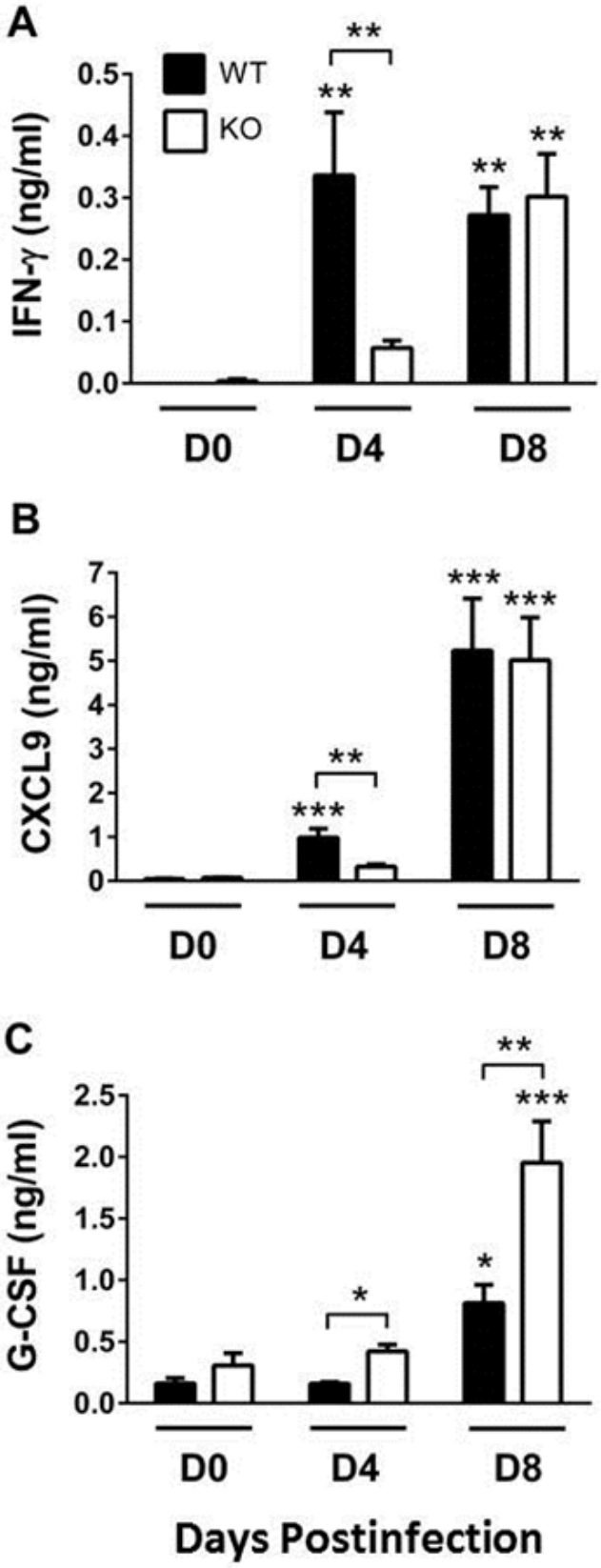

A
WT
KO
IFN-γ (ng/ml)
0.5
0.4
0.3
0.2
0.1
0.0
**
**
**
**
D0
D4
D8
B
CXCL9 (ng/ml)
7
6
5
4
3
2
1
0
***
**
***
***
D0
D4
D8
C
G-CSF (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
*
***
D0
D4
D8
Days Postinfection

Figure 9. Levels of plasma IFN-γ (A), CXCL9 (B), and G-CSF (C) in uninfected and *P. berghei* NK65-infected male WT and IL-3 KO mice. Infected animals were sacrificed on days 4 and 8 p.i. Data are presented as means ± SD from 4 to 5 mice per group analyzed individually. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) versus corresponding values for uninfected mice or (as indicated by the square brackets) versus corresponding values for mice of the other genotype (two-way ANOVA with Bonferroni posttest). The data are representative of two independent experiments.

from uninfected and *P. berghei* NK65-infected male WT and KO mice were counted to determine the % of neutrophils. There was no significant difference in the % of blood neutrophils between uninfected WT and KO mice (Table 3). After infection with *P. berghei* NK65, both WT and KO mice had increased % of blood neutrophils, but WT mice had a significantly greater increase than KO mice.

WT mice produce IL-3 in response to infection with *P. berghei* NK65

Plasma levels of IL-3 were undetectable by multiplex assays in both uninfected and infected WT mice, indicating that a local source of IL-3 is necessary in this model system. To begin determining at what point during the infection significant levels of IL-3 begin to be produced, IL-3 secretion by antigen-stimulated splenocyte cultures was examined using cells obtained from male WT mice at days 0, 2, 4, 6, and 8 p.i. (Figure 10). Levels of IL-3 were first detected in cultures containing splenocytes obtained at day 4 p.i. that were stimulated with *P. berghei* NK65 antigen but not control antigen or medium alone. By day 6 p.i., even cultures containing control antigen and medium contained IL-3, indicating extensive activation of cells *in vivo.*

Table 3. Percentage of neutrophils present in the peripheral blood of uninfected and *P. berghei* NK65-infected male WT and KO mice at 8 days p.i. (n = 5/group).

	% neutrophils	
	WT	KO
Uninfected	34.0 ± 8.9	32.3 ± 4.4
Infected	62.5 ± 15.1[a]	42.1 ± 11.5[a,b]

[a]Statistically significant difference compared to uninfected mice of the same genotype ($P < 0.05$).

[b]Statistically significant difference compared to similarly infected WT mice ($P < 0.05$).

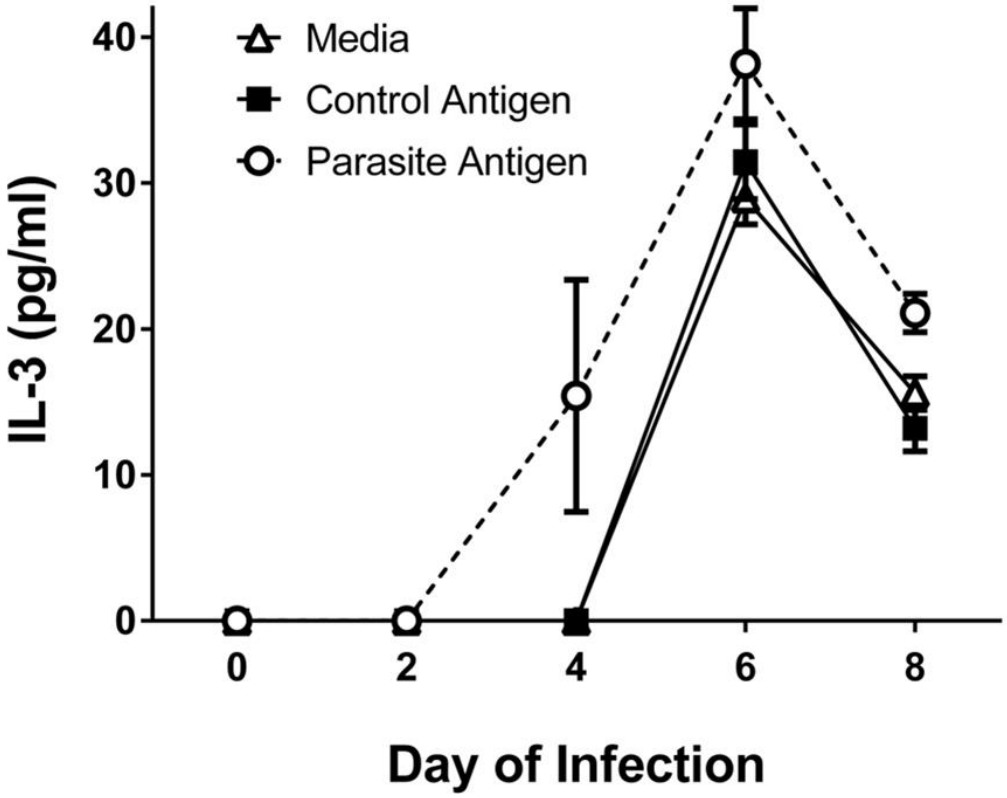

Media
Control Antigen
Parasite Antigen
IL-3 (pg/ml)
40
30
20
10
0
0
2
4
6
8
Day of Infection

Figure 10. IL-3 production by splenocytes isolated from *P. berghei* NK65-infected male WT mice. Unfractionated spleen cells were isolated from mice at days 0, 2, 4, 6, and 8 p.i. (n = 4 mice/group). Pooled cells were cultured in triplicate for 48 h in the presence of medium, control antigen, and parasite antigen, and supernatants were analyzed for IL-3 by ELISA. Data are presented as means ± SD from triplicate cultures.

Discussion

Although IL-3 is known to contribute to the host immune response to gastrointestinal nematodes, little work has been done exploring the role of IL-3 in the immune response to protozoan parasites such as *Plasmodium*. A recent genetic study showed an association between certain single nucleotide polymorphisms in the gene encoding IL-3 and protection against recurrent malaria attacks in humans; however, the phenotypes associated with these polymorphisms are unknown, making it impossible to determine whether IL-3 exerts a protective or detrimental effect (Meyer *et al.,* 2011). Earlier studies performed with the non-lethal rodent malaria *P. chabaudi* indicate that IL-3 may play a role in initiating the switch from Th1 to Th2 immunity by stimulating IL-4 production by non-B non-T cells (Helmby *et al.,* 1998), most likely of mast cell or basophil origin (Poorafshar *et al.,* 2000). Studies using the fatal strain *P. berghei* ANKA, which recapitulates cerebral malaria in mice, showed that the co-administration of anti-IL-3 and anti-GM-CSF antibodies prevented the development of cerebral malaria and prolonged survival by preventing increased TNFα production by macrophages (Grau *et al.,* 1988). To date, the results reported here represent the only work that has been done using IL-3 KO mice to explore the role of IL-3 in any model of rodent malaria.

Our results indicate that IL-3 plays a critical role in suppressing protective immunity to *P. berghei* NK65 infection as monitored by survival and parasitemia. Mortality caused by *P. berghei* NK65 differs significantly between males and females, with WT males succumbing to infection 8 – 13 days p.i. while WT and IL-3 KO females survive 3 – 4 weeks. Male IL-3 KO mice, however, have a survival curve more closely

resembling that of female mice, with nearly half of KO mice surviving 3 weeks or longer (Figure 3A-B). This suggests that IL-3 plays a detrimental role in the host response to *P. berghei* NK65 infection in male, but not female mice. These data concur with similar studies of *P. chabaudi* in IFN-γ receptor and IL-4 KO mice, in which male, but not female, KO mice had shortened survival compared to their WT counterparts (Zhang *et al.,* 2000). Orchidectomy was found to abrogate the shortened survival in male both IL-4 and IFN-γ receptor KO mice, while ovariectomy had no effect on the course of infection in females, suggesting that males and females respond to malaria infection via different pathways, and that the male response is moderated by testosterone (Zhang *et al.,* 2000). These and other studies (Klein, 2004) strongly support that in both rodents and humans, mortality rates are generally higher in males than in females and may relate to immunological differences in the sexes. Due to the fact IL-3 deficiency prolonged survival in males, but not females, many of the subsequent experiments were performed in males only.

Differences in survival between male WT and IL-3 KO mice showed a strong correlation with differences in parasitemia. Parasite levels in the blood were similar in WT and IL-3 KO mice at days 4 and 6 p.i. However, at days 8 and 10 p.i., when male WT mice begin to suffer significant mortality, parasitemia was greater in male WT mice than in corresponding KO mice. The increased parasitemia observed in male WT mice may not be the cause of death in these animals since male KO mice achieve higher parasitemias before succumbing 3-4 weeks p.i. (data not shown). Differences in parasitemia between male WT and IL-3 KO mice do not appear to reflect differences in migration of parasites into the bloodstream, since both mouse strains cleared parasites

from the peritoneal cavity by day 2 p.i. No significant difference in parasitemia was observed between female WT and KO mice, which correlates with their similar survival rates.

Malaria infection induces anemia in both humans and mice via a number of mechanisms: the destruction of pRBCs by the developing parasites, clearance of pRBCs by the host immune response, increased destruction of uninfected RBCs, and suppression of erythropoiesis (Lamikanra *et al.,* 2007). Our data shows that both male and female IL-3 KO mice develop more severe anemia than WT mice in response to malaria infection (Figure 6), and the reduced parasitemia in male IL-3 KO mice compared to WTs (Figure 3C) suggests that at least some of this increased anemia is due to more effective clearance of pRBCs.

Given that IL-3 has been suggested to promote erythropoiesis under certain conditions (Broughton *et al.,* 2012), we also investigated the possibility that impaired erythropoiesis is also a significant contributor to anemia in male IL-3 KO mice. Surprisingly, male IL-3 KO mice actually produced higher numbers of erythroid progenitors in both the bone marrow and the spleen than male WT mice in response to *P. berghei* NK65 infection (Table 2), suggesting that IL-3 may suppress erythropoiesis. Suppression of erythropoiesis has been reported for other cytokines, including IFN-γ, TNF-α, and IL-10 (Lamikanra *et al.,* 2007), suggesting that the elevated levels of IFN-γ in male WT mice (Figure 8A) may also be suppressing erythropoiesis in these animals. Previous studies have found lower levels of BFU-Es and CFU-Es in lethal malaria infections compared to non-lethal infections (Villeval *et al.,* 1990; Weiss, 1989), which agrees with our finding that the longer-surviving male KO mice have increased

erythropoiesis compared to male WT mice and suggests that a robust splenic erythropoietic response supports survival in malaria-infected mice.

The increased erythropoiesis seen in male IL-3 KO mice may also be a direct result of the KO mice's greater anemia and resulting increased demand for RBCs. Mice infected with *P. chabaudi* have been shown to produce erythropoietin (EPO) in response to malaria-induced anemia (Nairz *et al.,* 2012). EPO upregulates erythropoiesis in response to RBC destruction and the resulting reduction in blood oxygen tension (Nairz *et al.,* 2012). EPO has also been shown to play an anti-inflammatory role in malaria infection, and administration of exogenous EPO to *P. berghei* NK65-infected mice prolongs survival (Nairz *et al.,* 2012). It remains to be determined whether *P. berghei* NK65-infected IL-3 KO mice produce higher levels of EPO than WT mice to correspond to their greater anemia; if so, this could be one factor contributing to their prolonged survival.

The spleen plays several important roles in the host immune response to malaria infection, removing damaged and pRBCs from circulation, as a site for erythropoiesis and hematopoiesis, and as a secondary lymphoid organ where T cell and B cell responses can occur (Engwerda *et al.,* 2005). Our results showing increased splenomegaly in the longer-surviving IL-3 KO mice agree with the literature suggesting that the degree of splenomegaly correlates with the effectiveness of the immune response (Cadman *et al.,* 2008). Mast cells (Furuta *et al.,* 2006) and basophils (Poorafshar *et al.,* 2000) have both been shown to increase in the spleens of *Plasmodium*-infected mice, and mast cell derived TNF-α (Furuta *et al.,* 2006) and Flt3 ligand (Guermonprez *et al.,* 2013) have been shown to play important roles in the immunopathology of mice infected with *P.*

berghei NK65. In spite of the importance of mast cells in the host response to malaria infection, as well as the known ability of IL-3 to influence mast cell function, no difference in the number of mast cells per mm^2 of spleen tissue was detected between male WT and KO mice either before or 8 days after infection with *P. berghei* NK65 (Table 1). Both genotypes, however, showed a marked decrease in splenic mast cells postinfection, which may be due either to mast cell activation (degranulated mast cells cannot be detected using pinacyanol erythrosinate staining), or to the over-all increase in spleen size. Although IL-3 is also known to influence the proliferation and function of basophils, preliminary experiments have shown no difference in the number of splenic or blood basophils in *P. berghei* NK65-infected male WT and KO mice.

The spleen is made up of red pulp, a network of reticular cells and macrophages which perform the filtration functions of the spleen, and white pulp, where pathogen-specific B cell and T cell responses are generated (Engwarda *et al.,* 2005). The white pulp is separated from the red pulp by the marginal zone (Engwarda *et al.,* 2005). Hematoxylin-eosin (H&E) staining was performed on spleen sections in order to compare the relative areas of red and white pulp between uninfected and *P. berghei* NK65-infected male WT and KO mice (Figure 10). Spleens of uninfected WT and KO mice were similar, with well-defined areas of white pulp surrounded by a visible marginal zone separating them from the red pulp. After infection, however, the spleens of both WT and KO mice showed a dramatic loss of definition between the red and white pulp and shrinkage of the marginal zone, which agrees with previous studies on histological changes in the spleen caused by malaria infection (Cadman *et al.,* 2008; Engwerda *et al.,* 2005). Although more definitive studies of differences in splenic architecture need to be

completed, these data suggest that increased expansion of the splenic erythroid compartment in *P. berghei* NK65-infected KO mice in part accounts for the increased splenomegaly seen in KO mice.

Cytokines play a wide variety of roles in immunity to infection, recruiting and signaling between various effector cells as well as endothelial cells (Wiser, 2011). Since the complexity of the immune response to malaria infection and dearth of literature on the role played by IL-3 makes it difficult to predict which cytokines will be affected by the absence of IL-3, a multiplex assay was used to compare the levels of 32 major cytokines in plasma collected from *P. berghei* NK65-infected male IL-3 KO and WT mice at days 0, 4, and 8 p.i. Of these cytokines, 3 were found to be significantly different between *P. berghei* NK65-infected IL-3 KO and WT mice: IFN-γ, CXCL9, and G-CSF (Figure 8). These results do not definitively eliminate the remaining 29 cytokines as possible factors in the difference between the IL-3 KO and WT responses to *P. berghei* NK65 infection; many cytokines act locally rather than systemically and may not be present at detectible levels in plasma.

IFN-γ is an inflammatory cytokine produced primarily by lymphocytes including αβ T cells, natural killer (NK) cells, NKT cells, and γδ T cells (Inoue *et al.*, 2013). There have also been reports of IFN-γ production by myeloid cells (Inoue *et al.*, 2013), including mast cells (Marone *et al.*, 2000). IFN-γ production is the defining characteristic of Th1 cells, making it a critical factor in the balance between Th1 and Th2 immunity (Angulo and Fresno 2002.) The importance of INF-γ as a protective factor in the immune response to malaria infection has been well established (Angulo and Fresno, 2002). Therefore, we were surprised to find that the male WT mice had higher levels of IFN-γ at

day 4 p.i. with *P. berghei* NK65 than the longer-surviving male IL-3 KO mice (Figure 8A). This could reflect the fact that effect of IFN-γ can exert a protective or pathological effect depending on the strain of parasite and mouse. Although IFN-γ has been shown to be essential for immunity to *P. chabaudi* infection (Angulo and Fresno, 2002; Su and Stevenson 2002), IFN-γ has been implicated in the development of cerebral malaria in *P. berghei* ANKA-infected C57BL/6 mice (Su and Stevenson, 2002; Angulo and Fresno, 2002). The role of IFN-γ in the immune response to *P. berghei* NK65 infection is less clear: there is no significant difference in survival between BALB/c WT and IFN-γ KO mice infected with *P. berghei* NK65 (Ishih et al., 2008), however, treatment with anti-IFN-γ antibodies significantly prolongs survival in C57BL/6 mice infected with *P. berghei* NK65 (Yoshimoto et al., 1998). Therefore, it seems plausible that IFN-γ could contribute to immunopathology related to its inflammatory functions in this disease model. IFN-γ also promotes antibody class switching to the IgG2 isotype in mice; however, we found no difference in IgG2 antibody levels between *P. berghei* NK65-infected male WT and IL-3 KO mice (data not shown).

CXCL9, also known as monokine induced by IFN-γ (MIG), is a T cell chemoattractant produced by macrophages and other cells in response to IFN-γ (Liao *et al.,* 1995). Since CXCL9 is induced by IFN-γ production, it is unsurprising that CXCL9 expression in the plasma of *P. berghei* NK65-infected male WT and IL-3 KO mice is similar to that of IFN-γ, with WT mice expressing higher levels than KO mice at day 4 p.i. and WT and KO mice expressing similar levels at day 8 p.i. (Figure 8B). Like IFN-γ, CXCL9 has been shown to be essential to the development of cerebral malaria (Campanella *et al.,* 2008). CXCL9 was also found to be upregulated in the cerebral

microvasculature of BALB/c mice infected with *P. berghei* NK65 (Lacerda-Queiroz *et al.,* 2011). Although *P. berghei* NK65 infection does not cause the neurological symptoms of classical cerebral malaria (ataxia, paralysis, convulsions, and coma followed by death), *P. berghei* NK65-infected BALB/c mice did show increased cerebral vascular permeability and levels of leukocyte rolling and adhesion comparable to that seen in *P. berghei* ANKA infection (Lacerda-Queiroz *et al.,* 2011). This suggests that CXCL9, like IFN-γ, could contribute to WT mortality via inflammation-related immunopathology.

G-CSF is a growth factor that supports the proliferation, survival, and differentiation of neutrophils (Touw and van de Geijn, 2007). G-CSF also induces mobilization of neutrophils from the bone marrow and enhances neutrophil function (Touw and van de Geijn, 2007). Neutrophils are the most abundant leukocyte in the circulation and typically form the first line of defense against infection, phagocytizing pathogens and then undergoing apoptosis to be themselves consumed by macrophages (Mócsai, 2013). In addition, neutrophils are thought to help regulate the immune response by releasing inflammatory mediators as well as participating in the regulation of B and T cells, dendritic cells, and NK cells (Mócsai, 2013). Although classically considered essential for defense against extracellular pathogens such as bacteria and fungi, there is recent evidence suggesting that neutrophils may also play a role in the host defense against intracellular pathogens (Mócsai, 2013). In a mouse model of experimental cerebral malaria, *P. berghei* ANKA-infected mice developed a population of neutrophils expressing the high-affinity IgE antibody receptor FcεRI, which is normally expressed by mast cells and basophils (Porcherie *et al.*, 2011.) These $Fc\varepsilon RI^{+}$ neutrophils, which

migrated to the brain, were shown to be required for the development of cerebral malaria in infected mice, possibly by producing IFN-γ (Porcherie *et al.,* 2011).

Our own preliminary results showed an increase in the % blood neutrophils in *P. berghei* NK65-infected male WT and IL-3 KO mice, with a significantly higher percentage of neutrophils in WT mice at day 8 p.i. (Table 3.) Since white blood cell counts were not taken, it cannot be certain that the higher % neutrophils represent higher absolute numbers of neutrophils in the blood of infected male WT mice. Assuming that the lower % neutrophil counts in male IL-3 KO represent lower absolute numbers of neutrophils, the higher G-CSF production seem in male IL-3 KO mice could well represent a compensatory mechanism to increase neutrophil levels. Decreased neutrophil counts in male IL-3 KO mice could be due to impaired production of neutrophils in IL-3 KO mice, or increased loss of neutrophils due to phagocytosis and apoptosis. The latter possibility is intriguing since it could help to account for the enhanced parasite clearance seen in male IL-3 KO mice, although macrophages are generally considered the primary phagocytes responsible for clearance of *Plasmodium* parasites and pRBCs from the bloodstream (Angulo and Fresno, 2002).

It is important to note that IL-3 levels in blood or tissues are low or undetectable in normal mice using most assays (Neel *et al.,* 2004). This was supported by our findings that IL-3 was barely detectable in the plasma of *P. berghei* NK65-infected male WT mice (data not shown), indicating that IL-3 is produced and used locally rather than systemically in this model system. Our IL-3 kinetic study using spleen culture supernatants indicates that IL-3 production begins as early as day 4 p.i. (Figure 9); however, the fact that there are already detectable differences in the cytokine levels

expressed by *P. berghei* NK65-infected WT and KO mice at this time point suggests that IL-3 production begins even earlier. More sensitive assays will be needed to pinpoint the timing of IL-3 production as well as identify its cellular source.

The results of this study, which were recently published in the journal *Infection and Immunity* (Auclair et al., 2014), have established the novel finding that endogenous IL-3 significantly contributes to the susceptibility of male BALB/c mice to blood-stage infection with the lethal rodent malaria *P. berghei* NK65. This is supported by the findings that *P. berghei* NK65-infected male IL-3 KO mice have prolonged survival, decreased parasitemia, increased erythropoiesis, and increased splenomegaly compared to their WT counterparts. Although *P. berghei* NK65-infected IL-3 KO mice also experience more severe anemia than WT mice, this appears to be due to enhanced clearance of pRBCs and so is consistent with a detrimental role for IL-3 in the host response to malaria infection. Intriguingly, the influence of IL-3 on malaria infection does not appear to be mediated through mast cells or basophils, the primary mature effector cells that respond to IL-3. However, while we found no difference between *P. berghei* NK65-infected male WT and IL-3 KO mice in blood basophil or splenic mast cell numbers, our results cannot rule out the possibility that IL-3 is influencing the functions of these cells. More work is needed to elucidate the mechanism(s) by which IL-3 exerts its effects; however, several lines of evidence suggest that IL-3 contributes to a potentially damaging inflammatory immune response to *Plasmodium* infection. These include: suppressed erythropoiesis in *P. berghei* NK65-infected male WT mice compared to IL-3 KOs, higher levels of the inflammatory cytokines IFN-γ and CXCL9, and, pending confirmation of our preliminary results, higher % neutrophil counts. Taken

together, these observations not only suggest new avenues of research into the effect of IL-3 on malaria infection but into the role of IL-3 in the host response to disease. Some particularly promising areas for further study include repeating the male-only experiments (spleen histology, erythropoiesis, cytokines, and IL-3 kinetics) in female mice as well as determining the effect of IL-3 deficiency on the host response to different rodent *Plasmodium* species such as the non-lethal parasite *P. chabaudi.* Given that basophil and mast cells are such important mediators of the effects of IL-3, more work should be done exploring the activation and function of these cells in *Plasmodium*-infected WT and IL-3 KO mice. Finally, given the higher IFN-γ levels seen in *P. berghei* NK65-infected male WT mice compared to IL-3 KO mice, as well as the well-established importance of this cytokine during malaria infection, experiments could be performed to determine if anti-IFN-γ treatment could improve the WT response to *P.berghei* NK65 infection, or, conversely, if the administration of exogenous IFN-γ could decrease survival time in *P. berghei* NK65-infected IL-3 KO mice.

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