DISSERTATION

CD4+ T-CELL DERIVED IL-10 MITIGATES MALARIAL ANEMIA

Submitted by

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ABSTRACT

CD4+ T-CELL DERIVED IL-10 MITIGATES MALARIAL ANEMIA

Individuals living in malaria endemic areas develop effective anti-parasite immunity over several years of repeated exposure, but become resistant to severe disease after just one or two infections. This observation suggests that the acquired immune system plays a role in both processes, but may involve different mechanisms. Using the mouse model of malaria caused by non-lethal *Plasmodium yoelii*, we test the hypothesis that CD4+ T-cell derived IL-10 contributes to disease resistance by mitigating severe anemia. Here we show that IL-10 deficient mice develop significant anemia despite a very low parasite burden. Anemia in this model is mediated primarily by increased erythrocyte destruction and not from suppressed erythropoiesis. Wild type mice that have recovered from *P. yoelii* infection have an expanded population of IL-10 producing CD4 T cells, with the majority of these cells co-expressing IFN-g and display a Th1 phenotype. In the absence of IL-10, there is an increase in IFN-g+ T cells. We demonstrate that IL-10 competent CD4+ T cells protect athymic nude mice from anemia when compared to CD4 T cells taken from recovered IL-10 deficient mice. Utilizing an *ex vivo* system that tests the function of APCs in activating CD4+ T-cells, we also determined that APCs exposed to P. *yoelii in vivo* induced a greater population of CD4+ T-cells that express IL-10 compared to naïve APCs. We also demonstrate that IFN-γ is required, with the possible involvement of IL-10 and IL-12, for efficient IL-10 expression in CD4+ T-cells. Our findings suggest that one mechanism by which the acquired immune system contributes to resistance to severe anemia may be the

development of CD4 T cells that co-express IL-10 and IFN-g, thereby self-regulating IFN-g levels, which then inhibits pro-inflammatory mediated destruction of naïve red blood cells.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

Malaria is an infectious parasitic disease that continues to be a global health threat. Malaria is a major cause of morbidity and mortality, particularly in children under 5 years old, in subtropical regions of the world. To understand the mechanisms of immunopathogenesis during disease may help the development of immunomodulatory therapeutics and vaccines. This introduction is written to set the foundation for the following chapters based on this dissertation's hypothesis: CD4+ T-cell derived IL-10 mitigates anemia during malaria infection.

Malaria: World impact

Symptoms of malaria have been described as early as 2700 BC in Chinese medical literature (12). The origin of the modern name 'malaria' stems from the Italian term for the disease: "mala aria'. 'Mala aria', which translates to 'bad air' in English, comes from the miasma theory of disease where disease is thought to be caused by toxins in the air. The miasma theory was displaced by the germ theory, the idea that microorganisms cause disease, when the malaria parasite was identified in infected blood by Charles Lavern in 1880 (10).

Malaria, an infectious disease caused by parasites in the genus *Plasmodia*, remains a significant global health threat with the potential to infect over 3.3 *billion* people worldwide (1). Malaria is transmitted by the *Anopheles* mosquito and the vector is primarily found in tropical and subtropical regions, such as sub-Saharan Africa and Southeast Asia which coincide with areas the disease is found. The World Health Organization (WHO) reported in 2011 that malaria was present in 106 countries and an estimated 216 million cases of malaria were reported with 655,000 deaths were due to the disease (1). 91% of these deaths occurred in Africa and the majority of these deaths (86%) were children under 5 years old (1).

Economically, malaria decreases the national gross domestic product (GDP) in high transmission countries by an estimated 1.3% (1). In practical terms, the accumulated economic impact of malaria in high transmission countries has resulted in significantly lower GDP as compared to countries without malaria (1). Since many of the affected countries are already economically depressed, the decrease in GDP further sets these countries at a disadvantage.

Malaria can also be found outside endemic areas due to international travel to affected regions. There were 1688 reported cases of imported malaria in the US during 2010, which was a 14% increase over the number of 2009 cases (56). Approximately 400 people self-reported to have adhered to CDC recommended chemoprophylactic treatment for malaria, yet became infected, indicating suboptimal prophylaxis efficacy (56). In a study focusing on the number of imported cases of malaria in non-endemic industrialized countries from 1985 – 1995, 10,000 cases were estimated to be imported annually (64). Although the imported malaria cases comprise a very small percentage of the total number of cases worldwide, travelers are often susceptible to serious morbidity and mortality due to absent immunity to the disease (92, 100).

Malaria: The disease

Four *Plasmodium* species are associated with human disease: *P. falciparum, P. vivax, P. ovale*, and *P. malariae*. Of the four human disease causing species of *Plasmodium*, *P. falciparum* infection is associated with the highest rates of severe morbidity and mortality (92). *P. vivax*, the most prevalent malaria parasite worldwide, can cause severe disease, but is rarely fatal (41). After the *Plasmodium* parasite is introduced to the human host via mosquito feed, the parasite targets the liver where the parasite matures from the mosquito stage to the blood stage form of the parasite. The liver stage of the parasite is considered the 'incubation period' and this stage is clinically silent. The incubation period for *P.*

falciparum lasts from 9-14 days prior to the red blood cell stage of infection and it is the red blood cell stage of infection that initiates disease in the host (61). The incubation for *P. vivax, P. ovale*, and *P. malariae* are longer, around 15-16 days. *P. vivax* and *P. ovale* infected individuals may experience periodic relapses after the initial infection due to the persistence of dormant parasites in the liver (61).

Human *Plasmodium* infection can manifest as 'uncomplicated' or 'severe' malaria. Uncomplicated, or mild, malaria produces flu-like symptoms: fever, chills, and nausea, but does not require hospitalization. Severe malaria, on the other hand, can manifest in several ways: severe anemia, cerebral malaria, pulmonary edema, and acute renal failure (92). Metabolic abnormalities include acidosis and hypoglycemia. Patients can experience one or several symptoms at once and symptoms can develop rapidly.

In malaria-endemic areas, the mortality rate due to malaria for children aged one through four was estimated to be 25% (85). Of the different manifestations of severe malaria, cerebral malaria and severe anemia are most often associated with children under five years old. In high transmission areas, severe malarial anemia (SMA) is the leading cause of morbidity in children between one and three years old (62, 68, 77), with cerebral malaria most commonly found in children age 5 and older (76) .

Transmission of malaria can occur with transfusion of infected blood products, however, the focus of this dissertation will be on natural, vector-borne malaria transmission. The percentage of potential malaria-infected blood products in non-endemic countries are very low (45). Furthermore, it is difficult to determine, in malaria-endemic countries, whether the development of malaria post blood transfusion is due to contaminated blood products or due to natural infection.

Malaria: The parasite

An estimated 199 species of *Plasmodium* have been described as infectious to a wide range of mammals, birds, and reptiles (lizards, snakes, and worm-lizards) (57). Four species from the genus *Plasmodium* are human specific parasites: *P. falciparum, P. vivax, P. ovale*, and *P. malariae.* Simian strains of *Plasmodium* may infect and cause disease in humans, such as *P. knowlesi*, but they are currently considered a zoonotic form of malaria (13).These unicellular protozoan parasites are categorized under the phylum *Apicomplexa* and are transmitted to humans by mosquitoes from the genus *Anopheles*.

Both the human and mosquito hosts are required to complete the *Plasmodium* life cycle. The *Plasmodium* life cycle can be organized in three stages: mosquito, liver, and blood stage. The parasites are primarily in haploid form, except for a brief diploid stage within the mosquito gut.

Mosquito stage:

Plasmodium undergoes both sexual and asexual reproduction. Sexual reproduction, or sporogeny, occurs in the mosquito after male and female parasite gametocytes are taken up during a blood feed of an infected human. The male and female gametocytes fuse to become zygotes, after which the zygote embeds itself in the mosquito gut wall and develops into an oocyst where thousands of sporozoite form within. The oocyst then bursts, releasing the sporozoites where they travel to the salivary gland of the mosquito where it resides until inoculation into a new host.

Liver stage:

As the malaria infected mosquito feeds, up to a few hundred sporozoites, a motile asexual form of the parasite, are inoculated into the dermis of the host (39). The inoculated sporozoites randomly move within the dermis until they encounter a blood vessel, which they are able to enter in order to localize to the liver (3). Upon reaching the liver, the sporozoites infect hepatocytes where, during the next 5-16 days, the sporozoites divide into tens of thousands of merozoites, or haploid form. Upon release from the hepatocytes, the merozoites re-enter the blood stream. P. vivax and P. ovale have dormant liver stages which can manifest as relapses of disease for months or years after the initial infection.

Sporozoites can reside in the dermis for up to 6 hours post inoculation and a proportion of the inoculated parasite are taken up by lymphatics (102). Dendritic cells process and present the sporozoite to CD8+ T-cells in proximal lymph nodes, inducing protective immunity. Although CD8+ T-cells have been shown to be important during pre-erythrocyte parasite infection, murine studies have shown that these same cells provide little advantage during the blood stage of infection (96).

Blood stage:

After merozoites re-enter the blood stream, asexual log-stage growth of the merozoites occur. It is during the blood-stage of infection that clinical symptoms of malaria arise (61). The parasite enters red blood cells via multistep, receptor and ligand-mediated interactions. Although complex, parasite entry into the cell occurs rapidly, approximately 30 seconds after attachment (31). The estimated 48 hour multiplication rate of the *P. falciparum* during blood stage is 20.9 fold (79).

Plasmodium species employ antigenic variation, which allows the parasite to evade the immune system, prolonging infection. A small percentage of merozoites develop into gametocytes during the blood stage, which await uptake by a mosquito so the infectious cycle may begin again.

Malaria and severe anemia

Severe malarial anemia (SMA), has been defined by the World Health Organization (WHO) as a hemoglobin (Hb) concentration <50g/L or at a hematocrit (Hct) <15% (99). Based on an extensive literature review, Murphy, et al. estimated that severe malarial anemia (SMA) kills between 190,000 and 974,000 children under the age of 5 each year (65). There are three mechanisms that can explain malaria-associated anemia: direct lysis of red blood cells by the parasite, destruction of non-parasitized erythrocytes, and suppression of erythropoiesis. Results of a study in Thailand estimated that less than 20% of RBC loss during malaria infection was due to direct infection with the parasite(75). Suppressed erythropoiesis and increased erythrocyte destruction are likely orchestrated by host-mediated mechanisms and there is evidence that both contribute to severe malarial anemia (47).

Suppression of erythropoiesis

Erythropoiesis, the production of red blood cells, occurs exclusively within the bone marrow in humans and is a highly regulated process. Erythropoietin (EPO), produced by the kidney, increases in response to anemia, specifically the resulting hypoxia, which then stimulates erythropoiesis. Erythropoiesis can be monitored by measuring the appearance of reticulocytes, or immature red blood cells, that are released into the vascular system.

The normal lifespan of a red blood cell (RBC) is approximately 120 days, after which they are dismantled by the spleen. Components of the RBC, such as heme and iron, are recycled for new RBC formation.

Suppressed erythropoiesis can be due to either inadequate EPO production in response to anemia or deficient response to EPO by red blood cell progenitors. During severe malarial anemia (SMA), there is evidence that EPO production in adults is inhibited (49). In children, the population most affected by severe malarial anemia, EPO production is strong, suggesting inhibited response to EPO by RBC progenitors. Murine studies using *Plasmodium chabaudi* demonstrate that, despite high levels of EPO, erythropoiesis is suppressed (7), supporting the observation seen in children.

Clearance of uninfected erythrocytes

Approximately nine uninfected RBC are estimated to be cleared for every one infected RBC during malaria infection based on a mathematic model of infection (36). In a Thailand clinical study, less than 10% of RBC lost contained parasite (75). Possible mechanisms for increased clearance of uninfected RBCs include phagocytosis by activated macrophages and immunogenic changes to red blood cells that trigger immune recognition and cell clearance (38). Increased phagocytosis by macrophages could be indiscriminate due to improper activation or receptor mediated phagocytosis due to immunoglobulin and complement deposition on uninfected RBCs. Increased rigidity of uninfected RBCs have also been shown to increase during malaria-associated anemia, which also correlates with increased uninfected RBC clearance (67)

The immune response to malaria

Protective immunity from malarial disease, or clinical immunity, often develops before immunity to parasite infection (2, 16). Furthermore, protective immunity requires repeated exposures to the parasite and immunity is quickly lost in its absence (2, 17). Conversely, risk of disease was highest amongst populations with low to moderate malaria parasite exposure (85). Similarly, a study based in northern Ghana demonstrated that, in the year after drug clearance of malaria parasite, treated patients experienced higher rates of clinical malaria compared to their untreated cohort (49% vs 38% respectively; (72)). Together, these observations implicate the acquired immune system in protection against severe malarial disease, including severe malarial anemia.

Through both human clinical studies and animal model research, protection from malaria disease requires a coordinated effort by the adaptive arm of the immune system. CD8+ T-cells, aided by CD4+ T-cells, are critical during the liver stage of infection (71). During the blood stage of infection, CD4+ T-cells and a robust antibody response have been shown to be critical effector cells in controlling parasite growth. Through murine studies, CD4+ T-cells were demonstrated to both control parasitemia and immunopathogenesis (86). A balance of pro- and anti-inflammatory cytokines is critical for control of the parasite as well as protecting the host.

The role of antibodies in malaria

Plasmodium species parasites utilize antigenic variation to help evade the immune system during blood stage infection. Infected red blood cells express variant surface antigens (VSAs), most importantly *P. falciparum*'s PfEMP1 proteins. Although antibodies have been shown to both reduce parasitemia and severity of disease through murine

passive antibody transfer experiments, antigenic variation prolongs evasion of antibody driven recognition and clearance in the host. Because of antigen variation by the parasite, antibody is most likely effective later in infection when a broad antibody repertoire is developed. B-cells have been demonstrated to be required for complete clearance of the parasite in murine studies of malaria (93). It is unclear if significant antigenic variation takes place in murine malaria models. The observation that mice clear most murine infections within 30 days, and are solidly immune to re-infection, suggests that antigenic variation is limited.

The role of CD4+ T-cells in malaria

CD4+ T-cells fill several roles during malaria infection: assisting CD8+ T-cells during liver stage, acting as effector cells during blood stage, and assisting antibody production during blood stage of infection (28, 86). During blood stage malaria, CD4+ T-cells can kill parasite via inflammatory cytokines, specifically IFN-γ, and activating cells such as macrophages. In murine transfer studies, malaria-exposed CD4+ T-cells conferred protection against infection (90). In a recent study, CD4+ T-cells protected mice from lethal malaria infection and they were also required to coordinate B-cell antibody clearance of the parasite (89). These data demonstrate the importance of CD4+ T-cells during malaria infection.

The role of cytokines in malaria

Cytokines act as key regulators of the immune system where a balance is required to achieve a protective immune response while avoiding damage to the host. Cytokines are involved in almost all aspects of an immune response to antigen. Cytokines can regulate

the cells of the immune response, such as B- and T-cells, through coordination of pro- and anti-inflammatory cytokines (33).

During malaria infection, a strong pro-inflammatory response, including interferon-gamma (IFN-γ) and Tumor necrosis factor-alpha (TNF-α) (54, 73), is required to control the parasite. In contrast, elevated levels of pro-inflammatory cytokines IFN-γ and TNF-α correlate with greater disease (29, 30, 63, 78) These observations indicate that the clinical outcome of malaria infection may depend upon the regulation of the inflammatory response.

Murine models for malaria

Due to experimental limitations for human-based research, animal models have been utilized for malaria-related hypothesis driven research. Mice are considered the primary animal models for malaria. The advantages of mouse-based research include vast genetic and phenotypic information of inbred mouse strains, the ability to genetically manipulate mouse lines, and the opportunity to follow the progress of disease. Also helpful is the availability of natural rodent malaria strains. The most common rodent *Plasmodium* species used in research are: *P. chabaudi, P. yoelii, P. berghei*, and *P. vinckei (80).* Another benefit is that each strain manifests differently in morbidity and mortality, and these differences can be used to ask specific questions.

The murine *Plasmodium* strains can be separated by lethal and non-lethal phenotype. Lethal strains of *Plasmodium*, such as P. *berghei*, plus select strains of *P. vinckei* (*P. vinckei vinckei*), P. *yoelii* (P. *yoelii* YM, P. *yoelii* XL) and *P. chabaudi* (*P. chabaudi* CB), have been often used to test vaccines and drug therapies. Nonlethal strains, such as P. *yoelii* (P. *yoelii* 17XNL), *P. chabaudi* (*P. chabaudi chabaud*i AS, *P. chabaudi adami*) and *P. vinckei* (*P.*

vinckei petterei), have been primarily used to study mechanisms of the immune response (80, 101).

P. berghei ANKA is the most commonly used strain for studies relating to cerebral malaria (80). P. *chabaudi chabaudi* and P. *yoelii* are the most common strains used to study malaria-related anemia (82). For our studies, we use a non-lethal model for malaria: P. *yoelii* 17x NL parasite against C57/Bl6 background mice. Features of this model include a preference for reticulocyte infection, however mature red blood cells are also infected during the course of disease. Anemia is present, but not severe, which allows for the study of specific factors that mitigate anemia.

Therapeutic goals

Defining the optimal immune response for protection during *Plasmodium* infection would have long term implications for therapeutics. Most drug therapies associated with malaria infection work directly on the parasite. Unfortunately, parasite drug resistance is a growing problem requiring other avenues of treatment to be investigated. Furthermore, the reduction of malaria through prevention (i.e. mosquito nets) and drug therapy in currently holoendemic regions may leave a population that largely develops immunity through repeated exposure, at greater risk of severe disease. Understanding the factors that balance a protective immune response while avoiding harm to the patient would enable the development of effective vaccines or immunomodulatory drugs, enlisting the help of the immune system to resolve or protect against infection.

CHAPTER 2: IL-10 MITIGATES ANEMIA

Introduction

Severe anemia is the greatest cause of malaria-associated morbidity and mortality in children under 5 years old worldwide. Patients with severe malarial anemia often experience a degree of anemia that is disproportionate to their parasite burden, suggesting that additional mechanisms other than direct parasite lysis of red blood cells contribute to the anemia. A high pro- to anti-inflammatory cytokine ratio has been shown to be associated with severe malarial anemia. Specifically, the ratio of IFN-γ and TNF-α to IL-10 is frequently found to be elevated in severely anemic patients. Despite these observations in human populations, a causal role of IL-10 in severe malaria anemia has not been addressed in mice. IL-10 deficient mice experience greater morbidity than wild type mice when infected with murine strains of malaria, but anemia and erythropoiesis have not been described in these models. Here we show that IL-10 deficient mice develop significant anemia, as measured by RBCs/ul blood, despite a very low parasite burden during P. *yoelii* infection. Anemia, the reduction of red blood cell density, is the focus of this dissertation. Anemia in this model is mediated by a combination of increased erythrocyte destruction as well as suppressed erythropoiesis. Our findings suggest that one mechanism by which the immune system contributes to resistance to severe anemia may be due to IL-10 orchestrated protection of red blood cells.

Hypothesis

IL-10 mitigates anemia during malaria infection

Literature review relevant to Chapter 2

Properties of IL-10

IL-10, a powerful immunosuppressive cytokine, inhibits inflammatory immune reactions by inhibiting the activation of monocytes, dendritic cells, and macrophages, and their secretion of inflammatory mediators (33). The biologically active forms of IL-10 are homodimers which signal through the IL-10 receptor (9, 27, 33). IL-10 is expressed by various cells: B- and T-cells, NK cells, mast cells, eosinophils, dendritic cells, monocytes and macrophages (33).

Pro-inflammatory events require management to avoid immune-mediated tissue damage due to an excessive response as seen with autoimmune diseases. Psoriasis, a skin disease, is hallmarked by an overabundance of pro-inflammatory cytokine with diminished levels of IL-10 (33). Genetic abnormalities of IL-10 in humans are associated with ulcerative colitis and Crohn's disease (22, 94). Experimentally, IL-10 deficient mice develop severe enterocolitis, emphasizing its protective role in modulating the inflammatory response.

IL-10 has been shown to control the immunopathogenesis of several infectious diseases, including infection by protozoan parasites *Toxoplasma gondii* and *Trypanosoma cruzi* (25, 34, 35). A nonlethal strain of *Toxoplasma gondii* became lethal in IL-10 deficient mice (IL-10 KO) without an increase in parasitemia. Serum levels of proinflammatory cytokines IFN-γ and IL-12 were six-fold higher in the IL-10 KO as compared to wild type mice. Furthermore, *ex vivo* studies that exposed IL-10 deficient macrophages and spleen

cells to *T. gondii* resulted in significantly higher proinflammatory cytokines relative to stimulated IL-10 intact cells (25).

IL-10 in malaria

Low levels of IL-10 have been shown to be associated with greater disease during infection. In human clinical studies, low levels of IL-10 during malaria infection correlated with severe anemia (46) while high ratios of pro-inflammatory cytokines TNF-α and IFN-γ to IL-10 levels correlated with greater morbidity and mortality during malaria infection (46, 58, 70).

Studies using mouse models of malaria have shown that the absence of IL-10 results in elevated levels of pro-inflammatory cytokines, which correlate with greater mortality and disease (48, 52). Together, the human and mouse malaria studies indicate that IL-10 helps to mediate the inflammatory response to the parasite, allowing pro-inflammatory cytokines to orchestrate the control of parasitemia while preventing the same cytokines from causing harm to the host. Here we ask whether IL-10 mitigates anemia and whether the anemia is due to suppressed erythropoiesis or increased RBC destruction.

Mouse models of malaria have also shown that the absence of IL-10 is associated with increased morbidity and mortality (52). Morbidity is assessed by various measures of disease including weight loss, hypothermia, and hypoglycemia, however, the effect of IL-10 on anemia has not been clearly defined. Here we show that IL-10 protects against anemia in a murine model of uncomplicated malaria by modulating two mechanisms: ameliorating suppressed erythropoiesis and inhibiting destruction of uninfected red blood cells. Specifically, in the absence of IL-10, mice experience decreased erythropoietic activity and greater erythrocyte destruction relative to parasite burden. The latter is accompanied by a

substantially greater ability of IL-10 KO mice to control parasitemia. This model can be used to study the complex factors that need to be balanced for the control of infection and immunopathogenesis in severe malarial anemia. IL-10 has previously been shown to be protective against severe disease during malaria infection as measured by change in body weight, temperature, and blood glucose (11). The role of IL-10 in malaria-associated anemia, however, has not yet been described in a murine model and is the focus of this chapter.

Overview of Chapter 2 findings

A. *Plasmodium yoelii is controlled in the absence of IL-10, but the degree of anemia is disproportionately higher when compared to wild type mice*
B. *When parasitemia is equivalent between IL-10KO and B6 mice, IL-10KO mice experience greater anemia*
C. *IL-10 deficiency-associated anemia is due to a combination of suppressed erythropoiesis and increased RBC destruction*
    I. *Suppressed Erythropoiesis*
    II. *Increased RBC destruction*

Results

A. Degree of anemia is disproportionately high though parasite is controlled in absence of IL-10

IL-10 has previously been shown to be protective against severe disease during malaria infection as measured by change in body weight, temperature, and blood glucose (11, 48, 52). The role of IL-10 in malaria-associated anemia, however, has not yet been

described in a murine model. Using a mouse model for uncomplicated malaria, we asked if IL-10 deficiency was associated with a greater degree of anemia during P. *yoelii* infection. IL-10 deficient (IL-10 KO) and C57Bl/6 (B6) mice were infected with 10^6 parasitized red blood cells and assessed for parasitemia and anemia for 30 days.

IL-10 KO mice developed low levels of parasitemia, as measured by percent parasitized RBCs in blood, which peaked at less than 10% at approximately eight days post infection (Figure 2.1A). Age and sex matched B6 mice, however, developed parasitemias as high as 40% after two weeks, which was significantly higher than in IL-10 KO mice in all experiments (n = 6 experiments, 5 – 10 mice/group/experiment, $p < 0.05$, representative experiment Figure 2.1A). Surviving mice of both strains cleared their infections after approximately 21 days. We consistently found that the major differences in infection, both parasitemia and anemia, were detected in the first two weeks post-infection, thus in subsequent experiments infection was monitored through at least day 14.

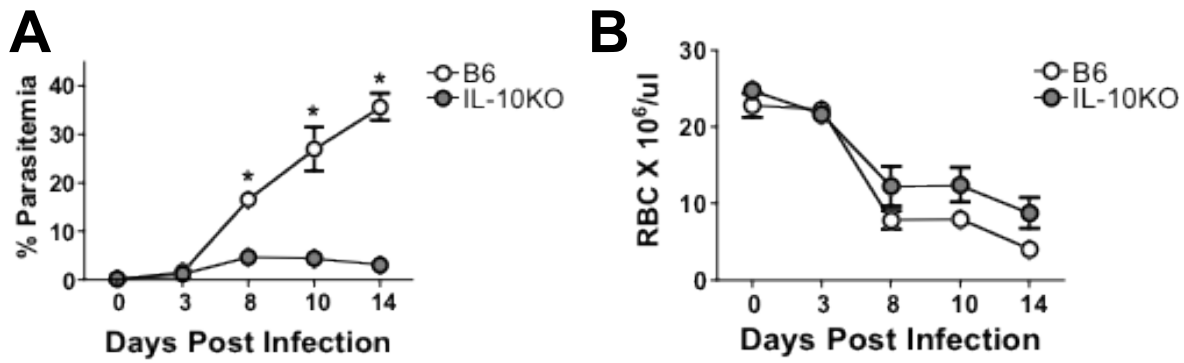

Figure 2.1 Parasitemia and anemia during P. *yoelii* infection of IL-10 KO and B6 mice. IL-10 KO and B6 mice were given the same dose of parasite: 10^6 infected red blood cells, 5 mice/group. Anemia and parasitemia were followed for 14 days. **(A)** Parasitemia was significantly lower in the IL-10 KO mice. **(B)** RBC numbers were similar between the two mouse groups: B6 (open circle) and IL-10 KO (filled circle) mice. Differences in parasitemia between both mouse groups were statistically significant ($p<0.05$ using two-way ANOVA).

The red blood cell counts for IL-10 KO mice did not decrease as low as B6 mice, but given the very low parasite burden, the degree of anemia appeared disproportionately high. In order to more objectively assess the relationship between anemia and parasitemia we measured the ratio between the maximum decrease in red blood cell count from pre-infection and the maximum degree of parasitemia through day 14 (ΔRBC count/10^6)/(%Parasitized RBC). A higher number indicates that the mouse is experiencing a greater degree of anemia relative to the parasite burden. In all experiments, the IL-10 KO mice had a consistently higher ratio of maximum red blood cell decrease to maximum parasite load. Table 2.1 summarizes 4 such experiments. This finding has been remarkably consistent over time and with different groups of IL-10 KO mice, whether purchased or bred in our facility. These data support the hypothesis that IL-10 KO mice develop greater anemia relative to parasitemia than their wild type counterparts.

Table 2.1 Ratio of maximum red blood cell change divided by the maximum parasitemia in 4 individual experiments. Ratio calculate by: (ΔRBC count/10^6)/(%Parasitized RBC). A higher value indicates greater anemia experienced relative to parasitemia. *All differencd between B6 and IL-10 KO mice were significant $p < 0.05$ using a one tailed t test.

B6	*0.12**	*0.2**	*0.4**	*0.4**
Number of mice	*5*	*10*	*10*	*10*
10 KO	*1.76*	*4.9*	*4.1*	*13.8*
Number of mice	*5*	*10*	*10*	*7*

B. Greater anemia experienced in the absence of IL-10 when parasitemia is equivalent between IL-10KO and B6 mice

In order to compare the degree of anemia between the two strains bearing the same parasite burden, we titrated the infectious dose so that the two strains developed a similar degree of parasitemia (data not shown). Figure 2.2A shows that when IL-10 KO mice are infected with 1000x the dose of parasite given to B6 mice, they develop similar levels of parasitemia (5 mice per group; Fig 2.2A; $p = 0.6$). The IL-10 KO mice, however, developed a significantly greater degree of anemia by day 2 post infection (PI) and remain more anemic than B6 mice through day 6 PI (Fig 2.2B; $p < 0.01$). This experiment was repeated twice more with similar results (5 mice per group, day 6 PI $p < 0.05$, repeated experiment data not shown). Together our experimental data demonstrates that IL-10 KO mice, while more resistant to parasitemia, suffer a disproportional degree of anemia when infected with P. *yoelii*.

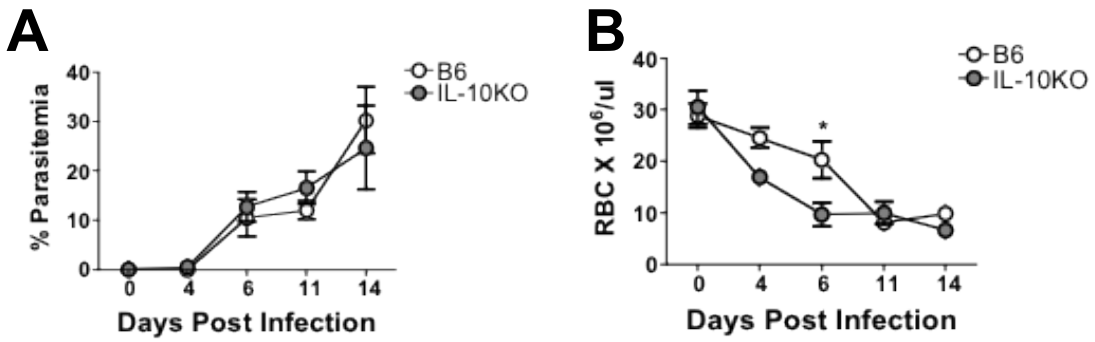

Figure 2.2: IL-10 KO mice experience greater anemia relative to B6 mice when parasitemia is normalized. B6 (open circle) and IL-10 KO (filled circle). 10^4 and 10^7 infected red blood cells given to B6 and IL-10 KO respectively, 5 mice/group. (**A**) Similar levels of parasitemia were achieved when IL-10 KO mice were infected with 1000x the dose of parasite given to B6 mice ($p = 0.6$). (**B**) IL-10 KO mice developed a significantly greater degree of anemia on day 2 and 6 PI ($p<0.05$ using two-way ANOVA).

C. IL-10 deficiency-associated RBC loss is due to a combination of suppressed erythropoiesis and increased RBC destruction

a. Erythropoiesis is inhibited in IL-10 KO mice

In the absence of external blood loss, anemia can either be due to red blood cell destruction and/or failure of erythropoiesis. Erythropoiesis is initiated with the production of erythropoietin (EPO) in the kidneys in response to low oxygen. Red blood cell precursors with EPO receptors respond to EPO by growth and differentiation (reviewed in (24)). Therefore, failure of erythropoiesis could be from impaired EPO production or impaired responses to EPO by red blood cell precursors.

We first measured EPO production in the serum of infected IL-10KO and B6 mice. Figure 2.3 shows pooled data from two experiments representing two separate infections. We measured serum EPO levels at the peak day of anemia (day 14 post infection) in both strains, and correlated EPO levels with red blood cell counts on the day of sacrifice (D14) as well as 4 days previous to sacrifice (D10; Figure 2.3A, 2.3B respectively). We not only

found that EPO concentrations were directly correlated with red blood cell counts in both strains, but that the linear regression lines from the two strains were superimposable. Therefore, we concluded that IL-10 KO mice were not impaired in their ability to produce EPO in response to anemia.

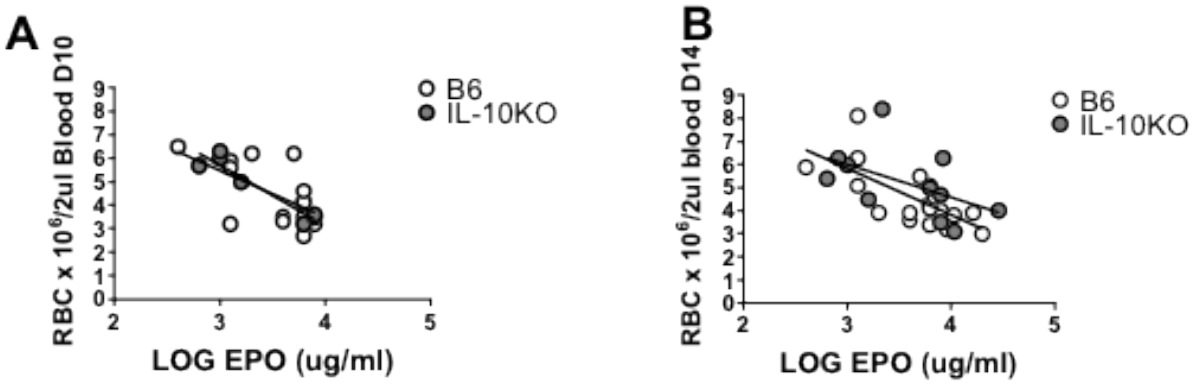

Figure 2.3: B6 and IL-10 KO mice make equivalent amounts of epo in response to anemia. B6 (open circle) and IL-10 KO mice (filled circle) were infected and sacrificed at day 14 post infection. Data pooled from two separate experiments (5 mice/group/experiment). The RBC count at day 14 **(A)** and day 10 **(B)** was plotted against the amount of epo detected in the serum on day 14. Linear regression analysis demonstrated that in all cases the slope was identical between B6 and IL-10 KO mice. Data was pooled from two separate experiments.

The main erythropoietic organ in B6 mice during malaria infection was previously identified as the spleen, while erythropoiesis in the bone marrow is suppressed (Reviewed in (6, 95, 103)). We compared erythropoietic response in the bone marrow and spleen in our experimental system by enumerating red blood cell precursors by flow cytometry using the murine erythroid specific surface receptor TER-119 (44). Ter-119 is expressed on the cell surface of early proerythroblast stage of RBC development and continues to be present on the mature RBC (Figure 2.4, (44)). A robust erythropoietic response was observed in the spleen during *P. yoelii* infection in contrast to a weak response in the bone marrow (data not shown), supporting previous findings demonstrating that the spleen as

the primary erythropoietic organ in mice. Since the primary erythropoietic organ of our experimental mice was the spleen, only the spleen data will be presented in this dissertation.

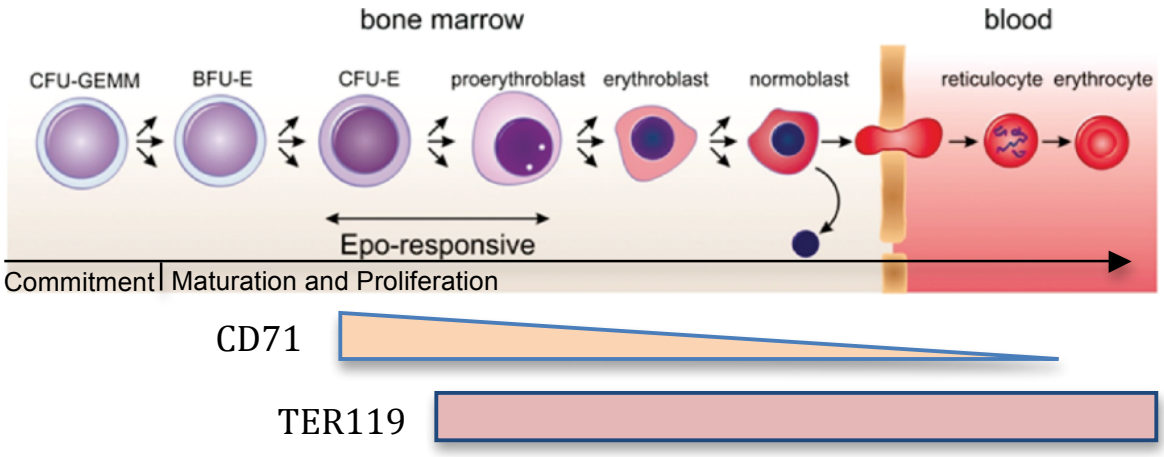

Figure 2.4: Erythropoiesis. Different erythropoietic stages of mice and humans (Figure adapted from (98)). Although the figure above depicts early events of erythropoiesis in the bone marrow, erythropoiesis in the spleen, the primary murine erythropoietic organ during malaria infection, is similar. CD71, a transferrin receptor, is expressed at high levels starting at the CFU-E stage of erythropoiesis and gradually declines and is largely absent on the mature RBC. Ter119 is present beginning during the proerythroblast stage of erythropoiesis and remains throughout the RBC's life.

Figure 2.5A shows pooled data from two experiments representing two separate infections (5 mice/group/infection). Both B6 and IL-10 KO mice made strong responses to anemia as evidenced by the 50 – 100 fold increase in TER-119+ cells in the spleen at day 14 post infection (Fig 2.5A). The number of TER-119+ cells in the spleen was significantly lower in IL-10 KO mice (B6 = 67 x 10^4, IL-10 KO = 38 x 10^4; $p < 0.05$) indicating erythropoiesis is impaired as shown in Figure 2.5A. Figure 2.5B represents the number of Ter-119+ cells in the spleen relative to red blood cell loss (Total Ter-119+ spleen cells)/(ΔRBC count) and shows that the IL-10KO mice had a significantly lower number of RBC precursors relative to circulating RBC loss.

To assess whether the reduced number of RBC precursors observed in IL-10KO mice was due to suppressed erythropoiesis, RBC precursor differentiation was assessed using the transferring receptor, CD71, and Ter-119 to identify early, mid, and late developmental stages (Fig 2.5C, 2.5D; (98)). CD71 is a useful surface protein marker for assessing RBC precursor development because levels of CD71 changes during maturation: CD71 expression is highest in early RBC precursors (CFU-E) and decreases through the reticulocyte phase ((50, 66, 84);Figure 2.4). Furthermore, Ter119 is absent on BFU-E and CFU-E cells and, thus, can be used to further characterize the earliest RBC precursors. In a paper by Wenger, et. al, the general maturation stages of erythropoiesis were identified (early to late) by comparing levels of surface CD71 and Ter119 as determined by flow cytometry. Morphology of sorted cells from each region was compared by microscopy and the morphologic characteristics correlated with the relative maturation regions outlined by flow cytometry. The study by Wenger, et al, provides a method for assessing changes in erythropoiesis by flow cytometry. In figure 2.5, three developmental stages were outlined (early, mid, late) based on surface levels of CD71 and Ter119. In brief, the following regions were assessed: Early (Ter119-CD71hi), Mid (Ter119+CD71hi), and Late (Ter119+CD71lo).

Figure 2.5D shows significantly fewer numbers of mid-stage erythrocytes in the IL-10 KO mouse group. To rule out apoptosis as the cause of lower erythrocyte numbers, Annexin-V binding was assessed within the different erythrocyte maturation stages (Fig 2.5E). The proportion of Annexin-V was equal amongst all maturation stages and between IL-10KO and B6 mouse groups, indicating that erythrocyte deficiency was not due to apoptosis.

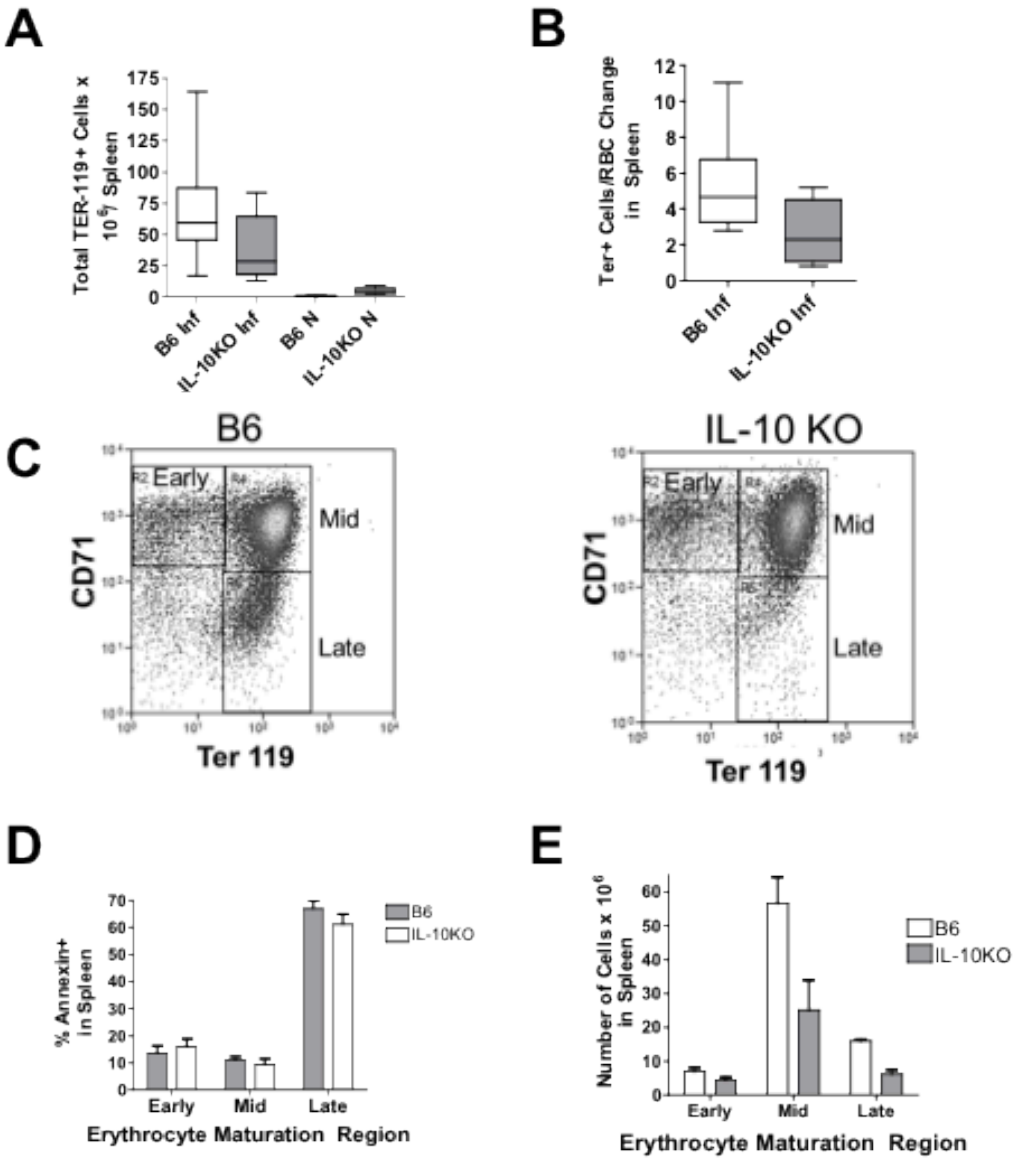

Figure 2.5 IL-10 KO mice experience greater degree of suppressed erythropoiesis. (A) The numbers of TER-119+ cells in the bone marrow of uninfected (N) and infected mice (Inf) were enumerated on day 14 post infection. Data pooled from two separate experiments (5 mice/group/experiment). B6 (white) and IL-10 KO (dark grey) mice. **(B)** Number of TER-119+ cells relative to RBC loss **(C)** Representative dot plots showing the different maturation stages assessed. **(D)** Number of splenic RBC precursors at different maturation stages for B6 (white) and IL-10 KO (dark grey) mice. **(E)** Proportion of Annexin-V+ cells within each maturation stage for both mouse groups. * $p<0.05$

b. IL-10 KO mice have increased red blood cell loss relative to parasitemia

We next asked if red blood cell loss also contributed to the disproportionate anemia during infection. First we tested the longevity of red blood cells in uninfected mice from both strains. CFSE labeled red blood cells were transferred to mice of each strain and the number of CFSE positive cells in circulation was measured over the next 11 days. Figure 2.6A shows that at the end of this time, greater than 90% of the transferred cells remained and there was no difference between the two mouse strains.

In order to assess the contribution of non-parasite mediated red blood cell lysis, we treated both strains of mice with pyramethamine, a drug that acts by interfering with folic acid processing, starting at the day of peak parasitemia (day 15; Figure 2.6B). Consistent with our previous observation, B6 mice had much greater parasite load than IL-10 KO mice at day 15 post infection. After three days of treatment, 5×10^8 CFSE-labeled naïve red blood cells were transferred intraperitoneally. Parasite was determined to be cleared by microscopy and flow cytometry. When direct parasite lysis no longer contributed to red blood cell loss, the kinetics of red blood cell clearance was identical between the two strains and significantly faster than in uninfected mice (Figure 2.6C). Whereas in uninfected mice, greater than 90% of transferred cells can still be detected after 11 days, in infected/treated mice, only 40% of the transferred cells can be detected at 11 days. These results indicate a significant degree of destruction of uninfected red blood cells in both strains. The similar degree of red blood cell loss despite the striking differences in parasitemia suggest a mechanism by which IL-10 KO mitigates anemia through protection of non-parasitized RBCs.

To compare the loss of circulating erythrocytes when parasite burden is equal between the B6 and IL-10 KO groups, IL-10 KO mice were infected with 1000x the dose of parasite given to B6 mice. Three days post infection, equal amounts of CFSE-labeled naïve red blood cells were transferred to both mouse groups. Although parasitemia was equal between IL-10 KO and B6 mice, both total RBC and CFSE-labeled RBC were significantly lower in the IL-10 KO mice (Figure 2.6D, E).

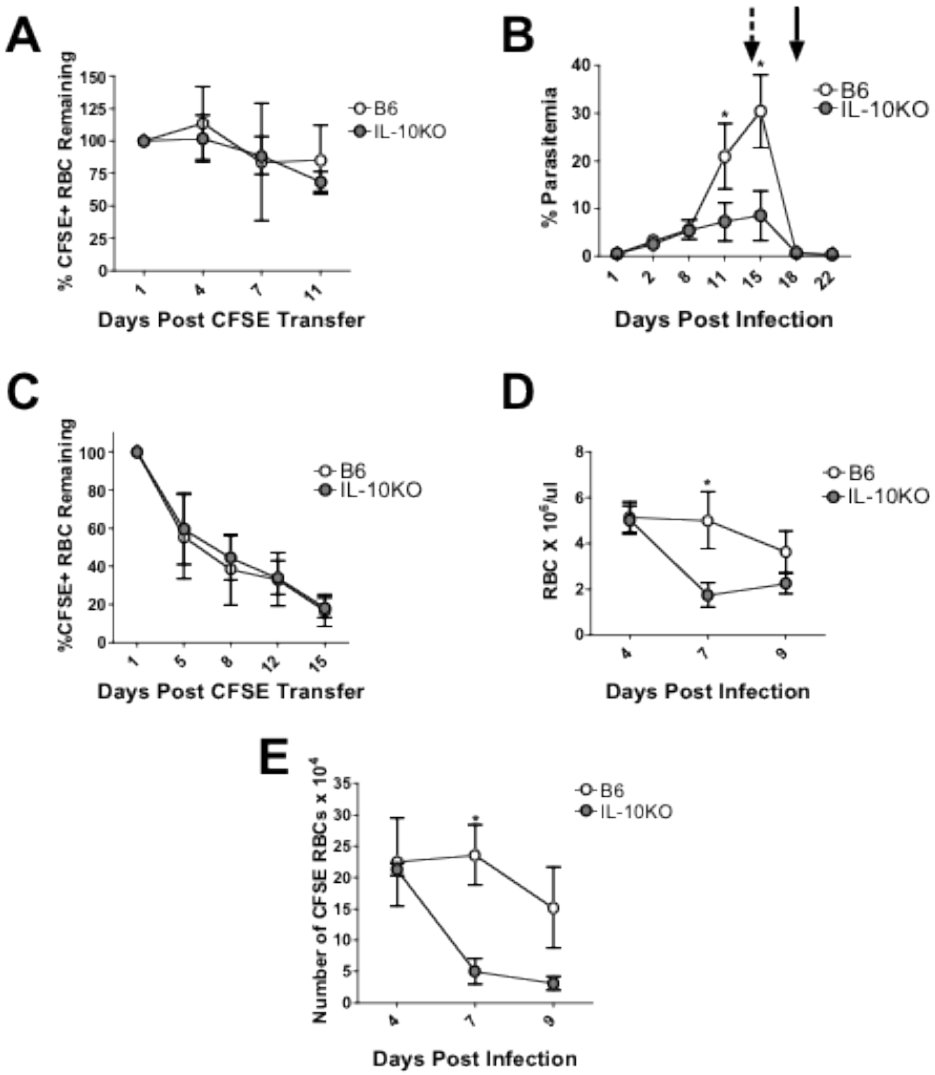

Figure 2.6: Red blood cells are cleared at similar rates in both strains despite a markedly different degree of parasitemia. CFSE experiments reflect 5 mice/group/infection (A) Kinetics of clearance of red blood cells in uninfected mice. 5 x 10^8 CFSE labeled RBC from B6 mice were transferred into uninfected B6 and IL-10 KO mice. The total number of CFSE+ red blood cells the next day were considered 100%, and the percentage of cells remaining each subsequent time point was calculated. **(B)** B6 and IL-10 KO mice were infected with 10^6 infected RBCs and treated with pyrimethamine starting at day 15 (broken arrow) for three days. CFSE labeled red blood cells were transferred at day 17 (solid arrow), and **(C)** the relative percent of CFSE-labeled RBC clearance calculated as above indicate similar levels. **(D)** RBC counts after IL-10 KO mice were infected with 1000x the dose of parasite given to B6 mice. IL-10 KO mice were significantly more anemic on D7. Arrow points to day of CFSE transfer. **(E)** Relative percent of CFSE-labeled RBC was significantly lower in IL-10 KO mice on days 7 and 9 PI. * $p<0.05$

To test whether increased anemia in IL-10 KOs could be due to antibody mediated RBC clearance, the level of RBC-associated IgG was quantified on days 5, 10 and 12 post infection. We found that IgG levels increased after day 5 equally in both strains, despite the significantly lower level of parasitemia in IL-10 KO mice (Figure 2.7). These results are consistent with the observation that red blood cells are cleared with equal efficiency in both strains, and support the idea that clearance during later stages of infection could be antibody mediated.

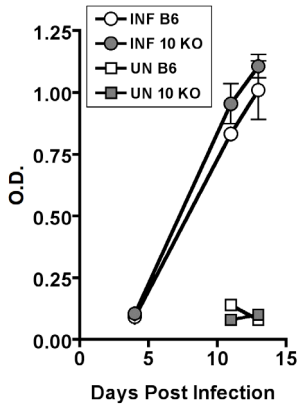

Figure 2.7: Amount of RBC-associated antibodies is similar in both strains despite a markedly different degree of parasitemia.
Antibodies on the surface of red blood cells appear during infection and are similar in both strains. The amount of IgG on red blood cells was measured after elution by ELISA.

Discussion

Human clinical studies have found that the low levels of IL-10 correlate with greater malaria-associated morbidity and mortality. Studies using murine models of malaria have shown IL-10 to be important in mediating disease by measuring changes in weight loss, hypothermia, and hypoglycemia (Couper et al., 2008; Langhorne et al., 2004; Li et al., 2003), however, the effect of IL-10 on anemia has not been reported. In this study, we find

that IL-10 impacts two mechanisms which contribute to malaria anemia: destruction of uninfected red blood cells and suppression of erythropoiesis.

We and others have observed that in the absence of IL-10, parasitemia is markedly decreased during infection when compared to wild type B6 mice. Although anemia has been reported in IL-10 deficient mice during infection with P. yoelii and P. *chabaudi* (6, 47), the severity of anemia that is associated with low parasitemia seen in these mice has largely been overlooked. Using a murine model for uncomplicated malaria, we asked whether IL-10 was protective against severe anemia.

First, we found that the ratio of maximum red blood cell loss to maximum parasite burden was a simple and consistent way to quantify the relationship between anemia and parasitemia in two strains with dramatically different parasite burdens. Our results showed that there was disproportionately high anemia relative to parasite levels found in IL-10 deficient mice. Furthermore, we found that normalizing levels of parasitemia resulted in greater anemia in the IL-10 KO mouse group during early stages of infection, most likely due to innate immunity. Infection of IL-10 KO mice by P. *yoelii* therefore provides another useful experimental model for severe malarial anemia.

Next we addressed the mechanism by which anemia occurs in the absence of IL-10. The two possible explanations include: 1) dysregulated erythropoiesis or 2) increased erythrocyte destruction. During infection, levels of erythropoietin (EPO) were found to be equivalent between both IL-10 KO and B6 groups, indicating that the signal to initiate erythropoiesis did not cause the disparity in anemia. When the total number of splenic RBC precursors were enumerated in both B6 and IL-10 KO groups, IL-10 KO mice had significantly lower numbers of RBC precursors relative to B6 mice yet equal levels of

Annexin V positive cells, suggesting suppressed erythropoiesis during infection and not increased apoptosis.

Suppression of erythropoiesis could be due to several factors. Proinflammatory cytokines IFN-γ and TNF-α have been experimentally shown to inhibit erythropoiesis (5, 59) and high levels of these cytokines have been associated with severe anemia (52, 53). We have previously shown IL-10 KO mice infected with P. *yoelii* exhibited greater levels of serum IFN-γ than B6 mice, but had equal levels of TNF-α (74). One explanation for the disparity of anemia in the absence of IL-10 could be the unchecked production of cytokines, such as IFN-γ, which may inhibit RBC production while stimulating RBC clearance.

In addition to inhibition of erythropoiesis, we also asked whether RBCs were lost through immune-mediated destruction of uninfected RBC. When levels of red blood cell destruction were assessed after parasite clearance with pyrimethamine treatment, the kinetics of red blood cell disappearance was identical between the IL-10 KO and B6 mice despite the significantly higher parasite experienced by the B6 mice. These results indicate a significant and comparable degree of destruction of uninfected red blood cells in both strains despite the marked differences in parasitemia. Furthermore, we found that levels of antibody associated RBCs rose by day 10 and were equivalent between the two mouse groups. The similar degree of red blood cell loss and RBC-associated antibodies, despite the significant differences in parasitemia, suggest a mechanism of immune-mediated destruction of RBCs and may further explain why IL-10 KO mice develop greater anemia during later stages of infection relative to parasite burden when compared with wild type mice. It will be useful to determine the isotype and specificity of the red-cell associated

antibody. This could be autoimmune, directed against parasite antigen bound to the red blood cell surface, or be immune complexes which are carried by red blood cells.

Severe malarial anemia (SMA) is thought to be due to a combination of direct lysis of red blood cells, dysregulation of erythropoiesis, and immune mediated red blood destruction. There are currently two murine models of SMA. One model is based on the observation that, in the absence macrophage migration inhibitory factor (MIF), severe anemia occurs during P. *chabaudi* infection (59). Severe anemia in the MIF murine model of infection is thought to be largely due to suppressed erythropoiesis. The second model is a low parasite SMA model that is achieved with repeated infections of Balb/C mice with *P. berghei,* followed by anti-malarial drug cure (18). In contrast to the MIF model, anemia was shown to be due to increased red blood cell clearance in the semi-immune SMA model.

Here, we have identified a third murine model of severe malarial anemia where IL-10 mitigates anemia by two different methods: IL-10 ameliorates immune mediated red blood cell destruction as well as suppression of erythropoiesis. It is possible that IL-10 may be the common factor that modulates severe malarial anemia observed in humans. One mechanism by which IL-10 could be mitigating anemia is by inhibiting the anti-erythrocytic effects of proinflammatory cytokines. Pro-inflammatory cytokines TNF-α and IFN-γ are capable to suppressing erythropoiesis. IFN-γ and TNF-α directly suppresses the growth of RBC progenitor CFU-E *in vitro* (60, 106).

Future Directions

In Chapter 2, we demonstrated that, in the absence of IL-10, anemia during *P. yoelii* infection was exacerbated due to a combination of suppressed erythropoiesis and increased RBC destruction. It will be important to identify the cellular source of IL-10 that

is critical for mitigating anemia as well as further understanding the mechanism behind erythrocyte loss during malaria infection. One approach would be to measure intracellular production of Il-10 in immune cells such as T-cells, Dendritic Cells, and B-cells during infection to determine the cell subtype that produces the greatest amount of the cytokine. A more targeted approach, however, can be taken since CD4+ T-cells from mice that have recovered from malaria make higher levels of IL-10. Furthermore, recent studies have demonstrating the importance of IL-10 derived from Th1 T-cells during diseases caused by Toxoplasma gondii, Lieshmania major and Plasmodium chabaudi.

In addition to characterizing the mitigating cells that produce IL-10, functional studies can be pursued. Working under the hypothesis that CD4+ T cell derived IL-10 is responsible for mitigating anemia during P. *yoelii, in vivo* studies can be utilized. For instance, the specific role of CD4+ T-cell derived IL-10 can be assessed after adoptive transfer of CD4+ T-cells from B6 or IL-10 KO mice into athymic mice. If CD4+ T-cell derived IL-10 is critical for mitigating anemia during infection, the athymic mice that receive the IL-10 KO T-cells would experience greater anemia. Understanding how IL-10 regulates anemia could provide critical targets for future immunomodulatory vaccination programs.

Materials and Methods:

Mice and experimental infection

Wild type C57BL/6J (B6) and IL-10 deficient B6.129P2-Il10tm1Cgn/J (IL-10 KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used after a 2-wk acclimation period. Mice were housed in the Painter Center, Colorado State University (Fort Collins, CO) and all experiments were approved by the Institutional Animal Care and

Use Committee. Only mice younger than 12 weeks old were used for these experiments. Non lethal P. *yoelii* 17X NL was used as described previously (91).

Red blood cell and parasite counts

The number of RBC/ul blood was used as a measure of anemia. To quantify the number of RBCs per volume of blood, 2 µl blood was obtained by tail nick and added to 998 µl of PBS with 2% FBS. 50 µl of this solution was added to 10^6 Caltag counting beads (Invitrogen, Carlsbad, CA) to quantify red blood cell numbers and the remainder was used for flow cytometric determination of parasitemia and reticulocyte counts. Naïve mice were simultaneously evaluated to ensure that anemia was not induced during the course of repeated blood sampling.

Parasitemia parasitemia determined after incubation of cells in 10µg/ml Hoescht 33258 (Sigma, St. Louis, MO) by the percentage of Hoescht positive cells. Flow cytometry was performed on a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA). Hoescht+ cells were enumerated using the 405 laser.

In order to quantify the degree of anemia relative to parasite burden, we calculated the maximum decrease in red blood cell count (pre-infection RBC count minus RBC count on each day of infection), and the maximum percent parasitemia as determined by flow cytometry. The ratio of these numbers (max RBC change/max parasitemia) is high if a small parasite burden causes substantial anemia, and comparatively low if it takes a greater parasite burden to cause the same degree of anemia.

Cell surface markers

Spleen and bone marrow cells were stained with antibodies to Ter-119 and CD71 (BD Biosciences, San Jose, CA). Apoptosis was determined by antibodies to Annexin-V (BD Biosciences, San Jose, CA). Non-specific antibody binding was blocked using CD16/32 anti-mouse antibodies (Fc block, BD Biosciences) prior to surface staining. Immunofluorescence was detected as described above.

CFSE-labeled RBC transfer

Naïve B6 mice were terminally bled and RBCs were resuspended at 5 x 10^7 cells/ml and final volume was noted. CFSE (Invitrogen, Carlsbad, CA) was added at a final concentration of 10μM and RBCs were incubated for 10 min on ice. RPMI 1640/10%FBS was used to quench CFSE staining by adding 5 times the RBC resuspension volume. Cells were then washed 3x in PBS. 5x10^8 CFSE labeled RBCs were injected intraperitoneally per mouse. CFSE RBC population in peripheral blood was monitored by flow cytometry with Caltag counting beads (Invitrogen). In some cases, mice were treated for three days with 5 mg/kg pyrimethamine in one experiment starting at day 15.

RBC antibody assay

5x10^6 red blood cells per sample were washed twice with 1.5ml 1x PBS then lysed at 4^{o}C for 30 minutes (lysis buffer: 50mM Tris HCL, 150mM NaCl, 1% NP-40, protease inhibitor cocktail, Sigma, St. Louis, MO). Lysed cells were spun for 1 minute at maximum speed in a microfuge. Clarified cell material was incubated with 50μl ProteinA coupled sepharose beads (GE Healthcare), while rotating, at 4^{o}C for 2 hours. Beads were washed

twice and bound antibody was eluted with 0.1M glycine-HCL, pH 2.5. Eluted antibody was immediately neutralized with 1M Tris, pH 8.0 and adsorbed on maxisorp 96-well plates (Nunc) overnight at 4°C. Wells were washed twice then blocked with PBS-10% FBS. Blocked wells were incubated with peroxidase labeled anti-mouse IgG antibody at 5ng/ml at 4°C for 1 hour. Wells were washed eight times with PBS-Tween-20 and TMB substrate (KPL) was added to each well. The reaction was stopped with 1M H_3PO_4 after 15 minutes. Samples were read at 405nm.

Measurement of serum erythropoietin

Erythropoietin was measured by ELISA (R&D Systems, Minneapolis, MN) on serum taken at sacrifice.

Statistical Analyses

Statistical significance was determined using two-way Anova unless otherwise noted.

CHAPTER 3: IL-10 DERIVED FROM TH1 CD4+ T-CELLS IS CRITICAL FOR MITIGATING MALARIA-ASSOCIATED ANEMIA

Introduction

Individuals living in malaria endemic areas develop effective anti-parasite immunity over several years of repeated exposure, but become resistant to severe disease after just one or two infections. This observation suggests that the acquired immune system plays a role in both processes, but may involve different mechanisms. In Chapter 2 of this dissertation, IL-10 was demonstrated to mitigate anemia during malaria infection. Using the same mouse model of malaria caused by non-lethal *Plasmodium yoelii*, we test the hypothesis that IL-10 producing T-cells arise as a consequence of infection, and contribute to disease resistance by mitigating severe anemia. Here we show athymic mice that received malaria specific CD4+ T-cells were better able to mitigate anemia when compared to athymic nude mice that received naïve CD4+ T-cells, suggesting that CD4+ T-cells that arise during infection have a protective effect. We also found that wild type mice have an expanded population of IL-10 producing CD4+ T-cells demonstrating a Th1 phenotype during mid- and late-infection. These IL-10 producing CD4+ T-cells protect athymic nude mice from anemia when compared to CD4+ T-cells taken from recovered IL-10 deficient mice. Our findings suggest that one mechanism by which the acquired immune system contributes to resistance to severe disease may be the development of IL-10 producing CD4+ T-cells.

Hypothesis

IL-10 derived from Th1 CD4+ T-cells is critical for mitigating anemia.

Literature review relevant to Chapter 3

CD4+ T-cell*s and malaria*

Through both human clinical studies and animal model research, protection from malaria disease requires a coordinated effort by the adaptive arm of the immune system. CD8+ T-cells, aided by CD4+ T-cells, are critical during the liver stage of infection (28, 86). During the blood stage of infection, CD4+ T-cells and a robust antibody response have been shown to be critical effector cells in controlling parasite growth. Through murine studies, CD4+ T-cells were demonstrated to both control parasitemia and immunopathogenesis (90). An early pro-inflammatory cytokine response is required to control parasite replication, however, excessive levels of pro-inflammatory cytokines, such as IFN-γ and TNF-α, are associated with severe disease (48, 52). Thus, a balance of pro- and anti-inflammatory cytokines is critical for control of the parasite as well as protecting the host.

IL-10 producing T-cells

There are multiple CD4+ T-cell subtypes that can express IL-10: Th1, Th2, Th17, and iTreg. The CD4+ T-cell subtypes are based on cytokine profiles as well as function (Figure 3.1). Th1 T-cells responsive against intracellular pathogens can be identified by IFN-γ upregulation as well production of T-bet, a transcription factor that transactivates IFN-γ expression. Th2 cells protect against extracellular pathogens, such as helminthes. Th17 cells act against extracellular bacteria and fungi. Inducible regulatory T-cells (iTreg) play a critical role in maintaining homeostasis amongst cells of the immune system and can

primarily be identified by expression of the transcription factor Foxp3. Inducible Tregs (iTreg) is an inducible population (i.e. induced by TGF-®) and is phenotypically similar to natural Tregs (104). Of the different IL-10 producing CD4+ T-cell subtypes, the primary CD4+ T-cell populations identified to be active during malaria blood stage of infection are iTreg and Th1 cells.

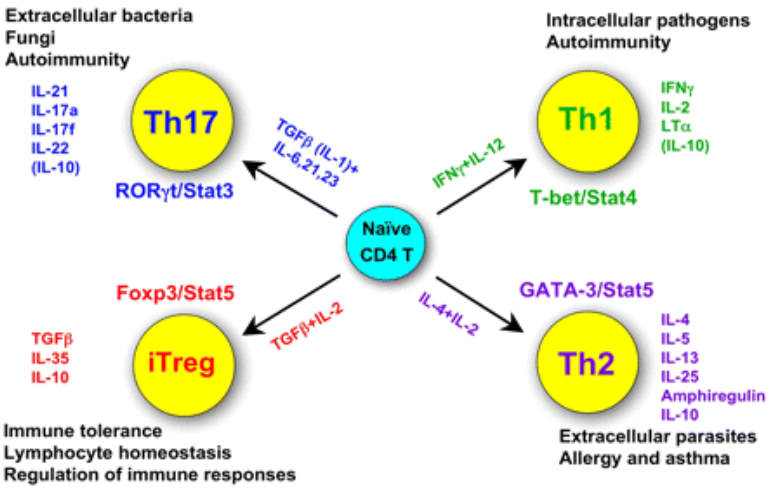

Figure 3.1: CD4+ helper T-cell subtypes with associated cytokine profiles Figure excerpted from: (104)

Evidence supporting the importance of iTregs during malaria infection has been published but the role of these cells remains inconclusive (32). One study showed that the depletion of these cells during lethal P. *yoelii* infection overcomes the lethal phenotype, indicating that Tregs may dampen parasite immunity, but a subsequent study failed to reproduce those findings (19).

In a study published by Walther, et al., higher levels of IL-10 producing Th1 CD4+ T-cells were associated with Gambian children who experienced uncomplicated malaria during *P. falciparum* infection. (97). Recently, Freitas do Rosário, et al. identified, using *P.*

chabaudi chabaudi and C57BL/6 mice, IL-10 producing Th1 CD4+ T-cells as the critical source of IL-10 and is responsible for ameliorating disease during infection as determined by weight loss, red blood cell density, and survival. For other intracellular protozoan parasites such as *Leishmania* and *Toxoplasma gondii*, IL-10 was recently found to be derived from conventional Th1 CD4+ T-cells (as defined by simultaneous expression of IFN-γ and T-bet) and not regulatory T-cells (37).

Overview of Chapter 3 findings

A. *CD4+* T-cell*s that arise during infection ameliorate anemia, but not parasitemia*
B. *CD4+* T-cell*s that specifically express IL-10 are critical for mitigating anemia*
C. *The majority of IL-10 producing CD4+* T-cell*s that arise during infection exhibit a Th1 phenotype*
D. *Absence of IL-10 results in elevated IFN-γ*

Results

A. *CD4+* T-cell*s that arise during infection ameliorate anemia, but not parasitemia*

Since CD4+ T-cells from mice that have recovered from malaria make higher levels of IL-10, and IL-10 KO mice experience greater anemia, we hypothesized that these IL-10 producing T-cells would protect from anemia. To test this, CD4+ T-cells from *P. yoelii* infected/recovered (I/R) or naïve B6 mice were purified and transferred to athymic nude mice. We chose to use a T-cell deficient model with a full complement of B cells (rather than RAG knockout mice) in order to specifically test the effect of CD4+ T-cell manipulation in the presence of an otherwise functioning immune system. One day after CD4+ T-cell transfer, the recipients were infected with 1 x 10^6 parasitized red blood cells (RBCs). This experiment was conducted three times. Figure 3.2 shows the development of anemia and parasitemia of recipient

mice in one experiment. The nude mice receiving T-cells from infected/recovered B6 mice (I/R CD4) developed a higher degree of parasitemia on days 9 and 12 post infection, yet anemia was not different as compared to nude mice that received T-cells from naïve B6 mice (Figure 3.2). In order to quantify and compare the relationships between anemia and parasitemia between the different mouse groups, we measured the ratio between the maximum decrease in red blood cell count and the maximum degree of parasitemia during days 0 to 15 days post infection: [(ΔRBC count/10^5)/(%Parasitized RBC)] (Table 3.1). A higher number indicates that the mouse is experiencing a greater degree of anemia relative to the parasite burden. The degree of anemia relative to parasitemia was significantly greater in the naïve CD4+ T-cell nude mouse recipients in all three experiments. These data suggest that CD4+ T-cells arise during infection and protect against anemia, but not necessarily against parasitemia.

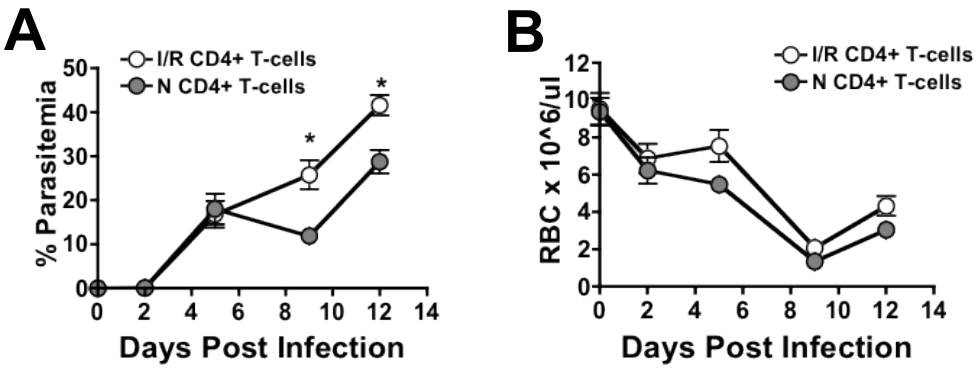

Figure 3.2: CD4 T-cells from infected/recovered mice protect against anemia but not parasitemia. Nude mice were given 5 x 10^6 CD4 T-cells IP from naïve B6 mice (white symbols) or mice that were infected 30 days previously (grey symbols) and infected the next day. Percent parasitemia **(A)** and red blood cell counts **(B)** are shown. Mice receiving T-cells from infected/recovered mice experienced significantly higher parasite burdens, but a lower degree of anemia ($p < .05$ using two way ANOVA).

Table 3.1: Ratio of maximum red blood cell change divided by the maximum parasitemia in 3 individual experiments. A higher value indicates greater anemia experience relative to parasitemia. *All differences between nB6 CD4+ and I/R B6 CD4+ T-cell recipient nude mice were significant. $p<0.05$ using a one tailed t test Calculation described in Methods

	Exp. 1	*Exp. 2*	*Exp. 3*
Naïve CD4	*2.8**	*2.7**	*3.1**
Number of mice	*8*	*5*	*7*
I/R CD4	*1.7**	*1.8**	*2.0**
Number of mice	*8*	*6*	*7*

B. IL-10 producing CD4+ T-cell*s that arise during infection exhibit a Th1 phenotype*

In light of recent studies demonstrating the importance of IL-10 derived from Th1 T-cells during disease, including a murine model for malaria, we hypothesized that IL-10 is responsible for mitigating anemia during P. *yoelii.* In a study published by Walther, et al., higher levels of IL-10 producing Th1 CD4+ T-cells were associated with Gambian children who experienced uncomplicated malaria during *P. falciparum* infection (97). Recently, Freitas do Rosário, et al. identified, using *P. chabaudi chabaudi* and C57BL/6 mice, IL-10 producing Th1 CD4+ T-cells as the critical source of IL-10 and these cells are responsible for ameliorating disease during infection as determined by weight loss, red blood cell density, and survival. Although Freitas do Rosário, et al. observed lower RBC density in the absence of CD4+T-cell derived IL-10, a different murine model for malaria was used. We hypothesized that the IL-10 responsible for mitigating anemia arises from Th1 cells in a P. *Yoelii* and C57Bl/6 model for malaria infection.

An IL-10 expressing CD4+ T-cell population, which was absent at day 3 PI, arose by day 15 PI (Figure 3.3A) and was maintained through day 30 PI (not shown). At days 15 and 30 PI, 89% and 91% respectively of the IL-10 expressing CD4+ T-cells were also $CD44^{hi}$, suggesting an activated T-cell phenotype . Interestingly, the majority of CD4+ T-cells expressing IL-10 simultaneously expressed IFN-γ (Figure 3.3A, C), which may suggest autoregulation of cytokine expression. We also find that these IFN-γ/IL-10 co-expressing CD4+ T-cells expressed higher levels of T-bet relative to cells expressing IL-10 alone, indicating that these co-expressers demonstrate a Th1 phenotype (Figure 3.3B). CD4+ T-cells that expressed IL-10 did not express Foxp3 and the total numbers of Foxp3+CD25+CD4+ T-cells did not change between the IL-10 KO and B6 groups during infection (Figure 3.4), consistent with a previous report(11). Together, these data show that the majority of IL-10 expressing CD4+ cells are Th1 T-cells.

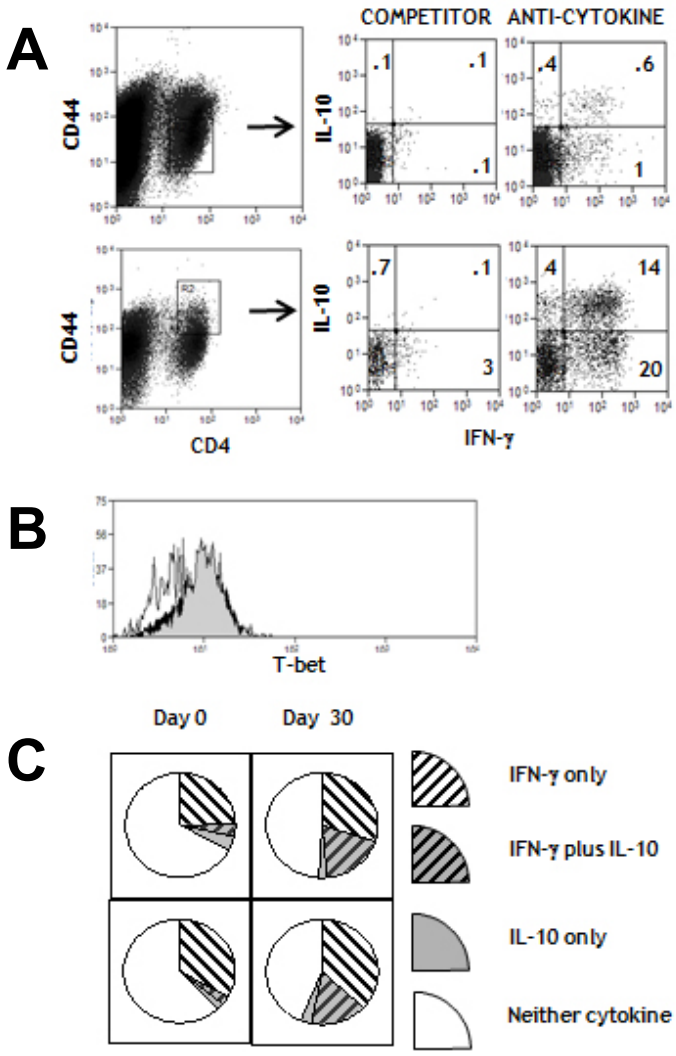

Figure 3.3: Production of IL-10 and IFN-γ during *P. yoelii* infection. (A) CD4+44hi T-cells produce the majority of IL-10 and IFN-γ at day 15 PI (as well as day 30, not shown). Spleen cells were stained with CD4 and CD44, and the CD4+, CD44 low and CD4+, CD44 high populations examined for IL-10 and IFN-γ production after stimulation with PMA/ionomycin and brefeldin. The panel labeled "competitor" depicts cells that were incubated with unlabeled anti-IL-10 and anti-IFN-γ at 5x the dose of the fluorochrome labeled antibody before the fluorochrome labeled antibody was added. This is used instead of isotype controls to determine the amount of non-specific staining. **(B)** Cells were stained with anti-IL-10, anti-IFN-γ and anti-T-bet. T-bet production was measured on each lymphocyte subset. IFN-γ producers, and IFN-γ/IL-10 producers had identical levels of T-bet, but IL-10 producers had low levels. **(C)** The distribution of cytokine producing cells at day 30 post infection reveals that the proportion of IL-10 secreting cells increased, but the majority of these cells also produced IFN-γ. The chart depicts the percentage of each type of cell in the CD4+CD44high population after stimulation with PMA/ionomycin with the top two and bottom two charts represent two different experiments.

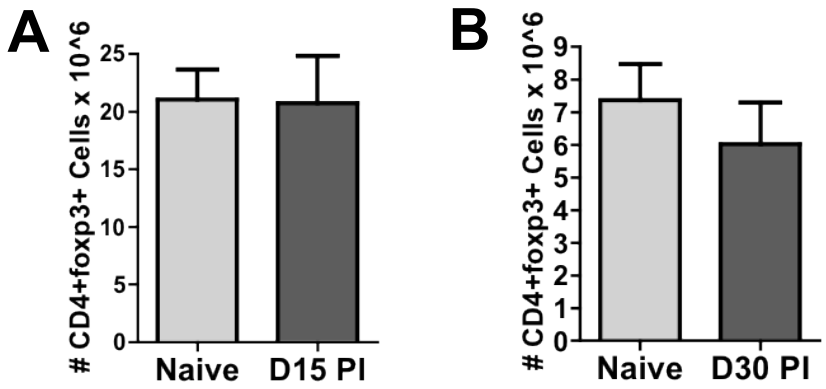

Figure 3.4: Levels of Foxp3 remained constant throughout infection. The number of CD4+foxp3+ cells in the spleen are equal between Naïve (light grey) and Infected (dark grey) mice. **(A)** Cells from naïve or 15 days post infection were stained with CD4 and examined for foxp3 production after stimulation with PMA/ionomycin and brefeldin. **(B)** Naive or 30 days post infection foxp3 cells were compared.

C. CD4+ T-cells that specifically express IL-10 are critical for mitigating anémia

Next we hypothesized that IL-10 produced by T-cells from I/R mice was necessary for the protective effect that was observed previously when compared to, Naïve CD4+ T-cells. To address this hypothesis, CD4+ T-cells from *P. yoelii* I/R (infected/recovered) IL-10 KO or B6 mice were transferred to nude mice and then infected with 1 x 10^6 parasitized red blood cells (pRBCs). The experiment was conducted twice. Figure 3.4 shows the development of anemia and parasitemia of recipient mice in one experiment. As seen with infected IL-10 KO mice in Chapter 2 of this dissertation, nude mice that only received IL-10 KO CD4+ T-cells experienced greater anemia relative to parasitemia as compared to nude mice that received B6 CD4+ T-cells, suggesting IL-10 specifically derived from CD4+ T-cells mitigates immune-mediated anemia during infection.

The nude mice receiving T-cells from IL-10 KO mice developed a lower degree of parasitemia on days 9, 12, and 15 days post infection while RBC loss was equivalent between the two groups (Figure 3.5). In order to quantify and compare the relationships

between anemia and parasitemia between the different mouse groups, we measured the ratio between the maximum decrease in red blood cell count and the maximum degree of parasitemia during days 0 to 15 days post infection: (ΔRBC count/10^5)/(%Parasitized RBC) (Table 3.2). A higher number indicates that the mouse is experiencing a greater degree of anemia relative to the parasite burden. In both of these experiments, mice receiving T-cells from IL-10 KO mice were more protected from parasitemia than those receiving B6 T-cells, but became more anemic relative to the parasite burden. These data suggest that IL-10 derived from CD4+ T-cells plays a role in is critical for mitigating anemia.

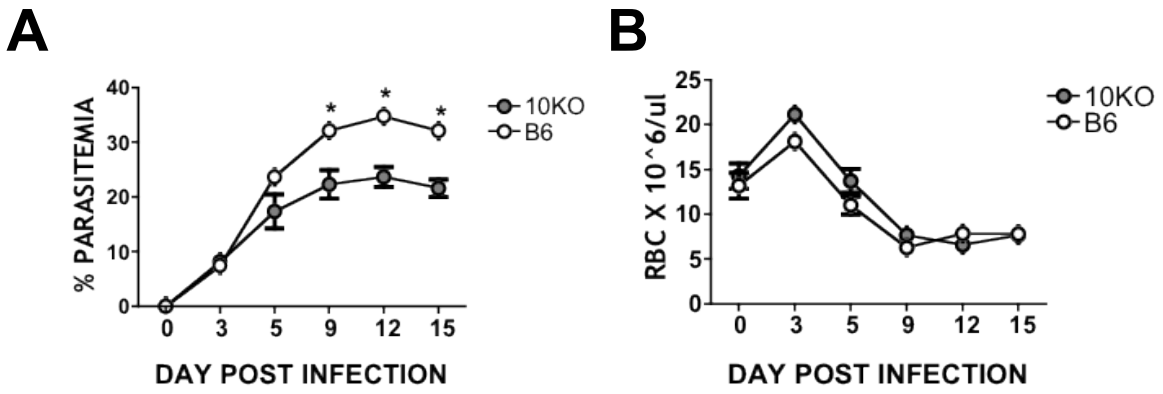

Figure 3.5: T-cells from infected/recovered IL-10 KO mice result in decreased parasitemia but a relatively greater degree of anemia compared with B6 mice. (A) Nude mice were given 5 x 10^6 CD4 T-cells IP from B6 mice infected 30 days previously (white symbols) or IL-10 KO mice (gray symbols) and infected the next day. **(B)** Degree of parasitemia was significantly different between the two groups. ($p < .05$, two way ANOVA)

Table 3.2: Ratio of maximum red blood cell change divided by the maximum parasitemia for B6 and IL-10 KO mice in 2 individual experiments. A higher value indicates greater anemia experience relative to parasitemia. *All differences between B6 CD4+ and 10KO CD4+ T-cell recipient nude mice were significant. $p<0.05$ using a one tailed t test

	Exp. 1	*Exp. 2*
B6 CD4+	*3.4**	*4.6**
Number of mice	*6*	*5*
10KO CD4	*6.0**	*6.3**
Number of mice	*6*	*5*

D. Absence of IL-10 results in elevated IFN-γ

We have previously shown that in IL-10 KO mice infected with P. *yoelii*, IL-10 KO mice exhibited greater levels of serum IFN-γ, although in those studies we did not address its cellular source (74). Recently, a technique called the integrated mean fluorescence intensity (iMFI) was introduced to measure the cytokine producing potential of T-cells in a vaccine model (15). iMFI incorporates both the percentage of cytokine producing T-cells, and the relative amount of cytokine per cell, which gives a more accurate depiction of cytokine production than looking at percentage of positive cells alone. Here, we show that the number of IFN-γ+ CD4+ T-cells multiplied by IFN-γ fluorescent intensity (iMFI) was greater in the IL-10 KO mice than in wild type mice (CD4+ T-cell competent mice). This was true for both spontaneous production of IFN-γ and for IFN-γ production stimulated by PMA (Table 3.3).

Similarly, T-cells from IL-10 KO mice transferred to nude mice made higher levels of IFN-γ than T-cells from B6 mice. We also examined serum cytokines in nude recipients of

T-cells (Table 3.3). The recipients of B6 CD4+ T-cells produced significantly higher serum IL-10 than nude mice receiving IL-10 KO CD4+ T-cells when measured at sacrifice, day 14 (937 and 177 pg/ml IL-10 respectively, $p < .05$). In contrast, the nude mice that received IL-10 KO T-cells produced higher serum IFN-γ than the B6 recipients (14 vs 6.3 pg/ml, $p < .05$). These findings demonstrate that the transferred T-cells either produced sufficient cytokine to be detected in the serum themselves, or influenced other cells to produce IL-10 and IFN-γ.

Table 3.3: Comparison of integrated mean fluorescence intensity of CD4-derived IFN-γ production between infected B6 and IL-10 KO mice (two different experiments: 1 and 2), and between nude mice receiving B6 or IL-10 KO CD4 T-cells (exp. 3 and 4).

*Experiments 1 and 2 are measurements of IFN-γ production by T-cells at day 12 post infection. Experiments 3 and 4 are measurements of IFN-γ production by T-cells from infected/recovered B6 and IL-10KO mice 12 days after transfer into, and infection of, nude mice.

†None = 4 hours of culture with brefeldin A and no stimulus, PMA = 4 hours of culture with brefeldin A, PMA, and ionomycin.

‡Number indicates integrated mean fluorescence intensity: (% of spleen cells that are CD4+CD44hiIFN-γ x the mean fluorescence intensity of these cells /100).

All comparisons between B6 and IL-10KO mice in boxes were significant $p < .05$

	Stimulus	*T-cell sufficient mice*			*Stimulus*	*Nude mice*	
		B6	10KO			B6-T	10KO-T
*Exp 1**	None†	142‡	137	*Exp 3*	None	68	284
	PMA	386	864		PMA	210	459
Exp 2	None	73	163	*Exp 4*	None	23	53
	PMA	115	219		PMA	73	100

Discussion

People living in malaria endemic areas develop resistance to severe illness before they develop resistance to infection. The factors involved in disease resistance are likely to be complex and multi-faceted. Here we identify one potential component of resistance to severe anemia: the development of IL-10 producing CD4+ T-cells.

IL-10, an important modulator of proinflammatory cytokines, can be expressed by multiple cell types from both the innate and adaptive arms of the immune system: B- and T-cells, NK cells, mast cells, eosinophils, dendritic cells, monocytes and macrophages. The magnitude and timing of IL-10 expression during *Plasmodium* infection can either result in immune mediated disease due to excessive proinflammatory mediators or unchecked parasite growth due to insufficient immune control (46, 58, 70). Identifying the cellular source of IL-10 that mitigates anemia during malaria infection could provide potential targets for future immunomodulatory therapeutics.

IL-10 has been shown to control immunopathogenesis in several murine models of disease, including parasite infections by *Toxoplasma gondii* , *Trypanosoma cruzi*, and *Plasmodium* spp. (25, 34, 35, 48, 52). IL-10 deficient mice succumb to death during infection in these models, despite parasite control, due to the overproduction of proinflammatory cytokines (25, 34). For example, IL-10 KO mice infected with a non-lethal strain of T. gondii experienced similar parasite levels to wild type mice, however succumbed to disease 1-2 weeks post infection with significantly higher levels of IL-12, IFN-g and TNF-a. Depletion of CD4+ T-cells in IL-10 KO mice prior to T. gondii infection reversed the increase in mortality and elevated proinflammatory cytokine production,

suggesting the critical role of CD4+ T-cells in mitigating immune-mediated disease during infection (25).

We previously published our observation that serum IFN-g, but not TNF-a, is significantly increased in IL-10 deficient mice during *P. yoelii* infection (74). In Chapter 2, we demonstrated that, in the absence of IL-10, anemia during *P. yoelii* infection was exacerbated due to a combination of suppressed erythropoiesis and increased RBC destruction. Proinflammatory cytokines IFN-γ and TNF-α have been experimentally shown to inhibit erythropoiesis (5, 55) and high levels of these cytokines have been associated with severe anemia (48, 49). It is possible that the disproportionate anemia we observed in IL-10 KO mice during P. yoelii infection is due to the uncontrolled elevation of IFN-g in the absence of IL-10 and we sought to further define the mechanism is Chapter 3.

In Chapter 3, using an adoptive transfer model, we identify the cellular source of IL-10 that mitigates anemia during parasite infection: CD4+ T-cells. IL-10 expressing CD4+ T-cells that arise during P. *yoelii* infection primarily consist of Th1 T-cells as defined by the production of IFN-γ with increased levels of intracellular T-bet and surface CD44+ expression (2, 15). Our data is consistent with a recent study identifying the importance of IL-10 producing Th1 T-cells two weeks after P. *chabaudi* infection, where a lower RBC density was observed in the absence of CD4+T-cell derived IL-10 (23). Similar IFN-γ/IL-10 producing T-cells have also been demonstrated in *Leishmania* and *Toxoplasma gondii* infections and were found to be protective against parasite growth. (4, 37).

In our study, the IFN-γ/IL-10 producing cells express high levels of CD44+ and express equivalent levels of T-bet when compared to T-cells producing IFN-γ only, consistent with Th1 T-cells. Because the cells that only produced IL-10 had lower levels of

T-bet, it seems likely that these T-cells are a different lineage, but it is not clear which of the two cell types are responsible for protection against anemia. Recently, Couper et al. identified IL-10 producing T-cells (Tr1 cells) early in infection (up to day 7 PI) that are critical for mitigating pathology (11). These cells did not appear to have increased levels of IFN-γ based on mRNA levels (11). It is our experience as well that IL-10 is not co-expressed with IFN-g in CD4+ T-cells at day 3 or 7 (data not shown), but is by day 15 post infection. Furthermore, adoptive transfer of IL-10 deficient CD4+ T cells in an IL-10 competent Nude mouse resulted in significantly higher serum IFN-g when compared to Nude mice that received wild-type CD4 T-cells, similar to our findings in IL-10 KO mice (74).

Together, the data from Chapters 2 and 3 suggest a model for immune mediated anemia during malaria infection that is orchestrated by CD4+ T-cells. During initial infection, robust IFN-g expression by CD4+ T-cells is required for parasite control. As infection progresses, Th1 cells co-express IL-10 by day 15 post infection, thereby limiting immune-mediated anemia. In the absence of CD4+ T-cell derived IL-10, IFN-g expression within Th1 cells is allowed to go unchecked, which correlates with elevated serum IFN-g and significantly greater anemia due to a combination of suppressed erythropoiesis and red blood cell clearance.

Future Directions:

In Chapter 3, we identified CD4+ T-cells as the cellular source of IL-10 that mitigates anemia during parasite infection. We then described Th1 cells as the primary subset of CD4+ T-cells that produce IL-10, which are detectable two weeks post infection. A logical next step for future experiments would specifically test whether IL-10 producing Th1 T-

cells, alone, are sufficient for mitigating anemia during infection. One potential experiment would utilize the green fluorescence protein (GFP) knockin mouse, known as the tiger (interleukin-ten ires gfp-enhanced reporter) mouse, which allows for real time tracking of IL-10 expression by flow cytometry (40). The significance of real-time tracking of IL-10 expression is that classic methods for intracellular IL-10 detection comes at the cost of cell viability. Cell viability is lost during the fixation step required for intracellular IL-10 labeling and the tiger mouse allows for the use of viable IL-10 expressing cells in functional studies, One could isolate IL-10 expressing Th1 cells as defined by the presence of CD44hi CD4+ cell surface molecules and intracellular GFP expression (IL-10) from tiger mice (WT phenotype) for transfer into Nude mice prior to infection. The role of IL-10 expressing Th1 cells could be compared to non-GFP Th1 cells and we would predict the IL-10 derived from Th1 cells are critical for mitigating anemia.

Another goal would be to identify the factors that upregulate IL-10 in Th1 cells during infection. Various molecular signals have been shown to induce IL-10 expression in Th1 cells. Characterizing the specific signals that upregulate IL-10 in Th1 cells during infection could provide an important step in understanding immune mediated anemia as well as provide potential targets for treatment development.

Materials and Methods

Mice and experimental infection

C57BL/6J (B6), B6.129P2-Il10tm1Cgn/J (IL-10 KO), and B6.Cg-Foxn1nu/J (nude) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used after a 2-wk acclimation period. Mice were housed in the Painter Center, Colorado State University (Fort

Collins, CO), and all experiments were approved by the Institutional Animal Care and Use Committee. P. *yoelii* 17X was used as described previously (74).

Red blood cell and parasite counts

Anemia during infection was assessed by adding 2 ul blood obtained by tail nick to 998 ul of PBS with 2% FBS. 50 ul of this solution was added to 10^6 Caltag counting beads to quantify red blood cell numbers, and the remainder was used for flow cytometric determination of parasitemia and reticulocyte counts. Naïve mice were simultaneously evaluated to ensure that anemia was not induced during the course of repeated blood sampling.

Parasitemia determined after incubation of cells in 10μg/ml Hoescht 33258 (Sigma, St. Louis, MO) by the percentage of Hoescht positive cells. Flow cytometry was performed on a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA). Hoescht+ cells were enumerated using the 405 laser.

In order to quantify the degree of anemia relative to parasite burden, we calculated the maximum decrease in red blood cell count (pre-infection RBC count – RBC count on each day of infection), and the maximum percent parasitemia as determined by flow cytometry. The ratio of these numbers (max RBC change/max parasitemia) is high if a small parasite burden causes substantial anemia, and comparatively low if it takes a greater parasite burden to cause the same degree of anemia.

Cell surface markers

Surface staining of cells include antibodies to CD4, CD44, CD45, Ter-119, and CD71 (BD Biosciences). Non-specific antibody binding was blocked using CD16/32 anti-mouse

antibodies (Fc block) prior to surface staining. Immunofluorescence was detected as described above.

Intracellular cytokines

Splenocytes were stimulated for 4 hours with PMA (50ng/ml) and ionomycin (0.5ug/ml) in the presence of brefeldin A (10ug/ml) and 2ME (50uM) in DMEM-5% FBS. Cells were surface stained, fixed in 4% paraformaldehyde for 20 minutes, and then permeabilized with 0.1% saponin in PBS. Cells were then stained with anti-mouse IFN-γ, IL-10, and T-bet antibodies (BD Biosciences) in .1% saponin. Cells were treated with 10 ug/ml of anti CD32 prior to antibody staining to prevent non-specific binding. Specificity of intracellular cytokines was confirmed using unlabeled antibody as a cold competitor how much? How long?. Immunofluorescence was detected as described above.

CD4+ T-cell transfer

CD4+ T-cells were positively selected from mouse spleens using directly conjugated anti-CD4 microbeads (Miltenyi). Because a significant amount of iron-containing pigment sticks non-specifically to magnetic columns, spleen cells were passed through a col umn before being incubated with the anti-CD4 beads. Purification was then conducted according to the manufacturer and purity was >90%. $5x10^6$ CD4+ T-cells were transferred intraperitoneally per mouse. Mice were infected 24 hours post CD4+ T-cell transfer.

Statistical Analyses

Statistical significance was determined using two-way Anova unless otherwise noted.

CHAPTER 4: IL-10 INDUCED IN TH1 CD4+ T-CELLS BY EXTRACELLULAR IFN-γ AND IL-10

Introduction

IL-10 producing Th1 CD4+ T-cells have recently been identified as key immunoregulators during the course of infectious diseases, such as Toxoplasma *gondii* and Leishmania. Recently, Th1 cells that express IL-10 were found at higher levels in Gambian children with uncomplicated malaria compared to children experiencing severe malaria. Furthermore, in a murine model of malaria infection, Th1 cell derived IL-10 mitigated disease. In Chapter 3 of this dissertation, we identified IL-10 expressing Th1 cells that arise during the course of P. *yoelii* infection. We also demonstrated the importance of CD4+ T-cell derived IL-10 in mitigating malaria-associated anemia. Chapter 4 focuses on identifying key molecules that upregulate IL-10 in CD4+ T-cells. Utilizing an *ex vivo* system that tests the function of APCs in activating CD4+ T-cells, we first determined that APCs exposed to P. *yoelii in vivo* induced a greater population of CD4+ T-cells that express IL-10 compared to naïve APCs. We also demonstrate that IFN-γ is required, with the possible involvement of IL-10 and IL-12, for efficient IL-10 expression in CD4+ T-cells.

Hypothesis

IL-12, IL-10 and IFN-γ induce IL-10 in Th1 T-cells during malaria infection

Literature review relevant to Chapter 4

Il-10 Th1 T-cell*s*

T helper cells are a subpopulation of CD4+ lymphocytes that act as cellular mediators of the immune system. Within the category of T helper cells, several different

subtypes have been identified: Th1, Th2, Th17, induced regulatory T-cells (iTreg) and the recently described T follicular helper cells (Tfh) (55). Each T helper cell subtype differs by specific function, cellular signaling, and cytokine expression profile. Their general mode of action is to recruit and activate other and recruit immune cells via cytokine secretion and direct cell contact.

Th1 cells facilitate cell mediated responses, primarily against intracellular pathogens (105) such as those that cause Listeriosis, tuberculosis, and Leishmaniasis (reference). Although Th1 cells are critical for protection against intracellular pathogens, dysregulated Th1 activity can cause harm. For instance, Th1 cells have been implicated in autoimmune diseases such as Type I diabetes, which is caused by immune-mediated destruction of islet cells in the pancreas.

Expression of IFN-γ and T-bet, a T-box transcription factor, are both hallmarks for Th1 cell identification. Th1 activation follows the recognition of specific antigen presented by antigen presenting cells (APCs). The cell-surface marker CD44 is a commonly used marker for identifying activated T-cells. During early stages of T-cell clonal expansion, CD44 is upregulated on the cell surface and is maintained at high levels thereafter.

Th1 cells were originally described as pro-inflammatory cells, while Th2 cells were considered anti-inflammatory, based on cytokine expression profiles. In the early 1990s, the discovery that Th1 T-cells expressed IL-10 simultaneously with IFN-γ came as a surprise, since the co-expression of anti- and pro-inflammatory cytokines appeared paradoxical and the phenomenon was novel. It soon became clear, through infectious disease research, that IL-10 acts to autoregulate the expression of IFN-γ within Th1 cells. IL-10 expression by Th1 cells has been found to be critical for survival during *Toxoplasma*

gondii infection (37). Conversely, during cutaneous Leishmania major infection in mice, the expression of IL-10 in Th1 cells prevented lesions from healing.

In 2009, a research study found that Gambian children experiencing uncomplicated malaria had CD4+ T-cells that coexpress IL-10 and IFN-γ at levels that were three fold higher compared to children experiencing severe malaria.. In 2012, Freitas do Rosário, et al. published their findings that Th1 T-cell derived IL-10 is critical for protection against severe immunopathology in a P. *chabaudi chabaudi* murine model for malaria. In Chapter 3 of this dissertation, we show that Th1 T-cell derived IL-10 is important for mitigating anemia. Understanding the signal for IL-10 upregulation in Th1 cells could help us identify the mechanism by which IL-10 Th1 cells are produced.

Regulation of IL-10 in Th1 T-cell*s*

The molecular signals required to induce IL-10 expression in Th1 cells are not completely understood. Initial studies demonstrated that both IL-12 and high levels of antigenic stimulation were required for IL-10 production in Th1 cells (81). In one study, IFN-γ together with IL-12 was found to create a positive feedback loop for IL-10 production in Th1 cells(83). In contrast to early findings, Levings, et al. found that both IL-10 and IFN-α were required to induce simultaneous IL-10 and IFN-γ production in CD4+ T-cells (51). Levings, et al. described these IL-10/IFN-γ producing T-cells as Tr1 cells based on the absence of IL-2 production, however, they did not evaluate the presence of T-bet, a definitive marker for Th1 cell differentiation. In light that there is much speculation of the validity of Tr1 T-cells as a discrete subtype of T-cells, it is possible that Levings' findings may apply to IL-10/IFN-γ Th1 cells. It should also be noted that cytokines IL-21 and IL-27 were independently found to enhance high levels of IL-10 in Th1 cells (21, 87).

In a different vein, Notch signaling has been shown to induce IL-10 in Th1 cells. Notch, a transmembrane receptor, is found on T-cells and its function is associated with cell development and differentiation (69). When T-cell Notch is stimulated by DLL4, a surface protein found on plasmacytoid dendritic cells, in the presence of IL-12 or IL-27, IL-10 production is induced (42). Notch added a different element to IL-10 upregulation in Th1 cells, compared to cytokine signaling, due to the cell-to-cell interaction. T-cells under CD3 stimulation expressed less IFN-γ when Notch signaling was disrupted, suggesting a possible role in IFN-γ expression during T-cell activation (69).

Since Th1 T-cells can affect disease outcome through IL-10 expression, it is important to understand how IL-10 is upregulated within these cells. In this chapter, we focus on determining the critical molecules responsible for upregulating IL-10 in Th1 cells in our model for malaria.

Overview of Chapter 4 findings

A. *P. yoelii*-exposed antigen presenting cells (APC), but not naïve APCs, induces IL-10 expression in CD4+ T-cells
B. IFN-γ stimulates IL-10 expression in CD4+ T-cells
    a. IL-10 or IL-12 may contribute, but are not required
    b. Notch pathway is not utilized

Results

A. P. yoelii exposed antigen presenting cells (APC), but not naïve APCs, induces IL-10 expression in CD4+ T-cells

Antigen presenting cells (APCs) are important activators of T-cells in the presence of foreign antigens. Our first hypothesis was: APCs that have encountered parasite *in vivo* will stimulate IL-10 in CD4+ T-cells more effectively than naïve APCs. To determine the role of APCs in IL-10 stimulation, we utilized an *ex vivo* system that requires CD4+ T-cells from mice transgenic for ovalbumin specific T-cell receptors, OTII mice (Figure 4.1). The other cellular component in this system is APCs that are isolated from the spleens of naive or infected mice. The APCs in this system are identified by surface receptor CDllc, an integrin that is expressed primarily on dendritic cells. Ovalbumin is added to the cell mixture to provide ligand for the OTII CD4+ T-cells. Since all OTII T-cells recognize ovalbumin (OVA), this system tests APC function.

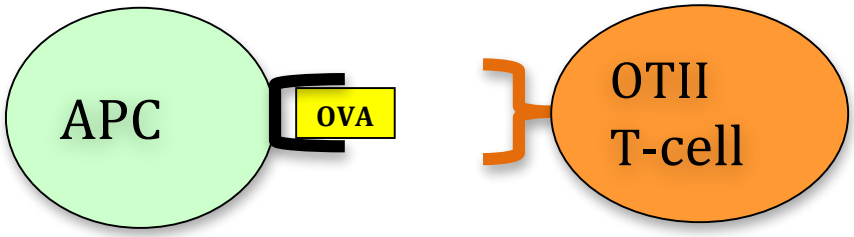

Figure 4.1: *Ex vivo* OTII assay to test APC function. CD4+ T-cells from OTII mice, which encode ovalbumin-specific T-cells, are co-cultured with APCs from experimental mice in the presence of free ovalbumin. Cells are cultured for 5 days, then restimluated with PMA for 4 hours. Intracellular cytokines are assessed by flow cytometry.

In the presence of either naïve or P. *yoelii* exposed APCs, the majority of IL-10 producing CD4+ T-cells also expressed IFN-γ (Figure 4.2A), which was also observed in previous *in vivo* experiments. Because the IL-10-only population was minimal in all OTII

experiments, the data focus in this chapter will be on IL-10+IFN-γ+ CD4+ T-cell data. In contrast to the IL-10+IFN-γ+ CD4+ T-cells (Fig 4.2A, C), the IFN-γ only CD4+ T-cells were equivalent when in the presence of either naïve or exposed APCs (Figure 4.2B). The experiment testing the relative roles of naïve vs. exposed APCs in stimulating IL-10 in CD4+T-cells was conducted three times and each time, the presence of exposed APC significantly increased the amount of IFN-γ+IL10+ CD4+ T-cells (Figure 4.2C). These data suggest that exposed APCs develop the ability to increase the IFN-γ/IL-10 co-expressing CD4 T-cell population relative to naïve APCs.

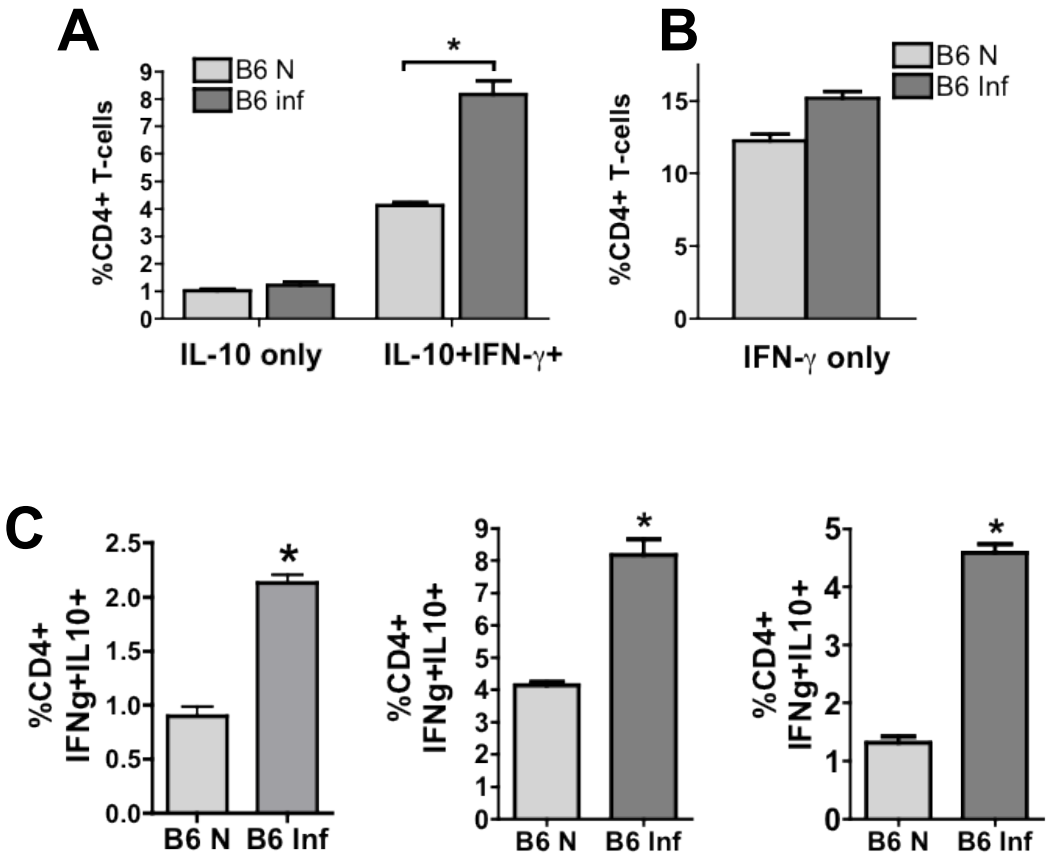

Figure 4.2 P. yoelli exposed **APCs increase the development of IL-10/IFNg producing CD4+ T-cells. (A)** Comparing CD4+ T-cells that only produce IL-10 to those that produce both IL-10 and IFNg in the presence of naïve (N) or parasite exposed (Inf) APCs. The majority of IL-10 producing CD4+ T-cells also produced IFNg, however there was a significantly greater percentage of IL-10+IFNg+ CD4+T-cells in the presence of exposed APCs. **(B)** keep the font and designation consistent only producing CD4+ T-cells did not differ in the presence of naïve vs. exposed APCs. **(C)** Levels of IL10+IFNg+ CD4+ T-cells in the presence of naïve vs. exposed APCs in three independent experiments. 3 mice per group were combined for APC enrichment and each condition was performed in triplicate in all

B. Ifn-γ induces IL-10 expression in CD4+ T-cell*s*

Several cytokines have been shown to upregulate IL-10 in IFN-γ producing CD4+ T-cells: IL-12, IFN-γ, and IL-10. Notch signaling, via DLL4 binding, is yet a different pathway that can upregulate IL-10. Here we determine which signals are critical for IL-10 upregulation in CD4+ T-cells.

Role of cytokines in CD4+ T-cell IL-10 production

Based on our observations that antigen presenting cells differentially induce IL-10 production in T-cells depending on their exposure to P. *yoelii* infection, we asked whether IL-10 produced by APCs were required for IL-10 expression in CD4+ T-cells. To test the role of APC derived IL-10, APCs from naïve or infected IL-10 KO mice were compared with wild type B6 mice (Figure 4.3). Like their B6 counterparts, exposed IL-10 KO APCs stimulated IL-10 production in IFN-γ CD4+ T-cells much more efficiently than naïve IL-10 KO APCs (Figure 4.3). Levels of IFN-γ only CD4+ T-cells were equivalent for all groups (data not shown). Interestingly, there were similar levels of IL10+IFN-γ+ CD4+ T-cells induced by both IL-10 KO and B6 APCs, suggesting that APC derived IL-10 is not important for IL-10 production in CD4+ T-cells.

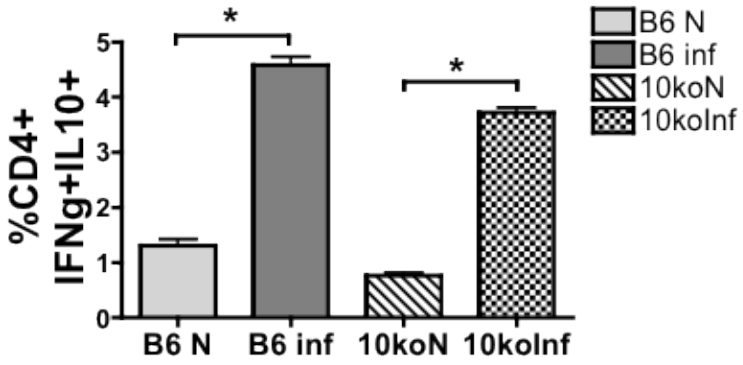

Figure 4.3: P. yoelli exposed **IL-10 KO and B6 APCs increase similar levels of IL-10/IFNγ producing CD4+ T-cells.** Comparing CD4+ T-cells that express both IL-10 and IFN**γ** in the presence of naïve or parasite exposed B6 or IL-10 KO APCs. Similar results in three independent experiments. 3 naïve and 5 infected (day 3 post infection) B6 and IL-10KO mice were combined for APC enrichment and each condition was performed in triplicate in all experiments. *p < .05, paired t test

Utilizing the *ex vivo* OTII system (Figure 4.1), we added cytokine antibodies during the 5 days the isolated APCs and CD4+ T-cells are in culture. Antibodies against IL-10, IFN-

γ, and IL-12 were added to the supernatant in order to determine the specific role for each cytokine in the stimulation of IL-10 in CD4+ T-cells (Figure 4.4). The experiment was conducted three different times and the effect of cytokine antibodies on IL-10 and IFN-γ expression were assessed. Antibodies to IL-10 or IFN-γ resulted in fewer T-cells that produce IL-10 and this was true for all three experiments (Figure 4.3A,B,C). Like previous experiments, almost all IL-10 expressing T-cells also expressed IFN-γ (data not shown). The effect of IL-12 antibody on T-cell IL-10 production was less consistent, with two out of three experiments demonstrating an effect (Figure 4.4 B, C, but not A).

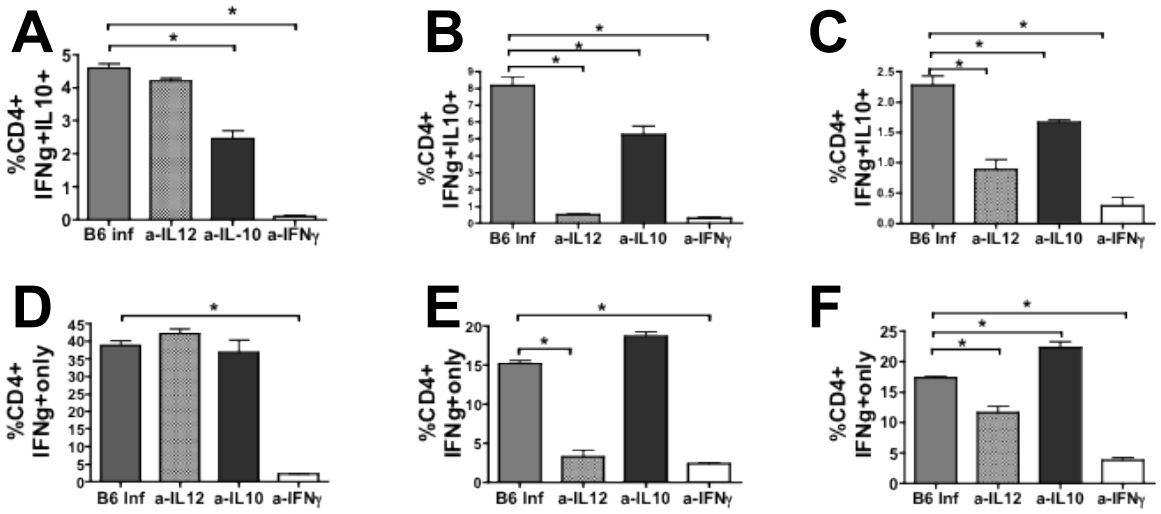

Figure 4.4: Ifn-γ and IL-10 antibody consistently reduces IL-10 and Ifn-γ expressing T-cell population. The effect of antibodies against IL-12, IL-10, and Ifn-γ on T-cell IL-10 and Ifn-γ expression. (**A, B, C)** Comparing cytokine effect on CD4+ T-cells that express both IL-10 and IFNγ in the presence of parasite exposed B6 APCs. **(D,E, F)** Comparing cytokine effect on CD4+ T-cells that express IFNg alone in the presence of parasite exposed B6 APCs. 3 naïve and 5 infected (day 3 post infection) B6 and IL-10KO mice were combined for APC enrichment and each condition was performed in triplicate in all experiments. *p < .05, paired t test

IL-12, IL-10, and IFN-γ can also affect those T cells that produce IFN-g only which was observed in our experiments (Figure 4.4D, E, F). IFN-γ antibody significantly reduced IFN-γ in T-cells for all three experiments while IL-12 antibodies reduced IFN-γ for two (Figure 4.4E, F). IL-10 antibody increased the IFN-γ+ T-cell population in one experiment (Figure 4.4.F), but not in two. Because of the effect on both IFN-γ and IL-10 production, the isolated effect on IL-10 production in T-cells is difficult to interpret.

During malaria infection, both pro-inflammatory and anti-inflammatory cytokines are upregulated. In human clinical studies, high ratios of pro-inflammatory cytokines to low IL-10 levels correlated with greater morbidity and mortality during malaria infection (46, 58, 70). Drawing from human studies, we compared the relative populations of IFN-γ to IL-10 expressing CD4+ T-cells. To compare the relative amounts, the IFN-γ only T-cell population was divided by the IL-10 population to produce a ratio of IFN-γ+ to IL-10+ T-cells (Figure 4.5). The higher the value, the fewer IL-10 producing T-cells relative to IFN-γ T-cells. Antibody against IFN-γ effectively reduced the IL-10 producing T-cells relative to IFN-γ in all three experiments (Figure 4.5). IL-10 and IL-12 antibody, however, each reduced IL-10 T-cells in only one experiment (Figure 4.5A and B, respectively). Together these data suggests that IFN-γ is an important cytokine for the production of IL-10 in CD4+ T-cells during infection. IL-10 and IL-12 alone, however, do not appear sufficient to consistently affect the IL-10 T-cell population.

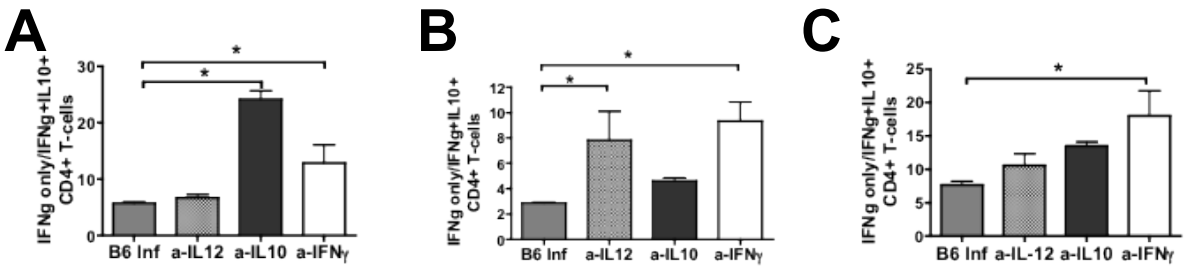

Figure 4.5: Ifn-γ antibody consistently reduces IL-10 expression relative to Ifn-γ in T-cells. The effect of antibodies against IL-12, IL-10, and Ifn-γ on T-cell IL-10 expression relative to Ifn-γ. **(A, B, C)** Comparing cytokine effect on CD4+ T-cells that express both IL-10 and IFN-γ in the presence of parasite exposed B6 APCs. 5 infected B6 mice (day 3 post infection) were combined for APC enrichment and used for each condition which was performed in triplicate. *p < .05, paired t test

Role of Notch in CD4+ T-cell IL-10 production

Notch signaling has also been shown to induce IL-10 in Th1 cells. When T-cell Notch is stimulated by DLL4 in the presence of IL-12 or IL-27, IL-10 production is induced. To test whether APC induction of T-cell IL-10 was facilitated by Notch signaling, the Notch ligand, DLL4, as well as a key protein required for Notch activation, gamma secretase, was targeted (42). Antibody to DLL4 or gamma secretase inhibitor (GSI) were added to the OTII system containing P.*yoelii* exposed APCs. Notch antibody was tested in this system, but resulted in massive cell death and thus, the data is not included.

The experiment was conducted twice and the effect of Notch signaling on IL-10 and IFN-γ expression was assessed (Figure 4.6). Anti-DLL4 antibody and GSI did not affect IL-10 expression in T-cells in either experiment (Figure 4.6A, B). Like previous experiments, almost all IL-10 expressing T-cells also expressed IFN-γ (data not shown). Neither anti-DLL4 antibody or GSI affected levels of IFNg-only producing cells either (Figure 4.6C, D). Together, these data indicate that Notch signaling does not play a role in IL-10 induction in T-cells in this system.

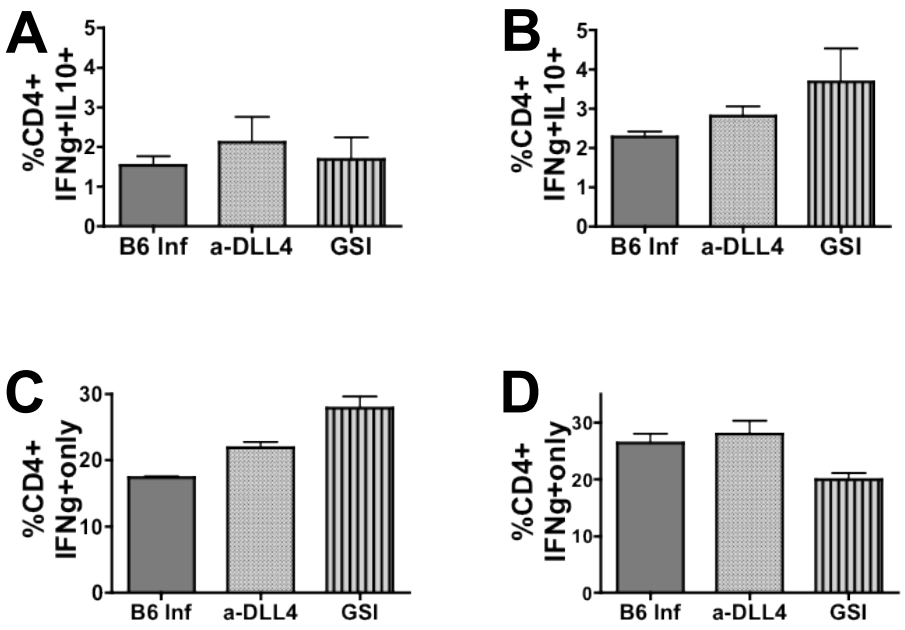

Figure 4.6: Disrupting Notch pathway does not affect IL-10 in T-cells. The effect of blocking Notch receptor binding as well as activation was achieved with anti-DLL4 antibody or gamma secretase inhibitor (GSI). A, B) Comparing two different Notch pathway disruptions on IL-10 expression in CD4+ T-cells in the presence of parasite exposed B6 APCs. C,D) Comparing Notch pathway disruption on CD4+ T-cells that express IFNγ alone in the presence of parasite exposed B6 APCs. The experiment was conducted twice and subfigures A/C and B/D are from the same experiment. 5 infected B6 mice (day 3 post infection) were combined for APC enrichment and used for each condition which was performed in triplicate. *p < .05, paired t test

Discussion

T helper cells include several different subtypes that act as cellular mediators of the immune system: Th1, Th2, Th3, Th17, induced regulatory T-cells (iTreg) and the recently described T follicular helper cells (Tfh) (55). Th1 cells are classically associated with IFN-g production and clearance of intracellular pathogens (8) while Th2 cells were initially thought to be responsible for IL-10 expression (20). Evidence now indicates that IL-10 autoregulates the expression of IFN-γ within Th1 cells during different models of infection,

which can affect disease outcome (4, 37). IL-10 expression by Th1 cells has been found to be critical for host survival during *Toxoplasma gondii* infection by limiting (37). Conversely, during cutaneous Leishmania major infection in mice, the expression of IL-10 in Th1 cells prevented lesions from healing (4). Th1 derived IL-10 has recently been shown to mitigate disease by suppressing an overabundant proinflammatory cytokine response during P. chabaudi infection (23).

In Chapter 2, we demonstrated that, in the absence of IL-10, anemia during *P. yoelii* infection was exacerbated due to a combination of suppressed erythropoiesis and increased RBC destruction. In Chapter 3, we identified the cellular source of IL-10 that mitigates anemia during parasite infection: CD4+ T-cells. We also found that the majority of IL-10 producing CD4+ T cells that arise during infection demonstrate a Th1 phenotype. In this chapter, we focused on testing the contribution of known upregulators of IL-10 using an in vitro model to further understand how Th1-derived IL-10 is modulated in our model of infection.

Utilizing an *ex vivo* system that tests the function of APCs in activating CD4+ T-cells, we first determined that APCs exposed to P. *yoelii in vivo* induced a greater population of CD4+ T-cells that express IL-10 compared to naïve APCs. We then examined the role of antigen presenting cell (APC) derived IL-10 by comparing IL-10 KO and B6 APC activation of CD4+ T-cells. The absence of APC derived IL-10 did not affect the IL-10+ T-cell population, indicating that APC IL-10 is dispensable for IL-10 production in T-cells. Early studies demonstrated that both IL-12 and high levels of antigenic stimulation were required for IL-10 production in Th1 cells (81). In addition, IFN-γ and IL-12 together was described as providing a positive feedback loop for IL-10 production in Th1 cells(83),

however IL-12 was required during the first three days post stimulation (26). Levings, et al. found that both IL-10 and IFN-γ were required to induce simultaneous IL-10 and IFN-γ production in CD4+ T-cells (51). Data in this chapter show that antibodies to IFN-γ resulted in dramatically fewer T-cells that produce IL-10, suggesting IFN-g is required for IL-10 expression. IL-10 antibodies added to the supernatant significantly also reduced the IL-10 producing Th1 population, however, when APC derived IL-10 was absent (IL-10 KO APC), there was no effect. Together, these data suggest that CD4+ T-cell, but not APC derived IL-10 is critical for IL-10 production by Th1 cells. Antibody to IL-12 produced inconsistent effects on IL-10 expression in CD4+ T cells, suggesting that IL-12 is not required, but may influence IL-10 expression, perhaps by the effect it has on IFN-g upregulation.

It should be noted that part of the complication when assessing factors that upregulate IL-10 lays in the ability for these same factors to affect other cytokines, such as IFN-γ, either directly or indirectly. In our studies, when testing the contribution of IFN-γ, IL-10, and IL-12 on IL-10 production, effects on IFN-γ production were simultaneously observed. To better define the relationship between IL-10 and IFN-γ producing T-cells, we measured the relative amount of IFN-γ+ T-cells compared to IL-10+ T-cells. We found that antibodies to IFN-γ consistently resulted in lower levels or IL-10+ T-cells relative to IFN-γ+ T-cells, suggesting that, although IFN-γ regulates T-cell IFN-γ production, the cytokine is also important in mitigating the IL-10 T-cell population.

Notch signaling has been shown to induce IL-10 in Th1 cells in a contact dependent manner via DLL4 binding, independent of IL-12 and IFN-g. We also tested whether the Notch pathway was utilized for IL-10 expression in T-cells. Using antibody to the Notch

receptor ligand, DLL4, or gamma secretase inhibitor (GSI), we found that neither altered IL-10 or IFN-γ in the T-cell population. In summary, we found that IFN-γ is critical for production of greater IL-10 expressing CD4+ T-cells relative to IFN-γ producers. Il-10 and Il-12 may support IL-10 production, but it is not absolutely required.

Together, the data from Chapters 2, 3, and 4 suggest a model for immune mediated anemia during malaria infection that is orchestrated by CD4+ T-cells. During initial infection, robust IFN-g expression by CD4+ T-cells is required for parasite control. As infection progresses, IFN-g or IL-10 (possibly both) are required for Th1 cells to co-express IL-10 by day 15 post infection, thereby limiting immune-mediated anemia. In the absence of CD4+ T-cell derived IL-10, IFN-g expression within Th1 cells is allowed to go unchecked, which correlates with elevated serum IFN-g and significantly greater anemia due to a combination of suppressed erythropoiesis and red blood cell clearance.

Future Directions:

In Chapter 4, we first determined that APCs exposed to P. *yoelii in vivo* induced a greater population of CD4+ T-cells that express IL-10 compared to naïve APCs. We also demonstrate that IFN-γ is required, with the possible involvement of IL-10 and IL-12, for efficient IL-10 expression in CD4+ T-cells. A logical next step for future experiments would explore other factors that affect IL-10 expression. In this chapter, we tested the affect of IFN-g, IL-10, IL-10, Notch and DLL-4. It would be useful to determine whether other proinflammatory or suppressive cytokines, such as TNF-a and TGF-B respectively, affect IL-10 expression in Th1 cells.

It would also be helpful to determine whether the timing of cytokine exposure affects IL-10 expression. In one study, IFN-γ together with IL-12 was found to create a

positive feedback loop for IL-10 production in Th1 cells(83) Interestingly, the timing of IL-12 exposure was critical. IL-12 was required within the first three days post T-cell activation for high levels of IL-10 production. Using our OTII culture system, we can easily manipulate the timing of cytokine exposure. Characterizing the specific signals that upregulate IL-10 in Th1 cells during infection could provide important insight in understanding the factors that cause immune mediated anemia as well as provide potential targets for treatment development.

Methods

Mice and experimental infection

C57BL/6J (B6) and B6.129P2-Il10tm1Cgn/J (IL-10 KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used after a 2-wk acclimation period. Mice were housed in the Painter Center, Colorado State University (Fort Collins, CO) and all experiments were approved by the Institutional Animal Care and Use Committee (IACUC). Only mice younger than 12 weeks old were used for these experiments. Non lethal P. *yoelii* 17X NL was used as described previously (91). In brief, $1x10^6$ parasitized red blood cells were injected intraperitoneally per mouse. (Insert technical name) OTII mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the Painter Center in accordance with protocols approved by the IACUC.

CD4+ T-cell and APC enrichment

OTII CD4+ T-cells were positively selected from single cell suspension spleens using directly conjugated anti-CD4 microbeads according to manufacturer (Miltenyi). Likewise, APCs from experimental mice were enriched with directly conjugated anti-CDllc microbeads

(Miltenyi). Purity of enriched cells were >90%. $5x10^6$ CD4+ T-cells were transferred intraperitoneally per mouse. Mice were infected 24 hours post CD4+ T-cell transfer

T-cell stimulation and analysis

$0.5x10^6$ enriched APCs were co-cultured with 1.5×10^6 OTII CD4+ T-cells for five days in DMEM-5% FBS, 2-ME (50uM), and 1ug/ml Ovalbumin (SIGMA). Anti-IL12, anti-IL10, and anti-IFN-γ were added at 5ug/ml. Anti-DLL4 and GSI were added at 5ug/ml and 20uM respectively (CHECK THIS). At the end of five days, cells were restimulated for 4 hours with PMA (50ng/ml) and ionomycin (0.5ug/ml) in the presence of brefeldin A (10ug/ml) and 2ME (50uM) in DMEM-5% FBS. Cells were surface stained, fixed in 4% paraformaldehyde for 20 minutes, and then permeabilized with 0.5% saponin in PBS. Cells were then stained with anti-mouse IFN-γ, IL-10, and T-bet antibodies (BD Biosciences) in 0.5% saponin. Cells were treated with 10 ug/ml of anti-CD16/CD32 prior to antibody staining to prevent non-specific binding. Specificity of intracellular cytokines was confirmed using unlabeled antibody as a cold competitor. Flow cytometry was performed on a CyAn ADP flow cytometer.

Statistical Analyses

Statistical significance was determined using two-way Anova unless otherwise noted.

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

Why does this research matter?

In response to the ongoing global impact of malaria, several large-scale organizations focus on reducing the presence of this disease. The Roll Back Malaria program (RBM), initiated in 1998, started as a collaboration between the World Health Organization, UNICEF, UNDP, and the World Bank in order to coordinate their efforts to control malaria worldwide. RBM efforts focus on vector intervention such as physical barriers (i.e. net tents) and mosquito population control (insecticides). In addition, RBM focuses on providing accessible drug treatment for infected individuals. This varied approach to combat malaria likely facilitated an estimated 20% decrease in malaria-associated mortality for children under 5 years old (88).

Although current approaches maximize currently available tools to combat malaria, the disease will likely continue to take its toll on world communities. Vector intervention provides a break in the infectious cycle of malaria, however a community can still be at risk of infection if there is inconsistent use of appropriate physical barriers. Furthermore, access to drug treatment has certainly increased due to targeted efforts to diminish the presence of malaria, yet there are still areas that the limited resources cannot reach. Even with treatment, there is a risk of the parasite developing drug resistance. Lastly, unless malaria is eradicated worldwide, reducing the prevalence of malaria may be detrimental long term, in the case of reemergence of the disease, due to the severe morbidity associated with non-immune individuals.

People living in malaria endemic areas often become semi-immune: resistant to disease, but not necessarily to the presence of parasite. Semi-immunity suggests that the immune system is important for long-term protection and the ability to prime the body's response to infection

with semi-immune characteristics may be a long term solution in the fight against malaria. Priming a semi-immune response to malaria would use the complex defenses of the body to diminish the effect of the parasite.

Severe malarial anemia is estimated to kill between 190,000 and 974,000 children under the age of 5 each year (65). The research presented in this dissertation focuses on the immunomodulatory component that is important in mitigating malaria associated anemia: IL-10 expressing CD4+ T-cells.

What does this research mean?

In Chapter 2, we present data suggesting that malaria-associated anemia is mediated by IL-10 by a combination of increased erythrocyte destruction as well as suppressed erythropoiesis. Our findings suggest that one mechanism by which the immune system contributes to resistance to severe anemia may be due to IL-10 orchestrated protection of red blood cells. In Chapter 3, we identified an expanded population of IL-10 producing CD4+ T-cells demonstrating a Th1 phenotype during mid- and late-infection. We also presented data indicating that antigen presenting cells develop the ability to induce IL-10 in CD4+ T-cells during infection and that IL-10 derived from these T-cells mitigate anemia. Our findings suggest that one mechanism by which the acquired immune system contributes to resistance to severe disease may be the development of IL-10 producing CD4+ T-cells. Chapter 4 focuses on identifying key molecules that upregulate IL-10 in CD4+ T-cells. We found that APCs exposed to P. *yoelii in vivo* induced IL-10 expression in T-cells. Furthermore, we find that IFN-γ, with possible IL-10 and IL-12 involvement, is important for stimulating both IFN-γ and IL-10 in T-cells. Together, the data in this dissertation provides insight during malaria infection, identifying

Th1 T-cell derived IL-10 as critical for mitigating anemia and IL-10 is induced by antigen presenting cells and cytokine signaling.

How does this dissertation contribute to the greater body of knowledge?

The balance between pro-inflammatory and anti-inflammatory cytokines can affect disease outcome. In human clinical studies, high ratios of the pro-inflammatory cytokines TNF-α and IFN-γ to low IL-10 levels correlated with greater anemia during malaria infection, but were unrelated to parasite burden [14,15,16,17,18]. A recent study found that an IL-10 promoter haplotype associated with increased circulating IL-10 was protective for severe malarial anemia [19]. These studies indicate that IL-10 helps to modulate the inflammatory response to the parasite, allowing pro-inflammatory cytokines to orchestrate control of parasitemia while preventing the same cytokines from causing harm to the host. A recent study found that children experiencing uncomplicated malaria had higher levels of FOXP-CD45RO+ CD4+ T-cells that coexpress IL-10 and IFN-γ. In a murine model for malaria, Th1 T-cell derived IL-10 was found to be critical for protection against severe immunopathology in a P. *chabaudi chabaudi* murine model for malaria.

Malaria-associated morbidity in murine models has been determined by various measures of disease including weight loss, hypothermia, and hypoglycemia, however, the effect of IL-10 on *anemia* has not been clearly defined. With data presented in this dissertation, we provide an explanation for malaria-associated anemia that pivots around Il-10 expressing Th1 cells. The absence of IL-10, either systemic or in CD4+ T-cells, increase IFN-γ levels in circulation as well as in T-cells. We also provide a glimpse into the signals the upregulate IL-10 in Th1 cells, such as IFN-γ and possibly IL-10 and IL-12.

IL-10 Th1 cells have been found to be important during the course of several infectious diseases. This T-cell subset is protective during *Toxoplasma gondii* infection while these same cells prolong the presence of cutaneous lesions during Leishmania infection. Understanding how IL-10 Th1 cells mediate malaria-associated anemia contributes to our understanding of how these cells balance the immune response. The immune response must to be robust enough to clear pathogens while preventing an excess response that will harm the host. Thus, the larger implication for deciphering the role of IL-10 Th1 cells is that we may better understand the immunopathogenesis of other infectious diseases to facilitate immunomodulatory therapies.

Future directions

Immediate future directions

In this dissertation, we identified a malaria specific subpopulation of Th1 cells that also express IL-10. Antigen presenting cells (APCs) that have been exposed to parasite more effectively induce IL-10 expression in T-cells when compared to naïve APCs. The upregulation of IL-10 is likely due to cytokine signaling, specifically IFN-γ and possibly IL-10 and IL-12. One avenue of study that would logically follow our findings is to further characterize the molecular mechanisms that induce IL-10 in Th1 cells. For instance, a broader range of cytokines could be tested for their role in IL-10 upregulation, since our studies were limited to Il-12, IL-10, and IFN-γ. It would be interesting to test the effect of IL-27, which shown to maximize IL-10 expression in Th1 cells by Rosario, et al during malaria infection (23). In addition, the intracellular regulation of IL-10 would be important to understand. Since IL-10 is produced by a variety of cells such as B- and T-cells, NK cells,

mast cells, eosinophils, dendritic cells, monocytes and macrophages, identifying unique mechanisms of IL-10 production in Th1 cells could provide targets for therapeutics.

Another avenue of study would pursue the specific importance of IL-10 derived from Th1 cells in malaria-associated anemia. In our studies, we studied the effect of IL-10 in the context of CD4+ T-cells. We were not able to conduct the functional studies using isolated IL-10+ T-cells in our system because the methods to detect intracellular cytokines is toxic to cells. The availability of transgenic mice could greatly facilitate our goals. Tiger mice, which encode GFP under the IL-10 promoter, would allow for the isolation of live cells actively producing IL-10. With the ability to isolate T-cells that express IL-10, adoptive transfer studies can be designed to better understand this population in the context of malarial anemia. One such experiment is isolate P. *yoelii* CD4+ T-cells that express IL-10 from Tiger mice and divide them into IL-10 expressing or non-expressing subpopulations. The two cell subpopulations could be independently transferred to two different athymic mouse groups and the infection kinetics in these mice could be assessed. We would hypothesize that the IL-10+ CD4+ T-cell recipient mice would more effectively mitigate anemia during malaria infection than those that received CD4+ T-cells that were not expressing the cytokine.

While the Tiger mouse could provide 'gain of function' perspective for IL-10+ T-cells in athymic mice, a targeted knockout mouse could provide 'loss of function' insight into the function of these cells. Cre-lox recombination, technology derived from a bacteriophage, facilitates the targeted excision of genes in laboratory mice. In brief, Cre recombinase recognizes and recombines Lox sequences. Lox sequences can be encoded in flanking regions of a gene of interest in a recombinant mouse. Cre expression, on the other hand,

can be engineered for production in specific cell types or under certain conditions. Specific to this dissertation, Cre expression under the CD4 promoter, combined with flox sequence flanking the IL-10 gene could eliminate the IL-10 producing CD4+ T-cell population. Thus, with the CD4+Cre,IL-10 flox mouse, the kinetics of infection could be studied in the absence of IL-10+ T-cells.

The big picture: potential applications

Understanding the factors that balance a protective immune response while avoiding harm to the patient would allow for the development of effective vaccines or immunomodulatory drugs, enlisting the help of the immune system to resolve or protect against infection. Immunomodulation therapy, or the manipulation of the immune system against disease, would have several advantages over antimicrobial treatments. Several advantages are found in the complex defense mechanisms used by the immune system. Unlike antimicrobials that usually target a specific step in the pathogen life cycle, the immune system responds with various cell types and mechanisms during the innate and adaptive immune response. While antimicrobials can lead to drug resistance, enhancing the body's ability to combat disease *de novo* could circumvent resistance. Furthermore, immunomodulation could protect against a range of diseases. For example, Il-10+ Th1 cells appear to be protective for both *Toxoplasma gondii* and malaria. One could imagine one therapy that induces IL-10 production specifically in Th1 cells that can be given during the blood stage of infection

Vaccines differ from immunomodulation therapy based on the timeline of infection. While immunomodulatation therapy is meant to manipulate the immune system during the course of disease in order to improve outcome, vaccines are given prior to infection in the

hopes of minimizing disease upon exposure. Rational vaccine design could target cells that mitigate disease during the blood stage of malaria. In a recent study, Darrah, et al., used two different vaccine adjuvants to determine whether IL-10 expressed by Th1 or APCs affected the cellular response to Leishmania in a murine model for infection (14). The adenovirus vector adjuvant increased the number of IL-10 producing Th1 cells in a dose dependent manner. The second adjuvant, the TLR 9 receptor ligand, CPG, induced IL-10 through innate cells such as macrophages or dendritic cells. The levels of IL-10, in turn, were found to inversely correlate with dendritic cell IL-12 expression. Together, these data demonstrate the potential to design a vaccine that specifically targets IL-10+ Th1 cells.

Currently, a vaccine that targets the liver stage of malaria infection, RTS,S/AS01, is undergoing promising clinical trials. In a human malaria challenge study, vaccination with RTS,S/AS01 conferred protection for approximately 41% of test subjects (43). Although promising, the efficacy of RTS,S/AS01 could be improved and Stewart, et al. demonstrated recently that using an adenovirus vector in tandem with RTS,S/AS01 significantly increased the number of T-cells that produce IFN-γ. It should be noted that IL-10 production was not assessed in this study however, based on the findings by Darrah, et al., it is possible that an IL-10+ Th1 population arises due to the adenoviral adjuvant. It would be interesting to further characterize the adenovirus induced T-cells and whether the adjuvant increases the efficacy of RTS,S/AS01.

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